Prion Diseases: A Genetic Perspective

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Prionenerkrankungen: Genetische Gesichtspunkte

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Zusammenfassung

Prionenerkrankungen: Genetische Gesichtspunkte

Sowohl beim Menschen als auch bei Tieren kann eine Gruppe übertragbarer neurodegenerativer Erkrankungen beobachtet werden, die nicht durch klassische Erreger hervorgerufen werden. Zu diesen Krankheiten zählen die Creutzfeldt-Jakob-Krankheit (CJD) des Menschen, die Bovine Spongiforme Encephalopathie (BSE) beim Rind, Scrapie bei Schaf und Ziege und die Chronic Wasting Disease (CWD) bei Wildwiederkäuern.
Die sogenannten Prionenerkrankungen sind vor allem durch die Akkumulation eines Proteinase-resistenten Proteins (“Prion”), einer Isoform des körpereigenen Prionproteins, vor allem im zentralen Nervensystem, gekennzeichnet. Es gibt eine Reihe von Hinweisen darauf, welche Aufgaben das Prionprotein erfüllen könnte, seine exakte Funktion ist aber noch nicht geklärt worden. Die Pathogenese der Prionenerkrankungen ist eng verbunden mit dem Vorhandensein des Prionproteins in einer Reihe von Zelltypen, ein Sachverhalt welcher mit Hilfe transgener Mausmodelle nachgewiesen wurde.
Das grundlegende Ereignis der Prionenpropagation ist die Umwandlung der normalen α-Helix-reichen Isoform des zellulären Prionproteins in eine andere, die vorwiegend aus β-Faltblättern besteht und sich vom zellulären Prionprotein in einer Reihe von biochemischen Eigenschaften unterscheidet. Die darauf folgende Akkumulation und Aggregation dieser Proteinase-resistenten Form führt zur Neurodegeneration. Es wurden mehrere Modelle entwickelt, um die Aggregation der Prionen zu erklären.
Es ist zudem bekannt, dass genetische Faktoren eine wesentliche Rolle im Konversionsprozess spielen, indem sie die strukturelle Stabilität des Prionproteins oder auch seine Expressionslevel beeinflussen. Darüber hinaus könnten sich Interaktionen des Prionproteins mit anderen Proteinmolekülen auf Pathogenese und die Phänotypen bei Prionenerkrankungen auswirken. Dies gilt auch für die Übertragbarkeit der Erkrankungen zwischen Individuen oder von einer Spezies zur anderen.
Die Existenz mehrerer Konformationen und Glykosylierungsmuster des Prionproteins führt zur Ausbildung verschiedener Prionen-Stämme (Strains). Der Phänotyp einer

Prionenerkrankung scheint demnach von der Struktur des körpereigenen Prionproteins einerseits und des infektiösen Prions andererseits abzuhängen. Genetische Faktoren sind hierbei von besonderer Bedeutung, da sie unter anderem die Konformation des Prionproteins beeinflussen können.
Die vorliegende Arbeit umreißt die grundlegenden Merkmale der Prionenerkrankungen, einschließlich der strukturellen Eigenschaften des Prionproteins und des Prions. Darüber hinaus werden unterschiedliche Modelle der Prionenaggregation unter besonderer Berücksichtigung der genetischen Implikationen diskutiert. Die verbreitetsten Prionenerkrankungen bei Mensch und Tier werden beschrieben und von einem genetischen Blickpunkt aus betrachtet. Zudem werden mutmaßliche Kandidatengene, die mit Prionenerkrankungen in Zusammenhang stehen könnten, vorgestellt.

Summary

Prion Diseases: A Genetic Perspective

In both humans and animals, a group of transmissible neurodegenerative conditions has been observed which are not caused by classical infectious agents. These disorders include Creutzfeldt-Jakob-Disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goats and chronic wasting disease (CWD) in cervids.

The so-called prion diseases are primarily characterised by the accumulation of a proteinase-resistant protein (designated “prion”) that is an isoform of the endogenous prion protein, mainly expressed in the central nervous system. There are several indications which tasks the cellular prion protein might fulfil but its exact function has not yet been clarified. The pathogenesis of prion diseases is closely linked to the presence of the prion protein in a number of cell types, an association which has been explored by using transgenic mouse models.

The basic event in prion propagation seems to be the transformation of the normal α-helix rich isoform into another that is mainly composed of β-sheets and differs from cellular prion protein in a number of biochemical properties. The ensuing accumulation and aggregation of this latter proteinase-resistant form leads to neurodegeneration. Several models have been proposed in order to explain aggregation.

Genetic factors are known to play a considerable role in the conversion process by influencing the structural stability of the prion protein or otherwise its expression levels. Furthermore, interactions of the prion protein with other endogenous protein molecules may have an impact on pathogenesis and phenotype in prion diseases. This also applies to the species barrier, i.e. to transmissibility of the diseases between individuals or from one species to an other.

The existence of several prion protein conformations and glycosylation patterns apparently leads to the development of multiple prion strains. Thus disease phenotype seems to be determined by the structure of both the endogenous prion protein and the infectious prion. Genetic factors are strongly associated with these aspects of prion disease as they have an effect on host prion protein conformation.

This work outlines the fundamental features of prion diseases including the structural properties of the prion protein and the prion. Different models of prion aggregation are furthermore introduced with a special reference to genetic implications. In addition, the most common prion diseases of humans and animals are characterised and viewed in a genetic perspective. Putative candidate genes that may be associated with prion diseases are discussed together with mechanisms by which they might exert their influence.

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Introduction

Prion diseases

Prion diseases are a class of neurological disorders also termed transmissible spongiform encephalopathies (TSEs) (see Table 1). Neuropathological findings in these diseases are generally associated with the formation of rods or “plaques” of a proteinase-resistant protein, vacuolisation and neuronal loss in tissues of the central nervous system (Prusiner *et al.*, 1983). Originally they had also been designated slow virus diseases or transmissible dementias because the nature of the agent causing these disorders was yet unknown.

A common characteristic of TSEs is their transmissibility from one being to the other within the same species. Transmission is independent of whether the illness originally occurred spontaneously or as a result of inherited predisposition. A range of factors, both host-specific and pathogen-specific, influence transmission dynamics. Prion properties, host genetic disposition and epidemiological factors such as infection dose, age at infection or route of infection are implicated in the resultant disease phenotype.

A number of questions concerning TSEs still remains to be answered. The nature of the infectious agent and the mechanism by which infectivity spreads is not yet entirely clear. Another unresolved question is in which way in PrP^{Sc} forms aggregates in the brain. Different models have been proposed but none could be completely affirmed by experimental procedures. It is furthermore unclear by which route the infectious agent enters the body. Apart from the oral route and transmission by surgical procedures (grafting, inoculation) several other possibilities have been discussed such as transmission via conjunctiva, wounds, skin, body fluids (blood, lymphocytes, milk) or other vectors, e.g. insects. For some TSEs like CWD or scrapie the route of transmission can only be presumed as detailed observations are lacking. Regarding scrapie it has been repeatedly doubted that it is an acquired prion disease at all. As the connection between genotype of the animals and susceptibility to disease is especially close in sheep it has been proposed that scrapie might be an inherited disease for which the predisposition is determined by the host PrP^{C} genotype. Dispositions for TSEs are known in humans and various in species, but it

is not yet clear if susceptibility to prion diseases might be controlled by the genotype in all species affected by these disorders.

Table 1: Prion diseases in different species

Species		Prion Disease
Human	Sporadic	sCJD
	Inherited	fCJD, GSS, FFI, BPI
	Acquired	iCJD, kuru, vCJD
Sheep		Scrapie
Cattle		BSE
Mink		TME
Felidae		FSE
Cervids		CWD

Historical Background of Human Prion Diseases

In humans the so-called Creutzfeldt-Jakob disease (CJD) is the most prominent spongiform encephalopathy. As early as 1920 a neurological illness that was accompanied by mental and sensory impairment and disturbance of the motor function was first described by Hans Gerhard Creutzfeldt who referred to the disease as “pseudosclerosis”. Only months later, Alfons Maria Jakob examined three patients with a resembling phenotype. He diagnosed the patients with a disorder he described as “spastical pseudosclerosis”. These latter patients were the first actual cases of human CJD. Although the case reported by Creutzfeldt does not meet the phenotypic criteria typical for CJD and probably refers to a different kind of disorder, the term CJD still remains in use.

By the criteria presently applied to neurological diseases such as clinical symptoms, neuropathological presentation and both biochemical properties of the agent and

genetic host factors, CJD can be classified into different forms. The disorder therefore can be caused by inherited genetic predisposition (familial form) or transmission of an infectious agent (iatrogenic form). CJD can also occur as a rare spontaneous event (sporadic CJD).

Furthermore, there are other human prion diseases such as Gerstmann-Sträussler-Scheinker syndrome (GSS) or Fatal Familial Insomnia (FFI) that are discernable with the help of various clinical and histopathological symptoms. These disorders are associated with specific mutations in the prion protein amino acid sequence.

Another neurodegenerative disease was recognized at the beginning of the 20th century when the epidemical course of a neurological syndrome was observed in the highlands of Papua New Guinea. Contrary to classical CJD, the illness termed kuru appeared to be orally transmitted through cannibalistic consumption of infected brain tissue among an isolated native tribe. Kuru was shown to transmit to chimpanzees (Gajdusek *et al.*, 1966), from which was concluded that the disease was caused by a slow virus with an exceptionally long incubation period.

The discovery of vCJD, the new variant of Creutzfeld-Jakob Disease in humans, in the 1990ies, revived the subject of spongiform encephalopathies. In contrast to classical CJD cases, vCJD patients were much younger and clinical symptoms differed from those hitherto observed in other CJD variants. It seems to be likely that vCJD has been caused by transmission of an infectious agent between two species, in this case from cattle to humans. Notably, in the 1980s a fatal prion disease of cattle termed Bovine Spongiform Encephalopathy (BSE) strongly occurred first in the United Kingdom and later on in a range of countries world-wide. BSE itself causes, amongst others symptoms, typical spongiform lesions in the brains of infected animals and leads to progressive ataxia. As vCJD brain neuropathology shows characteristics similar to lesions in brains of BSE animals it has been proposed that human illness is caused by infection with the same agent. There are also biochemical properties of the vCJD agent that differ from those observed in other CJD forms and are similar to the characteristics of the BSE infectious agent. As primary route of transmission from cattle to humans consumption of beef contaminated with infectious material is the most likely possibility.

From the early 60ies on there had been indications that spongiform encephalopathies are not caused by a slow virus but by an agent, possibly a protein, that was devoid of nucleic acid as the agent was not inactivated by nucleases or UV-

radiation. In the 1980ies the term “prion” (for proteinaceous infectious particle) was introduced in order to describe a protein that was isolated from the aggregates or plaques typically seen in spongiform encephalopathies. At first it was not clear whether this protein was encoded for by a viral nucleic acid or whether it might itself be the infectious agent. Only when it was demonstrated that the amino acid sequence of the protein was encoded by a chromosomal host gene it became obvious that its infectious properties were causative to the spongiform encephalopathies. It was also shown that the apparent differences between the ubiquitous cellular prion protein and the infectious form are due to posttranslational processing and structural conformation.

Historical Background of Animal Prion Diseases

In animals several spongiform encephalopathies other than BSE have been recognised to date. Natural scrapie in sheep and goats has been observed in European countries from as early on as the middle of the 18th century. Despite of the fact that it is present in a range of countries, even today it is unclear in which way exactly the disease is transmitted between animals and flocks.
As the emergence of the BSE epidemic coincided with an outbreak of scrapie on the British Isles it has been suggested that scrapie-contaminated meat and bone meal (MBM) was distributed to cattle. Nevertheless the origin of BSE could also lie in the occurrence of a sporadic BSE case and subsequent recycling of infectious material via MBM. There are characteristics observed in some types of BSE and scrapie agent that are very similar to each other although neither of both possibilities could hitherto be excluded.
Epidemics of spongiform encephalopathy are known to occur in mink kept on fur producing farms which led to the introduction of the term Transmissible Mink Encephalopathy (TME) (Marsh and Bessen, 1993). The disease first described in the 1940ies seems to be orally transmitted through consumption of contaminated feed. Interestingly, there are two different forms observed in affected animals which can be distinguished by the clinical presentation. Furthermore, in cervids such as elk or white-tailed deer, Chronic Wasting Disease (CWD) has been described in parts of the United States of America, both in captive animals as well as in free-ranging deer. It was first detected in 1967 and recognised as a TSE nine years later. CWD seems

to have developed independently from other TSEs though the origin of the disease remains unclear.
Another disorder called Feline Spongiform Encephalopathy (FSE) has been detected in wild and domestic cats (Leggett *et al.*, 1990). It causes typical neurological disturbances and spongiform changes in the brain. Research on FSE has been scarce as the threat of transmission to human beings through the oral route is low, at least in the western hemisphere. TSEs can also be found in zoo animals such as tiger, puma, bison, kudu, oryx etc. (Kirkwood and Cunningham, 1994). It is possible that the so-called zoological spongiform encephalopathy (ZSE) includes CWD, FSE or BSE according to the species of the affected animal. Prion diseases in zoo animals seem to be caused by the distribution of feedstuff contaminated with the infectious agent.

The Prion Protein

One characteristic of prion diseases is the presence of proteinase K-resistant protein aggregates in the brain of affected individuals. These amyloid structures observed in prion diseases were found to be composed of protein molecules with a weight of 27-30 kDa. The proteins were therefore initially termed PrP^{27-30}.
Oligonucleotides corresponding to the N-terminus of PrP^{27-30} were prepared by Oesch *et al.* (1985). With the help of these probes it was possible to select a corresponding cDNA clone from a scrapie-infected hamster brain library. Southern blotting with PrP^{C} cDNA demonstrated that the protein was encoded by a single chromosomal gene. DNA both from scrapie-affected and normal brain exhibited common restriction patterns.
Additional evidence that PrP^{C} and the PK-resistant PrP^{27-30} are encoded by the same gene was found when amyloid plaques in brain tissue sections of humans and animals were stained with PrP antibodies (Kitamoto *et al.*, 1987). The highly intense PrP staining of the amyloid PrP^{27-30} plaques indicated that a common single gene encoded both proteins. Differences in protein properties such as proteinase K resistance must have been caused by posttranslational processes relevant for structural conformation as amino acid sequences of the PrP^{C} isoform and PrP^{27-30} were identical (Borchelt *et al.*, 1990). These results did argue against the theory of PrP^{27-30} as a viral protein, a hypothesis that had been suggested beforehand. After

the discovery of PrP^C and its gene, experiments were carried out to define its characteristics (see Table 2).

Table 2: Properties of PrP^C and PrP^{Sc} (Hunter et al., 1997, modified)

	Normal	Abnormal
Name	PrP^C	PrP^{Sc}
PK	Sensitive	Partially resistant
Detergent	Soluble	Insoluble
Length	~250 amino acids	~250 amino acids
Structure	α-helix and loops	β-sheet
Glycosylated	Two sites	Two sites
Molecular weight (-PK)	33-35 kDa	33-35 kDa
Molecular weight (+PK)	Degraded	27-30kDa
Antigenicity	Bind to same antibodies	Bind to same antibodies
Location	Cell surface, GPI-anchored	Fibrils, deposits
Expression	Various tissues	Various tissues, pronounced in brain, central nervous system (CNS), lymph nodes, spleen, tonsils
Expression in disease	Protein levels constant	Protein levels increase
Turnover	Rapid	Slow

The precursor of the 33-35 kDa PrP^C is encoded by a single copy gene and comprises two signal peptides, one at the C-terminal end, the other one at the amino-terminus. Both of these signal peptides are cleaved off during protein processing and a glycosylphosphatidyl inositol (GPI) anchor, which fastens the glycoprotein to the cell plasma membrane, is attached to the carboxy-terminus of the protein (Rieger *et al.*, 1999). N-glycosylation might occur at positions 181 and 197 of the amino acid

chain at the C-terminus. Occupation of these sites can vary, so that diverse oligosaccharide side chains can be attached to the prion protein (Rudd *et al.*, 2001). Interactions between protein molecules or within the protein at residues 1-90 can thus be prevented by the glycans attached at position 181 and 197. They shield parts of the protein, which leads to sterical prevention of interaction. Side chains at positions 177, 181 and 185 remain open as potential sites of interaction with other molecules as long as no glycan is attached to them. Interestingly, a copper-binding octapeptide repeat sequence is located between codons 50 and 91 (Hornshaw *et al.*, 1995). It is therefore possible that glycosylation status of the prion protein influences its ability to bind metal ions, one possible function of PrP^{C} in the organism.
Within eurkaryontic cells, synthesis of the prion protein is accomplished at the rough endoplasmatic reticulum that contains several forms of the protein. The carboxy terminal transmembrane protein is termed ^{Ctm}PrP, the amino terminal protein ^{Ntm}PrP and the secretory protein is called ^{Sec}PrP. This last form is identical to classical PrP^{C} (Rieger *et al*., 1999). The transmembrane forms of PrP^{C} usually constitute but 10% of total PrP^{C} that is produced. They span the lipid membrane once by help of a central hydrophobic region (amino acid positions 111-134). Either the C-terminus (^{Ctm}PrP molecules) or the N-terminus (^{Ntm}PrP molecules) is thus located on the extracytoplasmic side of the membrane (Stewart and Harris, 2003). Via Golgi granules and secretory vesicles PrP^{C} is transported to the cell surface where it is attached by its GPI anchor. Endocytosis of PrP^{C} is possible either through caveolae-like domains or clathrin-coated pits with the putative involvement of the laminin receptor (LR). It has been suggested that PrP^{C} can be transported back to the cell surface by uncoupling vesicles. As GPI-anchored proteins can be spontaneously transported to plasma membranes PrP^{C} could also be transferred to other cells in this fashion (Ilangumaran *et al*., 1996).

Structural Properties of the Prion Protein

The mature form of PrP^{C} consists of two distinct domains, one of them a globular core domain of approximately 100 amino acids. Its N-terminal domain, also designated “tail”, which contains about 100 residues, is disordered and flexible. This structure is common to mammalian prion proteins (Riek *et al.*, 1996, 1998, Calzolai *et al.*, 2000, Lopez Garcia *et al.*, 2000). The structure of mouse, Syrian hamster, human

and cattle PrP^C differs only by strictly local conformational variations, namely atom position, variable surface charge distribution and dynamic properties (Calzolai *et al.*, 2000). The C-terminal globular domain of PrP^C comprises two α-helices linked by a disulfide bridge (DeArmond *et al.*, 2002). Another α-helix can be found in the N-terminal "tail" along with two short antiparallel sheets (see Figure 1).

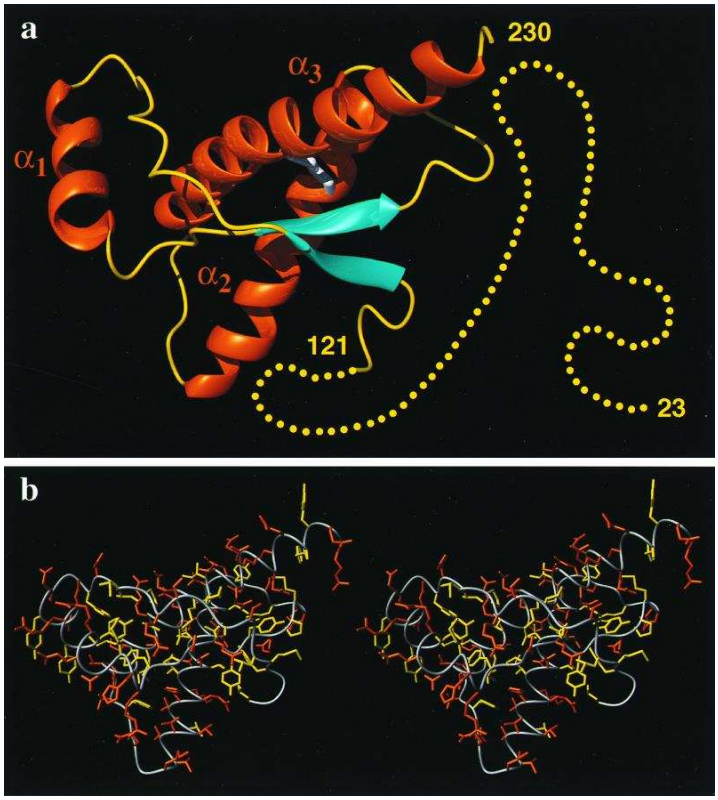

Figure 1: Structure of the human prion protein

(a) Three-dimensional structure of the intact human prion protein, hPrP(23-230). The helices are depicted in orange, β-strand cyan, the segments with nonregular secondary structure within the C-terminal domain yellow, and the flexible "tail" (23-121) is represented by yellow dots.
(b) Stereoview of an all-heavy atom presentation of the globular domain, with residues 125-228, in hPrP(23-230) in the same orientation as in (a). The backbone is shown as a grey spline function through the C^α positions, hydrophobic chains are yellow, and polar and charged side chains are orange (Zahn *et al.*, 2000).

The variable domain furthermore contains an octapeptide region, a segment of several repeats of an eight–amino acid sequence which shows affinity to copper (Hornshaw *et al*., 1995, Collinge, 2001). The stability and conformation of the protein might in part depend on its glycosylation properties and interactions between amino acids comprised in local structures. The presence of salt bridges and sulfide bonding is associated with stabilisation.

Prion Protein Distribution

At first, PrP^C was considered to be exclusively expressed as a neuronal protein in various regions of the brain. More recent experimental results are nevertheless indicating that PrP^C can be evidenced in a much wider range of tissues in a hamster model (Bendheim *et al.*, 1992). Despite of this, the amount of PrP^C that can be detected in non-CNS tissues is generally much lower than in neuronal tissues.
In immunomorphical studies it was possible to locate PrP^C in the synaptic domain of nerves in the hamster brain where it seems to colocalise with presynaptic synaptophysin (Fournier *et al.*, 1995). Another experimental approach in a rodent model showed that PrP^C could be detected at the presynaptic as well as the postsynaptic level (Haeberlé *et al.*, 2000) depending on the type of neuronal cells in which it was found. Purkinje cells (PCs) contain a considerably higher amount of PrP^C than is found in cerebellar inhibitory interneurons. PCs also display PrP^C at their postsynaptic dendrites in contrary to other classes of neurons in the cerebellum. This neuron-specific expression of PrP^C may represent differential involvement of PrP^C in synaptic function. In humans PrP^C is also expressed specifically in the postsynaptic domain of neuromuscular junctions of the skeletal muscle (Askanas *et al*., 1993, Gohel *et al.*, 1999).
As for non-neuronal tissues PrP^C could be found in the lungs of hamsters (Bendheim *et al.*, 1992) in secretory granules. Kidney has also been tested positive for the presence of PrP^C (Fournier *et al.*, 1998). PrP^C has furthermore been observed in epithelial cells of the stomach in humans and hamsters and in hamster intestine (Fournier *et al.*, 1998). This pattern of distribution might be of importance for prion disease infection via the oral route. Moreover, secretory granules of epithelial cells are known to contain PrP^C (Fournier *et al.,* 1998; 2000). The stellate cells of the liver represent another tissue in which PrP^C is expressed (Ikeda *et al.*, 1998). In activated

hepatic stellate cells both PrP^C RNA and the protein itself can be detected on the plasma membrane.
The distribution of normal PrP^C within cells and within tissues naturally implicates putative functions of the protein.

Putative Functions of the Prion Protein

Distinct knowledge of PrP^C function has yet to be obtained. The distribution of PrP^C in the body may point at such possible tasks. As PrP^C is attached to the cell surface it was supposed to act as a receptor or as part of a transmembrane signalling pathway. Interaction with extracellular ligands would be enabled by PrP^C domains oriented to the extracellular space. The hypothesis of PrP^C as a receptor protein is furthermore based on the finding that endocytosis of PrP^C can be mediated by clathrin coated pits, a mechanism that is known to be characteristic of transmembrane receptors such as transferrin or low-density lipoprotein receptors (Shyng *et al.*, 1994).
Stuermer *et al.* (2004) showed that in T cells PrP^C, by cocapping with reggie rafts, triggered signal transduction across the cell membrane. This might imply a role for PrP^C in immune system signalling. Another signalling pathway involves the tyrosine kinase Fyn. PrP^C-dependent activation of Fyn has been observed in a murine neuronal differentiation model (Mouillet-Richard *et al.*, 2000) suggesting an association of PrP^C with cell maturation.
Brown *et al.* (1999) were able to show that the murine PrP^C possessed activity similar to that of superoxide dismutase (SOD). The SOD-like activity of the protein was impaired by deletion of its copper-binding octarepeat sequence. This result suggested that PrP^C could influence oxidative stress resistance at cellular level. In another approach Brown *et al.* (2001) purified mouse PrP^C from brain and cultured cells and analysed its capability to bind copper. The more copper the protein was able to bind the higher was its protective effect against oxidative stress that had been experimentally imposed by PrP106-126, a neurotoxic peptide which inhibits SOD-like activity. This is in accordance with the findings of Wong *et al.* (2001). In their experimental study they measured SOD-like activity of PrP^C purified from brains of scrapie-affected mice in comparison to control animals. In PrP^C of scrapie-infected brain homogenates, SOD activity was significantly reduced to about 10% of control values. Also total SOD activity in brain homogenates was significantly impaired in

scrapie-infected brains. It was furthermore evident that the changes in antioxidant activity were accompanied by impairment of the brain metal metabolism. Cu, Zn, Mg and Ca were observed to be significantly reduced in the scrapie-infected animals. Neuroprotective functions of PrP^C in case of stress have been examined by Kim *et al*. (2004). Expression of PrP^C was achieved by transfection of immortalised cells derived from PrP^C knockout neuronal cell lines. Stress was caused by induction of apoptosis or serum deprivation. The presence of PrP^C was found to protect cells from death or at least delayed it. Mitochondrial dysfunction was observed in the cells after serum deprivation. Once again PrP^C was able to prevent damage of mitochondria, possibly via cytochrome *c* release into the cytosol. These findings point to a neuroprotective function for PrP^C as it rescued the stress-induced cell death and mitochondrial damage. This function might be impaired in prion disease and consequently be involved in pathogenic mechanisms in addition to negative influences of prion aggregate formation.

The new method of ultra-immunomorphology which was employed to detect PrP^C in a range of different tissues afforded results that pointed to an alternative function of the prion protein in the body. The presynaptic location of PrP^C in nerve terminals in hamsters suggested a role in synapse activity (Fournier and Grigoriev, 2001). Neurotransmitter vesicles might be coupled with PrP^C or the protein might be involved in intracellular vesicle trafficking. Indeed, PrP^C was found to colocalise with synaptophysin and synapsin (Spielhaupter and Schaetzl, 2001). Both of these proteins are known to be associated to small synaptic vesicles.

Modulation of PrP^C binding to such synaptic components seems to be achieved by oxidative stress (Morot-Gaudry-Talarmain *et al*., 2003). Copper (II) thereby acted as a mild oxidative agent and altered prion protein binding by synaptosomal ligands. These changes were positively correlated with changes in immunoreactivity of calcineurin B, a molecule involved in calcium regulation, in synaptic components. Impairment of PrP^C function therefore might not only have negative effects on copper regulation but also perturb the calcium regulation system of cells leading to pathological modifications.

In order to further investigate the function of PrP^C in copper homeostasis Sakudo *et al.* (2003) analysed copper levels in murine neuronal cells deficient for the prion protein gene (*Prnp*) transfected with *Prnp* and/or the doppel gene (*Prnd*). The expression of both genes itself did not influence copper levels. Oxidative stress was

induced by serum deprivation. The cellular copper concentration significantly dropped in all transfected cell lines with exception of those that were solely transfected with *Prnp*. PrP^C appeared to prevent the decrease of copper concentration. Therefore it is possible that PrP^C stabilizes cellular copper homeostasis under oxidative stress. Nevertheless it is not clear whether this is the only function PrP^C fulfils in an organism. Additionally, the effect of PrP^C was inhibited by overexpression of the doppel protein (Dpl) in cells. This indicates that PrP^C and Dpl can interact, a further corroboration of what has been suggested by PrP^C knockout experiments in mice.

In an approach to identify binding partners of PrP^C, a yeast two-hybrid screen with murine prion protein and a murine neuronal cDNA library were employed (Spielhaupter and Schaetzl, 2001). Several proteins that interacted with PrP^C were detected and those with high homology to already known proteins were selected. The three outstanding interaction partners were the murine growth factor receptor-bound protein 2 (Grb2), murine synapsin Ib and another protein of 162 amino acids in length. The latter protein had not been described beforehand so that it was termed prion interactor 1 (Pint1). To test whether the interaction was limited to yeast cells the proteins were expressed in mammalian cells. Co-immunoprecipitation assays were used to confirm the interaction of Grb2, synapsin Ib and Pint1 with the full-length PrP(23-231). Both synapsin and Grb2 were furthermore found to interact with two regions in the N- and C-terminal domains of PrP^C, in contrast to Pint1 which interacts with a region in the C-terminal domain. Further co-immunoprecipitation experiments revealed that Grb2 also specifically interacts with authentic PrP^C. Expression of Pint1 was determined in several organs including brain, heart, thyroid, muscle cells and liver, an expression pattern in part in congruence with PrP^C mRNA distribution. Synapsin and PrP^C apparently co-localise at nerve endings indicating a role of both proteins in the lifecycle of synaptic vesicles. Grb2 is implicated in intracellular signalling cascades, a function that has already been suggested for PrP^C. Thus interaction with Grb2 and synapsin 1 points to an involvement of PrP^C in these two important physiological functions. Both proteins appear to act as interaction partners and are therefore putative candidates for the existence of genetic variation in PrP^C action and sensitivity to prion diseases. The fact that PrP^C can be found at presynaptic as well as postsynaptic positions poses the question whether PrP^C fulfils

multiple functions which might differ according to PrP^C location within the body or which might be tissue-specific.
Another interaction partner of PrP^C seems to be tissue-type plasminogen activator (t-PA). Recombinant human PrP^C specifically stimulated plasminogen activation by binding kringle domains of t-PA (Epple *et al.*, 2004). The stimulating effect was restricted to t-PA that bound PrP^C by lysine-binding sites. These findings suggest that in the CNS, PrP^C might act as a co-factor in the regulation of plasminogen activation.
Recent studies have suggested that there might also exist prion-like mechanisms, i.e. the ability to convert the structural conformation of other molecules, in further proteins with regard to synaptic function. Si *et al.* (2003) revealed that a neuronal protein, a member of the cytoplasmatic polyadenylation element binding protein (CPEB) family, exhibited an unusual amino acid composition at its N-terminus. It could be demonstrated experimentally that it showed prion-like properties in yeast, especially the ability to convert other CPEBs to a conformational state similar to its own. It was furthermore suggested that CPEB has the ability to form aggregates. The converted form of the protein was also able to stimulate mRNA translation in synapses. According to Si *et al.* the prion-like mechanism could activate mRNA in a local fashion and be responsible for the maintenance of long term synaptic change implicated in memory storage.
Taken together, the functions of prion proteins and prion-like switches might be more far-reaching than hitherto thought.

The “Protein-only” Hypothesis

The first indication that proteins are at least involved in the development of neurological disorders like CJD and scrapie arose when experiments with scrapie agent showed that infectivity was dependent on the presence of a protein (Prusiner, 1982). Since experiments hinted at the absence of nucleic acid in the infectious agent the possibility emerged that the protein itself might be causative for infection. Prusiner (1982) introduced the term prion (for proteinacious infectious particle) and the protein-only hypothesis that postulates the sole responsibility of the prion for pathogenesis. For a long time the structural properties of the infectious particle had not been clarified. The agent could have been a nucleic acid encoding the proteins embedded in the proteins in a virus-like manner, a polynucleotide in association with

proteins or solely a protein without nucleic acid being involved. There was also a number of mechanisms postulated by which the infectious particles could be replicated, among others mechanisms utilized by viruses or modification of the cellular protein isoform on a posttranslational level (Prusiner, 1991).
Although the widely-accepted protein-only hypothesis has been able to explain why the infectious particles are resistant to nucleic acid-damaging procedures, such as treatment with nucleases and UV radiation, there are questions that remain to be answered. Firstly, different disease phenotypes can be observed relating to the existence of multiple strains of prions. How can such phenotypic diversity occur if not through the involvement of nucleic acid? Secondly, the existence of a “species barrier” that impairs prion disease transmission between species cannot be traced back to variation of a genome as in other types of pathogens.
In order to deal with these difficulties, the so-called “unified theory” was introduced by Weissmann (1991). He postulated that a small nucleic acid might encode strain-specific information leading to variable disease phenotypes though the major infectious factor would still be the protein. In case the “unified theory” was to be appropriate, standardised strain characteristics would be disturbed if the nucleic acid was destroyed. This has not yet been experimentally demonstrated for the prion agent, so that today, most scientists accept the „protein-only“ theory.
Recently, a study of recombinant mouse PrP provided further evidence in favour of the “protein-only” hypothesis (Legname *et al.*, 2004). Synthetic murine PrP was polymerized into amyloid fibrils that produced neurologic dysfunction in transgenic mice intracerebrally inoculated with the fibrillar aggregates. These latter animals expressed normal murine PrP^{C} on a PrP knockout background. Proteinase-resistant PrP was detected in the brain extracts of the transgenic mice. Furthermore, inoculation of wildtype and transgenic mice overexpressing PrP with such brain extracts led to the transmission of disease. These findings indicate that prions can form solely from recombinant PrP and that no exogenous agent other than PrP^{C} appears to be necessary for prion propagation

Prion Strains

A range of prion strains has been distinguished from each other by means of their diverse proteinase K cleavage sites and glycoform ratios (Collinge *et al.*, 1996), as wells as distinct incubation times and neuropathological patterns in inbred mouse lines (Bruce *et al.*, 1991). Through PK digestion the prion agent is cut into fragments whose length is probably dependent on protein conformation. The pattern of relative glycosylation of the protein also becomes visible by removal of the N-linked glycans in proteinase K treatment and ensuing gel electrophoresis. The higher the increase in banding intensity compared to the original banding, the higher the gycosylation at specific sites (Rudd *et al*., 2001). The relative glycosylation site occupancy of host PrP^{Sc} mirrors that of the donor PrP^{Sc} when infectious agent is transmitted from one animal to another. It is distinct from that of host PrP^{C}. Similarities in glycan amount and location might allow prion proteins to travel to neurons or brain regions (DeArmond *et al*., 1997). If this is true, PrP^{Sc} might be transported in a glycosylation-dependent fashion to specific locations in the brain. Consequently, PrP^{Sc} would target PrP^{C} that possesses a rather similar glycosylation pattern because both conformational forms would collocate.

Strain-specific “lesion profiles” are deduced according to vacuolisation patterns, i.e. distribution and extent of spongiform changes in the CNS (Fraser and Dickinson, 1968). With the help of such lesion profiles it was possible to identify several scrapie strains in mice. After inoculation of the animals with BSE agent from different cattle sources a much more uniform pattern was observed, indicating that strain diversity in BSE prions is low (Bruce *et al*., 2002). The lesion pattern was nevertheless considerably distinct from that in scrapie-infected mice. In conclusion, infectious agent obtained from different groups of BSE-affected cattle, which was employed in the study, seemed to have originated from the same source.

Moreover, amount and rate of the conversional change appears to vary between specific prion strains (Mulcahy and Bessen, 2004). In mink two different strains of TME, designated hyper (HY) and drowsy (DY), according to the clinical symptoms, have been observed. Strain typing of both TME variants was performed in Syrian Hamster. HY animals exhibited hyperexcitability and ataxia while DY animals showed progressive lethargy (Bessen and Marsh, 1992). Incubation periods were considerably longer in the DY strain than in the HY strain (65±1 compared to 168±2

days). Furthermore, PrP^{Sc} isolates from the brains exhibited distinguishable properties such as different gel migration pattern after PK digestion and different type of ß-sheet secondary structure (Bessen and Marsh, 1992, 1994, Caughey *et al.*, 1998). PK digestion also led to a faster degradation of DY PrP^{Sc} and inactivation of its infectivity than of HY PrP^{Sc}. Experimental cell-free PrP conversion showed that the HY strain caused a faster and higher level of PrP^{Sc} formation than the DY strain. Strain-specific factors therefore might be able to influence conversion characteristics, also depending on host factors e.g. PrP^{C} primary structure or presence and function of chaperone molecules.

In humans, two different types of the sporadic form of CJD have been identified by Parchi *et al.* (1996) through immunoblot analysis. After PK-digestion the remaining core fragments of PrP^{Sc} from sCJD brains exhibited two distinguishable migration patterns on gel electrophoresis. This was due to primary cleavage sites at residues 82 (type 1 pattern), 97 (type 2 pattern) and a number of secondary PK cleavage sites. In forms of the CJD acquired by peripheral infection it was also possible to see a type 3 pattern with shifted banding in western blotting (Collinge *et al*., 1996). The distribution of PrP^{Sc} fragments in vCJD was considerably different to type 1 and type 2 patterns and could be distinguished from both by specific band intensities. The vCJD agent possessed a highly consistent glycosylation pattern, which was not comparable to either CJD strain type 2 or 3. This indication that sCJD and vCJD are caused by different strains of prion agents was further corroborated by transmission experiments of CJD and BSE in mice. While mice inoculated with CJD exhibited the classical CJD pattern, the glycosylation pattern induced by BSE prions closely resembled that observed in the new variant of CJD. These findings constitute considerable evidence that the prion disease BSE could have been transmitted to human beings. Transmission between species is limited by a phenomenon termed “species barrier”.

The Species Barrier

When prion diseases are transmitted between different mammalian species an effect can be observed which is commonly termed “species barrier” (Pattison, 1965). Though inter-species transmission might be possible, incubation times in primary transmission are increased compared to those observed in transmission within the same species. After passage of the infectious agent in the same species incubation times are narrowed down to those seen in intra-species transmission.

The “height” of a species barrier can be estimated through measurement of the comparative drop in mean incubation period between first and second same-species passage (Collinge, 2001). Species barriers were first thought to arise through differences in the amino acid sequence of host PrP^{C} and the infectious agent. Experiments in transgenic mice expressing chimeric PrP genes demonstrated that substitutions in the amino acid chain of PrP^{C} influence disease susceptibility between species. Mice whose PrP^{C} contained amino acid substitutions encoded by the Syrian hamster PrP gene were preferably targeted by chimeric PrP^{Sc}, while nontransgenic mice as well as nontransgenic hamsters were infected much less efficiently (Scott *et al.*, 1993). It was suggested that the greater homology between the agent and host PrP^{C} enhanced prion propagation in the animals.

It then became obvious that factors other than amino acid sequence of the donor PrP^{Sc} and recipient PrP^{C} were influential with regard to the “species barrier”, especially such that are modulating structure and conformation of the proteins. A certain conformation of PrP^{Sc} in the infectious agent deduced from one species might preferably infect a species that possesses PrP^{C} inclining to the same PrP^{Sc} conformation. In prion strains that can cross species barriers relatively easily, PrP^{Sc} might be in congruence with PrP^{Sc} conformations that are favoured by PrP^{C} structure in a wide range of host species.

The strain type of the infectious agent has increasingly gained in importance in connection with the species barrier (Bruce *et al.*, 1994). Distinct glycosylation types observed in different strains might have an influence on conformational properties of PrP^{Sc} and therefore on the ability of interspecies transmission.

Prion Protein and Prion Disease

The impact of host PrP^C amino acid sequence in connection with observed clinical cases of prion disease came to notice when polymorphisms in the murine and ovine prion protein were found to influence scrapie incubation times (Westaway *et al.*, 1987; Goldman *et al*., 1994). In humans, an association between polymorphisms in the PrP^C gene and CJD was revealed by Windl *et al.* (1996).

To further investigate the role of the PrP^C concerning TSEs, knockout experiments in mice were conducted. A first approach (Bueler *et al.*, 1992) showed that PrP^C-deficient mice developed normally and did not exhibit impaired behaviour. However, contrary to wildtype controls it was not possible to infect these PrP^C knockout animals with scrapie (Bueler *et al.*, 1993). The importance of these results was furthermore corroborated as heterozygous mice showed enhanced resistance to disease.

In other mouse models considerable defects such as altered circadian activity rhythms and sleep patterns (Tobler *et al.*, 1996) and loss of cerebellar Purkinje cells and ataxia (Sakaguchi *et al.*, 1996) were observed. Another knockout approach resulted in impairment of GABAA receptor-mediated fast inhibition as part of a disrupted synaptic neurophysiology in the animals (Collinge *et al.*, 1994). A latter study suggested that ataxia and loss of Purkinje cells in knockout mouse models could not be attributed to the absence of PrP^C in the body but to the upregulation of Dpl, which resembles PrP^C (Moore *et al.*, 1999). Nevertheless it was demonstrated by these murine knockout models that the presence of PrP^C is crucial for TSE infection.

The depletion of endogenous neuronal PrP^C even protected mice with neuroinvasive prion infection from developing clinical symptoms of the disease (Mallucci *et al.*, 2003). In this study, transgenic mice generated on a PrP knockout background expressed endogenous PrP^C at 1- to 3-fold wildtype levels, so that scrapie infection could take place. Their neuronal PrP^C expression was stopped by Cre recombinase mediated depletion at approximately 12 weeks of age. This led to an approximately 3-fold life span in comparison to animals that did not express Cre recombinase. The mice did not show neuronal loss despite of ongoing gliosis and accumulation of proteinase-resistant PrP^{Sc} in the brain. These findings indicated that the presence of neuronal PrP^C is required for the occurrence of clinical scrapie in the transgenic

model, which again highlights the importance of PrP^C for the pathogenesis of prion diseases.

Conversion of PrP^C to PrP^{Sc}

The commonly accepted “protein-only” hypothesis states that the causing agent for prion diseases is an infectious isoform of PrP^C (Prusiner, 1982). The non-pathogenic form PrP^C apparently alters its normally α-helix rich conformation to an isoform that contains mainly β-sheet structures (Pan *et al.*, 1993) (see Figure 2).

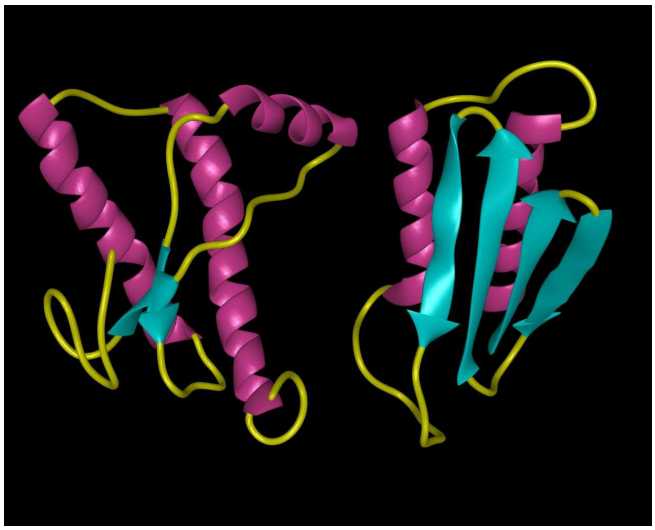

Figure 2: Model of recombinant PrP (right) and PrP^{Sc} (left) (Prusiner, 2001)

The model to the left depicts Syrian hamster recombinant PrP90-231, which presumably resembles that of the cellular isoform PrP. α-helixes A (residues 144 through 157), B (172 through 193) and C (200 through 227) are purple with loops in yellow; residues 129 through 134 in strand S1, and residues 159 through 165, in strand S2, are blue. A plausible model of the tertiary structure of human PrP^{Sc} is shown on the right side. S1 β-strands (residues 108 through 113 and 116 through 122) and S2 β-strands (residues 128 through 135 and 138 through 144) are blue. α-helixes B (residues 178 through 191) and C (residues 202 through 218) are purple, with yellow loops.

The variable N-terminal “tail” thereby changes its structure by extending the β-sheets The mechanisms underlying this conversion have been poorly understood until now. Transition might either take place spontaneously or could be triggered by the

presence of PrP^{Sc}. There are different models available to explain such conformational conversion (see Figure 3).

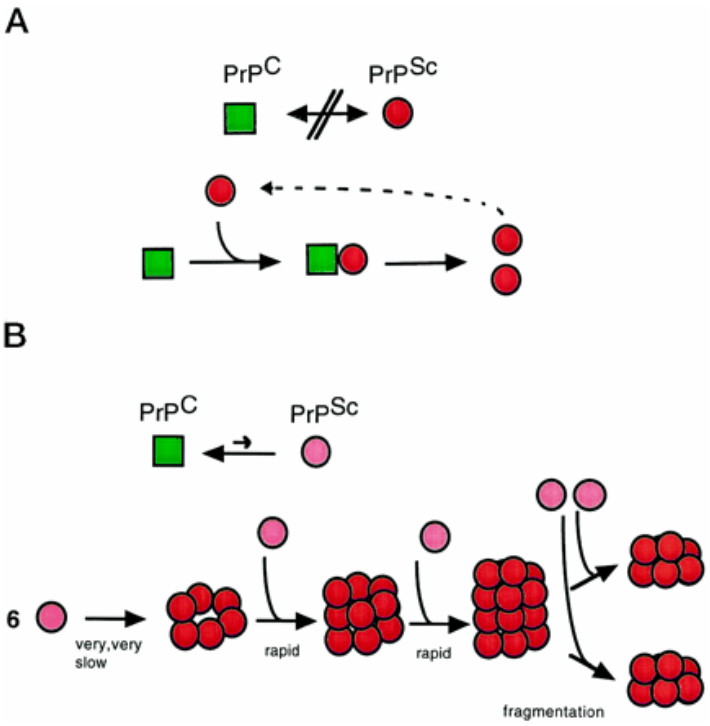

Figure 3: Models for the conformational conversion of PrP^C to PrP^{Sc} (Weissmann, 1999)

A "refolding" model
B "nucleation" model

The "refolding" theory (Figure 3, A) interpreted PrP^{Sc} formation as a result of interaction between the infectious PrP^{Sc} and PrP^C. The process would be kinetically controlled and led to an exponential conversion cascade (Prusiner *et al.*, 1991). The "nucleation" model (Figure 3, B) regarded the formation of PrP^C from PrP^{Sc} as reversible. The infectious isoform remains stabilised only by the presence of a PrP^{Sc} aggregate or "seed". First steps leading to aggregation would be slow, with speed increasing as more and more monomers are added to the seed (Brown *et al.*, 1991). Transmutation could be aided by a chaperon, designated protein X, whose binding sites are located in the C-terminal region of PrP^C (Telling *et al.* 1995, Kaneko *et al.*,

1997). Experimental studies revealed that substitution of amino acids in the PrP^{C} sequence stopped the protein from being converted to PrP^{Sc} (Kaneko *et al.*, 1997). The mutant forms of PrP^{C} seem to bind to protein X thus prohibiting the formation of PrP^{Sc}. A latter study (Cordeiro *et al.*, 2001) comes to the conclusion that this chaperon might not be a protein at all but a nucleic acid or protein-nucleic acid complex.
This hypothesis was further substantiated as Deleault *et al*. (2003) discovered that specific host-encoded RNA molecules stimulated the *in vitro* amplification of PrP^{res}. This protein-resistant protein showed similarities to PrP^{Sc}. Tests were conducted *in vitro* in the absence of other cellular factors to exclude the possibility of additional interactions. Nucleic acids of the host might thus play a role in prion disease pathogenesis. The question whether these RNA factors involved in conversion might have further effects, e.g. in the generation of prion strain diversity, has yet to be answered.

Prion and Prion Aggregation

The infectious prion, PrP^{Sc}, cannot be chemically distinguished from PrP^{C} but there are properties that allow differentiation. PrP^{C} is sensitive to enzymatic degradation by proteinase K, contrary to the infectious prion. The latter is partially resistant to proteolysis, and after PK digestion forms a fragment designated $PrP^{27\text{-}30}$ (Oesch *et al.*, 1985). In contrast to PrP^{C}, PrP^{Sc} cannot be solubilized in detergents (Meyer *et al.*, 1986). PrP^{C} shows rapid turnover in the organism while PrP^{Sc} accumulates in the brain of affected subjects (Borchelt *et al.*, 1990). Furthermore, PrP^{C} distribution in the brain differs from that of PrP^{Sc} as both isoforms localise in specific regions (Taraboulos *et al.*, 1992). In the course of disease the core fragment of PrP^{Sc} ($PrP^{27\text{-}30}$) polymerises into amyloid-like deposits or rods (Prusiner *et al.*, 1983), which are found in the brain of individuals affected by prion disease.
Not only in prion diseases but also in other neurodegenerative diseases such as Alzheimer's or Parkinson's illness the accumulation of proteins has been observed in a range of different tissues. In the group of diseases termed amyloidoses, fibrillar structures composed of proteins are therefore encountered in the extracellular space. Nevertheless it is yet unclear by which mechanism and pathogenic species i.e. conformational state of the proteins or aggregates neurodegeneration is caused.

Protein molecules fold into their specific conformational state after the polypeptide chain has been synthesised at the ribosomes (Dobson, 2001). This final conformation is basically determined by the amino acid sequence of the protein although the folding process is accomplished with the help of chaperones and catalytic enzymes. Chaperones prohibit undesirable interactions of other molecules with the unfolded protein or accelerate certain stages of the folding process. As misfolding of proteins can take place in the transition phase there are protective mechanisms. They prevent negative effects like accumulation and aggregation of damaged or misfolded proteins (Sherman and Goldberg, 2001). For example, heat-shock proteins (Hsps) can diminish prion aggregation, dissociate aggregates, degrade abnormal proteins etc. In prion diseases and other disorders in which protein aggregation can be observed these protective mechanisms seem to be impaired. Aggregation processes can thus occur if the correct conformational state of the proteins is not achieved. This effect can be caused by mutations of the amino acid chain, abnormal interaction with metal ions, alteration of pH levels, temperature or other environmental factors. Chemical processes such as oxidation can furthermore be involved in aggregation (Stefani and Dobson, 2003). Reasons for an increasing presence of misfolded proteins could be enhanced protein synthesis or otherwise reduced clearance of the molecules.

Aggregation and Toxicity

The ability to aggregate into fibrils does not seem to be an exclusive property of disease-related proteins. Guijarro *et al.* (1998) demonstrated that a domain of the globular protein bovine phosphatidylinositol 3-kinase (PI-SH3), an enzyme that is not associated with disease, can form amyloid fibrils. Toxic effects have been demonstrated for aggregated forms of PI-SH3 and the HypF Protein (HypF-N) which is found in *Escherichia coli* (Bucciantini *et al.*, 2002).
The main factor that influences aggregate formation seems to be protein core structure. Protein side chains are nevertheless supposed to be involved in structural variation of amyloids and in the assembly of protofibrils, the precursors of amyloid fibrils (Stefani and Dobson, 2003). Conversion from one conformational state into another is apparently taking place via a number of intermediates. It has been an object of discussion in which way aggregates are formed.

Lorenzo and Yanker (1994) were able to show that a specific aggregation state is necessary for toxicity of the amyloid in Alzheimer's disease (AD). An amorphous form of β-amyloid aggregates did not lead to neurotoxic effects in a rat cell culture model. The amorphous aggregates were regarded as analogous to diffuse plaques. A fibrillar form analogous to AD compact plaques of the amyloid was found to mediate neuronal loss of synapses. Synapse number was not influenced by the amorphous β-amyloid. Furthermore, fibril-binding dye (Congo red) prevented the formation of fibrils and, consequently, neurotoxicity. Both forms of the amyloid are known to be present in human AD though only one form of fibrillar aggregation elicits toxic effects. This indicates that structure of aggregates should be of importance for the mechanism leading to synapse loss and ensuing neuronal death.

The results of this study suggested that fibril formation is necessary for neurotoxicity in amyloidoses. Nevertheless there has been increasing experimental evidence that not only full-formed fibrils are toxic to cells but that pre-fibrillar intermediates have even greater neurotoxic properties (Stefani and Dobson, 2003). Supramolecular organisation of the amyloids was found to be an important factor in this context. Cytotoxicity was higher for rapidly–formed aggregates that did not possess fibrillar structure compared to organised fibrillised aggregates. Mature fibrils appeared to have no toxic effects on cells. The more pronounced toxicity of pre-fibrillar aggregates might be due to the better accessibility of certain protein regions. Hydrophobic side chains and putative sites of interaction are supposed to be buried in the native state and also in fibrillar structures. Mature fibrils seem to be more resistant to protein degradation which also indicates a lower ability to interact with other molecules. Protofibrils might thus be more appropriate to interact with cell components because of the disordered nature of their surface. Exposed groups of amino acids on the protein surface may more easily form interaction sites in a combinatorial way. Binding partners or receptors of a wider range of molecules might thus be more eligible for interaction.

Results of several studies suggest that the neurotoxic effects of aggregated proteins are caused by direct interaction between these structures and cellular components (Stefani and Dobson, 2003). An experimental study by Thomas *et al.* (1996) demonstrated that β-amyloid can interact with endothelial cells in blood vessels. Moreover, free oxygen radicals probably mediated the detrimental effects of the aggregates. Slight endothelial damage was thus prevented by the presence of

superoxide dismutase (SOD). β-amyloid aggregates might therefore interfere negatively with the oxidative stress response of cells. Apart from the possibility of direct interaction it is not to be excluded that major accumulation of protein aggregates itself might cause neurodegeneration. A low clearance rate of the aggregates might be responsible for the occurrence of clinical symptoms.
Another aspect of protein aggregation is loss of function of the initially correctly folded proteins. In the case of prion diseases, the exact function of PrP^C has yet to be discovered but an involvement in cellular stress response has been proposed. Thus a large number of misfolded proteins might not only have negative effects by their structural state but also by the failure to provide protective cell reaction.
Pre-fibrillar structures are known to interact with cell membranes (Hirakura *et al.*, 2002), which supposedly leads to destabilisation and inhibition of membrane-associated protein function (Zhu *et al.*, 2000). One mechanism by which interaction between protofibrils and cell membranes might take place would be comparable to that of bacterial toxins. Oligomerisation of these toxins takes place in the membrane leading to the establishment of pore-like structures that destabilise the bilayer and hamper ion balance. This so-called “channel hypothesis” predicts that a pre-fibrillar aggregate could interact with the membrane in a two-step mechanism (Kourie and Henry, 2002). Electrostatic interaction of complementary charged residues would be followed by insertion of hydrophobic aggregate regions into the membrane interior. The defective folding of the protein could, according to the hypothesis, have cytotoxic effects. By exposure of hydrophobic areas, interaction with the bilayer might lead to the formation of pore or channel structures described for a range of proteins, among them β-amyloid (Zhu *et al.*, 2000). Oxidative stress may furthermore favour the impairment of ion balance between cell and extracellular space. This does not exclude the possibility that oxidative stress itself could have cytotoxic effects by damaging proteins.
Protective mechanisms exist in order to prevent detrimental effects elicited by protein misfolding, e.g. proteasome degradation and detoxification by heat-shock proteins. Possibly there is a certain binding capacity of molecular chaperones. If this capacity were to be exceeded a “chaperone overload” would allow the accumulation of the defective proteins and, finally, their accumulation. Indeed, abnormal expression of molecular chaperones has been observed in the brains of AD patients. Therefore defence mechanisms against protein misfolding may be modulated through

mutations in the encoding genes. Genetic factors thus may have an influence on the ability of these mechanisms to prevent accumulation and aggregation of proteins and the toxic effects of the resulting protein structures on cells.

Influence of Genetic Mutations

In order to investigate the fundamental influence of genetic mutations on amyloid formation, the *in vitro* aggregation process of muscle acylphosphatase (Acp) was studied by Chiti *et al.* (2002). Mutant proteins carrying amino acid substitution were designed. It was taken into consideration that conformation (α-helixes or β-sheets) and hydrophobicity of the protein was not to be disturbed by these substitutions, i.e. they were to lie in charged or hydrophilic regions. Unfolding and aggregation of the mutant proteins were monitored in the study. Conformational stability was decreased but not to a considerable extend when compared to the destabilising effect of substitutions in hydrophobic regions (Chiti *et al.*, 1999). Concerning protein aggregation, modification of net charge through mutations leads to altered aggregation rate. In particular, mutations that increased overall net charge of the protein slowed the aggregation process, while mutations that reduced the net charge favoured aggregation. The net charge thereby is not restricted to local regions as is observed in hydrophobic interactions and secondary structural implications. Consequently, changes in aggregation rate rather resulted from overall net charge alteration than charge of specific areas of the amino acid chain. Nevertheless, a range of mutations found in human amyloid diseases reduce protein net charge which implicates that such alterations may be of importance in at least some amyloidoses.

To explore the effects of mutations in human PrP^C on structural conversion Vanik and Surewicz (2002) designed a recombinant protein variant carrying a mutation at residue 198. The substitution of Phe by Ser (F198S) is associated with Gerstmann-Sträussler-Scheinker disease in humans. The mutant protein exhibited considerably reduced thermodynamic stability at different pH levels. When conformational conversion to β-sheet-rich structure was induced by acidic environment, the transition of the protein carrying the substitution was about 50 times faster than that observed for a wildtype control. Furthermore, the F198S form of PrP^C was more unstable than the wildtype and tended to undergo spontaneous conversion to mainly β-sheet

structure. The mutant form was also found to display increased resistance to proteinase K treatment. These findings indicate that partial destabilisation by amino acid substitutions is an important factor influencing the conformational transition to β-sheet-rich oligomers. Partial instability caused by such mutations seems to be critical for spontaneous conversion, an effect that appears to favour PrP^{Sc}-induced transition of PrP^{C} in cell-free assays (Kocisko *et al.*, 1994).

One of the models proposed for aggregate formation suggests that aggregation starts with an initial seed of a misfolded monomeric protein to which further monomers are attached. This continuous addition of correctly folded monomers, which misfold when attached to the existing aggregate, leads to further growth of the complex structure (Mobley *et al.*, 2003). It is also not to be excluded that protein aggregation is accomplished via several intermediates. In such a model, independent intermediates that are themselves not misfolded attach to one other. The misfolding step is only taking place in case a misfolded seed is presented or if intermediates are joined together to form a larger aggregate. Mobley *et al.* (2003) explored both models under premise of two-dimensional (also called areal) aggregation of prion proteins. In their study monomeric growth, as proposed in the first model, would take place too slowly when compared to experimental data on incubation times. Therefore aggregation via intermediates was considered more probable.

There are nevertheless other models of the PrP^{Sc} structure than the two-dimensional one. Electron crystallography analysis by Wille *et al.* (2002) led to the construction of a β-helix model in which two of these structures exist in a parallel orientation. Together they form the key element of the aggregation process. Crystals of N-terminally truncated PrP^{Sc} (PrP^{27-30}) and a miniprion termed $PrP^{Sc}106$ were prepared and analysed by electron microscopy. Image maps of both proteins were compared to localise N-linked sugars. Afterwards the parallel β-helix model was designed in order to meet requirements like the high β-sheet content, dimensions of the protein monomers and location of the sugars. Parallel orientation of fibril had beforehand been investigated in connection with AD. In AD β-amyloid is produced during the course of the illness. Antzutkin *et al.* (2000) analysed fibrils formed by the β-amyloid peptide by multiple quantum solid-state NMR (MQNMR). A parallel orientation of fibrils was consistent with data derived from MQNMR data and successive simulations.

In order to investigate the formation of putative intermediates in PrP^{Sc} fibrillisation this process was simulated by means of Syrian hamster PrP^{C} containing a D147N mutation (DeMarco and Daggett, 2004). As the pH level was decreased the proteins increased in β-structure while the helical structure was reduced. A new extended region manifested itself in the protein structure. This increase in extended structure was located at the N-terminus of the protein. The area was unstructured in original PrP^{C} but adopted β-sheet structure during the simulation. The conversion process led to an increase of hydrophobic residues in the protein. According to the study protofibrillisation includes the attachment of further monomers to an already existing aggregate forming a spiralling protofibril (see Figure 4).

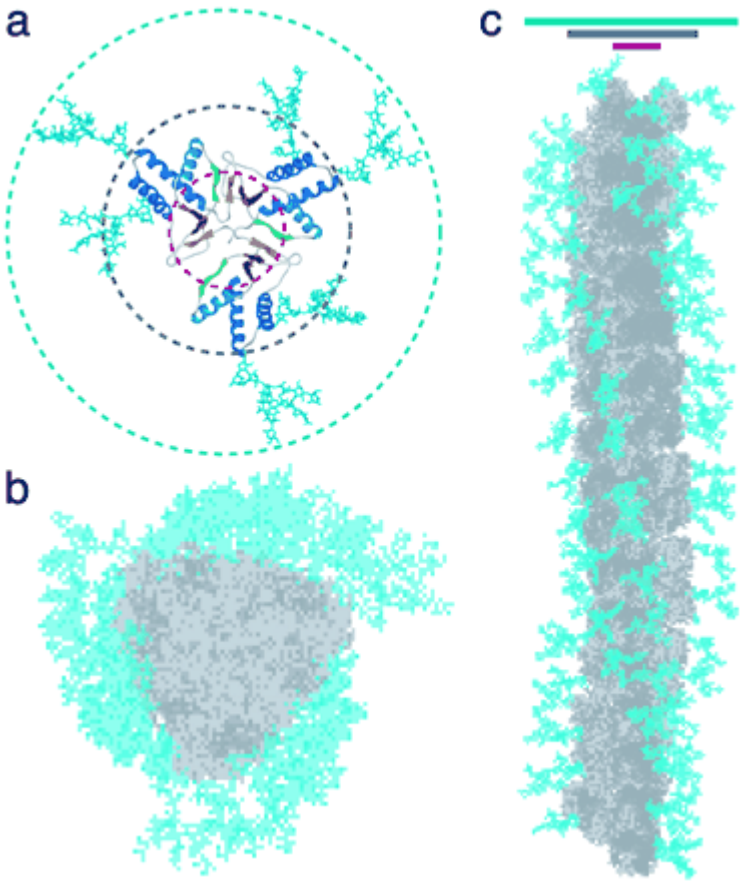

Figure 4: Dimensions of PrP protofibril model and higher-order oligomers

(*a*) A diglycosylated PrP^{Sc}-like trimer with circumferences (dashed circles) of the β-extended core (magenta), all protein atoms (grey), and the diglycosylated protofibril (cyan).
(*b*) Same view as in (*a)* of a 48-mer protofibril with the protein surface shown in grey and the sugars shown in cyan.
(*c*) Side view of a 48-mer protofibril. Bars at the top indicate diameters of the 35-Å extended β-core (magenta), 65-Å protein diameter (grey), and a 110-Å diglycosylated protofibril (cyan).

Specific regions of PrP^{C} have been implied in stabilisation and conversion of its structure. Particular emphasis has been assigned to the role of PrP^{C} helix 1 which is located between residues 144 and 153. Hydrophobic regions in both α-helices and β-sheet structures can interact with the core domain in proteins influencing stability of their secondary structure (Minor and Kim, 1996). Surprisingly, helix 1 of PrP^{C} considerably differs from this concept. It exclusively contains hydrophilic residues, which impairs its ability to interact with the core structure. The helix itself derives its stability from two internal salt bridges between amino acids Asp and Arg but forms no

external bridges with other regions of the protein. A favourable ordering of charges seems to interact with the dipole moment of the helix providing additional stabilisation. Morrissey and Shakhnovich (1999) proposed the so-called β-nucleation model in order to fit these unusual properties of PrP^{C} helix 1 into an aggregation model. Accordingly, unravelling of the helix leads to conversion to PrP^{Sc}. Residues of helix 1 are then added to the core β-sheet aggregate which is parallel. In the model this mechanism is supposed to be catalysed by PrP^{Sc} and stability of the aggregate upheld by salt bridges between helix 1 residues and other protein molecules. The importance of helix 1 in the conversion process was further underlined by a cell culture experiment by Vorberg *et al.* (2001). PrP^{C} mutants in which the first or second β-strand and the α-helix 1 was deleted were supposed to be converted to PrP^{Sc}. Contrary to the deletion of the first β-strand, the removal of both the second β-strand and helix 1 resulted in different processing and cellular localisation of the protein in comparison to wildtype. All mutants considerably impaired conversion in a cell culture system, while the removal of helix 1 led to complete inhibition of PrP^{Sc} conversion. In order to further investigate the role of PrP^{C} helix 1 in this process Speare *et al.* (2003) created mutant forms of PrP^{C}. The aspartic acids at residues 144 and 147 were replaced, so that salt bridges formed in these areas would be removed. No difference was observed between the mutant and wildtype in a cell-free conversion assay. Consequently, salt bridges do not appear to considerably stabilise helix 1 of PrP^{C}. Thermal denaturation of the proteins nevertheless revealed differences between the PrP^{C} variants. Comparison of thermal unfolding curves of mutant and wildtype proteins suggested that mutants unfold differently and more reluctantly. This was also true concerning states of denaturation and reversibility of the reaction, which was not observed for the wildtype protein. Cell-free experiments demonstrated that aspartic acids at position 144 and 147 do not play an essential role for conversion. When conversion was tested without detergents and denaturants mutant PrP performed the process with up to 4-fold higher efficiency compared to the wildtype. In conclusion, though helix 1 salt bridges have no intrinsically stabilising effects on PrP^{C}, its aspartic acids might prevent the wildtype protein from converting to PrP^{Sc}. Ziegler *et al.* (2003) showed by CD and NMR studies of mammalian prion proteins that the helix 1 region favours the helical state even under varying conditions. Salt content and pH level can be altered and organic co-solvents supplied without loss of helicity. Consequently, extension of the helix 1 region does not seem

to be the initial step in the conversion from the wildtype PrP^{C} to PrP^{Sc} but more likely prevents conversion processes.

Another region that was implicated in the aggregation process of PrP^{C} is the copper-binding octapeptide region. Zahn (2003) conducted NMR and dynamic light-scattering experiments in recombinant human PrP(23-230) as a model for natural PrP^{C}. NMR data indicated that binding sites, which are involved in the aggregation of hPrP(23-230), are contained within the octapeptide region (residues 60-91). These *in vitro* experiments demonstrated that the aggregation states of PrP(23-230) and (assuming similar behaviour *in vivo*) PrP^{C} are pH-dependent. The protein aggregation appears nevertheless to be due to homo-oligomeric interaction at the site of the octarepeats. Thus the octapeptide region might modulate aggregation in copper-dependent conformational change.

There exists a range of areas that appear to play a role in the process of conformational shift and aggregation of PrP^{C}. Fibril formation might furthermore be influenced by glycosylation of the protein. Unglycosylated and glycosylated fragments (residues 175-195) from helix 2 of the human PrP^{C} were prepared in a study by Bosques and Imperiali (2003). The fragment encompassed a glycosylation site (Asn-181) and a cysteine at residue 179. When dissoluted, both forms of the peptide showed random coil structure, which after incubation changed to aggregated β-sheets. In a reducing environment fibril formation was favoured for both peptides though the glycosylated form was found to be more stable. Thus N-linked glycosylation appears to reduce the rate of fibrillisation. Inhibition of this process might be caused by alteration of Cys-179 redox properties. As a consequence the peptide would be stabilised as a homodimer linked by a disulphide bond, which showed considerable resistance to form fibrils. The role of Cys-179 in this context was further investigated by the construction of C179S, a mutant protein of the unglycosylated form. A high liability to aggregation was observed for the peptide, which implicates Cys-179 in fibril formation. In the native PrP^{C} part of residues 175-195 is shielded by helix 3, a conformational state stabilised by disulphide bonding at Cys-179. Reducing environment leads to exposition of the area and consequently to fibrillisation which effect assigns an important role to the disulphide bond. The glycosylation of the protein could in different ways be influential for the forming of fibrils, e.g. steric effects could impair intermolecular interaction necessary for

fibrillisation. Otherwise, oxidation potential of the cysteine residue could be altered which might lead to formation of a cysteine dimer instead of fibrils.
Taken together, the ability to aggregate seems to be a generic property of proteins. Even proteins that are not associated with disease can form structures that impair cell function. Furthermore, the substantial toxic effects seem to be caused by pre-fibrillar states that may interact with the cell membrane, possibly involving oxidative stress. The cellular protective mechanisms against protein misfolding seem to be overwhelmed in the amyloidoses, which might be also due to genetic factors.
There are several models proposed for fibril structures in prion diseases. Some evidence in favour of the existence of parallel β-helices has been put forward although other models could also be feasible. It has been suggested that specific regions of the protein e.g. helix 1 play a special role in the conversion process by exceptional stabilising properties. Though this has not yet been clarified, the existence of salt bridges or disulphide bonds within the protein or between several molecules may influence the liability to convert conformation state. In this context modulation of the conversion process by protein glycosylation has been mentioned. It seems to be likely that a number of factors such as amino acid sequence, stability of α-helix structures and glycosylation determine the intrinsic liability of PrP^C to convert to a structure rich in β-sheet. The presence and action of molecular chaperones and cellular defence mechanisms might then modify accumulation and aggregation of the misfolded proteins. Aggregation process and the formation of fibrillar structures together with oxidative stress and loss of function may then lead to cytotoxic effects, formation of stable aggregates and cell death through apoptosis.

Influence of Genetic Polymorphisms on PrP^C conversion

The existence of a species barrier implies that genetic factors of the host, in particular those that determine PrP^C properties, have an impact on disease pathogenesis and susceptibility. Moreover, the conformational transition could also be influenced by the genetic disposition of the host.
Experimental studies confirmed that polymorphisms within the human prion protein gene (*PRNP*) interfere with PrP^C conformation. Petchanikow *et al.* (2001) carried out dichronism studies of human PrP^C fragments polymorphic at residue 129. In acidic environment the PrP^C fragments (PrP 109-139) with either methionine or valine at

residue 129 both showed β-sheet, β-turn and unordered structures. The methionine-containing peptide PrP109-136M had more β-sheet structures and less random coils than the valine-comprising PrP109-136V. This constitutes a significantly different content of ordered and unordered structural properties between both PrP^{C} isoforms. Furthermore, amyloid fibrils can be formed by both PrP109-136M and PrP109-136V. The former peptide produces a much higher amount of amyloid than the latter. Parchi *et al.* (2000) found an additional effect of the same polymorphism. Analysis of the proteinase K cleavage pattern resulted in the identification of different structural regions in human PrP^{Sc}. An important role was consequently attributed to the polymorphism at codon 129 as to regulation of the extent of β-sheet transformation in PrP^{Sc}.

Studies aimed at determining the structure of Syrian hamster PrP^{C} in different pH values showed that a different polymorphism at Asp-178 decreases the conformational change from PrP^{C} to PrP^{Sc}. In case Asp-178 is protonated, interaction with Tyr-128 is impaired and the conversion to PrP^{Sc} is favoured (Alonso *et al.*, 2001). This is especially notable, as a mutation to Asn-178 appears to be the cause of CJD or FFI in humans as well. Phenotypic expression of these medical disorders is nevertheless controlled by a polymorphism at codon 129 of the human prion protein gene (Goldfarb *et al.*, 1994).

In sheep there exist different allelic variants of PrP^{C} and correlation between PrP^{C} genotype and prion disease susceptibility phenotype can be observed. Rezaei *et al.* (2002) investigated the conformation of various PrP^{C} forms. Susceptibility variants of the ovine PrP^{C} exhibited higher thermal stability than resistance variants, and the structure of their unfolding intermediates also differed. It was possible to demonstrate that resistance variants when unfolded showed coil structure whereas susceptible variants on their part possessed β-sheet structure. The rate of unfolding is considerably different between the ovine PrP^{C} allelotypes. Thus polymorphisms in the prion protein amino acid sequence can have an important influence regarding the susceptibility to disease, at least in some species.

Routes of Infection and Pathogenesis

The ability of prion diseases to cross species barriers was highlighted by the occurrence of a new variant of CJD (vCJD) in the UK (Will *et al.*, 1996). It quickly emerged that the neuropathological features of the new variant CJD differed from hitherto known human prion diseases like sporadic CJD with regard to plaque type and in the comparatively early onset of disease. The examined lesions moreover closely resembled the florid plaques seen in brains of scrapie-infected sheep. It was demonstrated that transgenic mice expressing the bovine PrP^C easily succumbed to vCJD while no difference in clinical symptoms could be observed (Scott *et al.*, 1999). Serially passaged BSE and vCJD inoculation led to the same neuropathological features regarding spongiform degeneration, plaque type and localisation. These findings indicate that it was possible for vCJD to be transmitted from BSE-infected cattle to humans. The hypothesis implying that the causing agent might be the same as in BSE was further corroborated by mouse experiments in which vCJD was transmitted (Bruce *et al.*, 1997). Neuropathological features and lesion profiles were strikingly similar to those manifesting in mice infected with BSE but differed from those called forth by other prion diseases such as natural scrapie. Despite of this evidence that BSE had crossed the species barrier from cattle to humans it was not clear by which route of infection the causing agent had been transmitted from one species to the other. In laboratory studies of prion diseases the infectious agent is usually inoculated into the animal brain. Under natural conditions, however, infection must take place through a peripheral route. Aside from intravenous injection and corneal grafts it is possible to induce prion diseases through oral administration of infectious feed. In order to investigate the circumstances of transmission, calves were orally dosed with BSE brain homogenate. They consequently succumbed to disease, which demonstrated the efficiency of the oral route of infection (Wells *et al.*, 1998). Prion infectivity following oral challenge is normally detected first in the gastrointestinal tract. Membranous epithelial cells (M-cells) in Peyer's patches have already been identified as candidates for transepithelial transport of prions from the intestinal lumen into the intra-epithelial pockets. These spaces contain lymphocytes and dendritic cells (Neutra *et al.*, 1996, Aguzzi *et al.*, 2003). Lymphocytes appear to be involved in the pathogenesis but are not necessarily the main means of transport of infectivity (Aguzzi *et al.*, 2003). During the course of disease prions can be

detected in lymphatic organs like the spleen, particularly in follicular dendritic cells (FDCs). At an even later stage prions are found along peripheral nerves in a PrP^C-dependent fashion. Infectivity seems to route through the spinal cord prior to attaining the CNS. To date it is not known whether prions can reach sympathetic nerve endings directly from FDCs.

The actual transport from the lymphatic to the central nervous system is yet unclear. Brandner *et al.* (1996) inoculated scrapie prions both intracerebrally and intraocularly into PrP-knockout mice that had been grafted with neuroectoderm of PrP-overexpressing embryos. Intracerebrally infected mice readily succumbed to disease showing typical scrapie pathology along the optic nerve, then generalised pathology and death. The intraocularly challenged mice developed no signs of disease in the graft. As grafts gave rise to an immune response that might have influenced the prion spread the same procedure was carried out with immunotolerant knockout mice. No signs of disease were observed in the immunotolerant animals. Thus it could be shown that the presence PrP^C is indispensable for CNS prion propagation.

Transmission and pathogenesis of TSEs appears to be largely dependent on the expression of PrP^C in certain tissues, especially in the gastrointestinal tract. The nature of the cells that are responsible for the invasion of the lymphatic system from the gut lumen is yet unclear. It is nevertheless likely that prion pathogenesis is the result of a chain of PrP^C-expressing cells leading from the intestine to the CNS from where widespread prion propagation can occur.

Prion Diseases in Humans and Genetic Implications

The human PrP^C is encoded by a single gene (*PRNP*) on chromosome 20, which possesses two exons, of which one comprises the complete open reading frame (ORF) of the gene. The precursor of human PrP^C consists of an amino acid chain of 253 acids. In NMR structure studies of recombinant human PrP^C it was possible to determine the globular domain, which extends from residue 125 to 228 and comprises three α-helices at residues 144-154, 173-194 and 200-228. The β-sheet extends from residues 128-131 and 161-164 (Zahn *et al.*, 2000).

To generate the mature PrP^C the first 22 amino acids are cleaved off the precursor (Collins *et al.*, 2004). Glycosylation sites that have been mentioned above are positioned at amino acids 181 and 197, respectively. One N-terminal copper–binding

octarepeat region of PrP^C was found to consist of a nonapeptide that is positioned between residues 51 and 91. A second site was detected between residues 96 and 111 (Jackson *et al.*, 2001), which includes binding sites for metal ions, preferably for copper.

The basic structure of human PrP^C is similar to that of other mammalian prion proteins. It comprises three alpha-helices and a short ß-sheet plus a flexible N-terminal tail. In human beings PrP^C is expressed throughout the body, where the highest titers are found in neuronal tissues (Martins and Brentani, 2002).

A number of neurological disorders have been described in humans that are linked to certain conformational changes of PrP^C. With the help of phenotypic characteristics they can roughly be distinguished into sporadic, familial and acquired diseases. It is important to note that there is no possibility to absolutely classify human TSEs. Terms like sCJD or vCJD are employed to describe a certain spectrum of both clinical symptoms and neuropathological data. Untypical cases occur in all human TSEs, which hampers a strict definition of each syndrome.

The pronounced heterogeneity in human prion disease led to the specification of a group of determinants that are applied as tools for diagnosis. Criteria are primarily epidemiological factors (age at onset and duration of disease), genetic disposition (codon 129 genotype and/or causative *PRNP* mutations), clinical presentation and neurological and immunohistochemical findings. Clinical presentation is mainly focussed on the presence or absence of cognitive symptoms/dementia, ataxia and cerebellar symptoms, psychiatric symptoms, visual and sensory symptoms, and sleep disorder. Further diagnostic tools are electroencephalography (EEG) tests, magnetic resonance imaging (MRI) examination and the detection of 14-3-3 proteins in the cerebrospinal fluid (CSF). Neuropathologic criteria are primarily vacuolisation, PrP^{Sc} accumulation and astrogliosis as well as loss of neurons in certain tissues and areas of the CNS. Classification of vacuoles and PrP^{Sc} plaques according to morphology is also important. Location and degree of these neuropathologic changes in the CNS furthermore have to be considered. Western blotting techniques and PrP immunostaining are employed in these analyses. With the help of data available for these different disease characteristics a diagnosis is possible under the premise that not all of the symptoms are recognised in a patient. It is furthermore necessary to incorporate the temporal pattern in which the clinical signs manifest themselves. The same symptoms can appear in different forms of CJD, only in a deviant chronology.

Sporadic CJD

Sporadic Creutzfeldt-Jakob Disease (sCJD) is observed in about 0.5 to 1 case per million human beings world-wide (Belay, 1999). The first clinical features are headache or dizziness but also involve psychiatric symptoms such as depression, anxiety, behavioural change and emotional imbalance (Beisel and Morens, 2004). Furthermore, impairment of cognitive and visual functions can occur. Later on progressive dementia and myoclonic contractions in muscles can be observed before ataxia, dysarthria and delirium lead to coma and finally death. EEG is usually normal or non-specifically abnormal in the first stages of disease. As it progresses, periodic waves of 1 Hz frequency with high amplitude appear over a diminished background. Another sign of sCJD can be high-amplitude delta waves in an irregular pattern (Beisel and Morens, 2004).

Epidemiologically, the distribution of sCJD cases does not show a specific geographical or temporal pattern, which is contrary to what is seen in other human prion diseases, especially in vCJD (Beisel and Morens, 2004).

Phenotypes observed in patients with sCJD vary considerably, depending on their genetic predisposition and strain type. Predisposition and phenotypic appearance of the disease are mainly thought to be determined by a polymorphic site at codon 129 of the prion protein gene (Palmer *et al.*, 1991). At this position either methionine or valine can be encoded. Homozygosity for methionine seems to favour the conversion of PrP^{C} in humans. In about 80% of sCJD patients in Europe this homozygous genotype has been encountered (Ironside, 1998).

Two PrP^{Sc} types (type 1 and type 2) can be distinguished after proteolytic digestion and Western blotting. Together with the genotype at codon 129, six distinct subtypes of sCJD were proposed by Gambetti *et al.* (2003). They have been termed sCJDMM1/sCJDMV1, sCJDVV2, sCJDMV2, sCJDMM2, sCJDVV1 and sporadic fatal insomnia (sFI). Clinical symptoms as well as histopathologic properties were seen to vary between those subtypes. Apparently, there is a preferred interaction between codon 129 genotype and PrP^{Sc} type.

The majority (95%) of M129M sCJD cases succumb to the PrP^{Sc} 1 type in contrast to 86% of M129V and V129V sCJD victims who posses the PrP^{Sc} type 2 (Parchi *et al.*, 1996, 1999). After proteinase K treatment two cleavage sites at residues 82 and 97 of the prion can be observed which led to distinction of PrP^{Sc} type 1 and type 2. In

addition to these primary fragments there are groups of secondary fragments starting at different residues (Parchi *et al.*, 2000). The cleavage sites of the secondary fragments are associated with PrP^{Sc} type and codon 129 genotype. A smaller PK-resistant PrP^{Sc} fragment of either 12 or 13 kDa has also been identified but the effect on disease phenotype is yet unclear (Zou *et al.*, 2003).

The most common sCJD phenotype – also called “classical” CJD, myoclonic CJD or Heidenhain’s variant phenotype - is found in M129M or M129V patients with PrP^{Sc} type 1 (Parchi *et al.*, 1999) (sCJDMM1 and sCJDMV1). For sCJD subtype 1 initial clinical symptoms of the disease can be observed at a mean age of 65 years (range 42-91 years). Duration is rather short with a mean of 4 months (range 1-18 months) (Parchi *et al*., 1999). A typical clinical sign in the early phase is cognitive impairment. Later on, ataxia, psychiatric and visual defects emerge. Heterozygous patients preferably show ataxia compared to cognitive distortion. As the illness advances symptoms become more severe leading to terminal coma and death (Parchi *et al*., 1999). Homogeneously distributed spongiform change is seen in the brain along with astrogliosis, loss of neurons and fine vacuoles. Distribution of proteinase-resistant PrP^{Sc} follows a punctuate (so-called synaptic) pattern.

Subtype 2 (sCJDVV2) is also called ataxic variant of sCJD. The epidemiological data for this subtype is similar to those of subtype 1. Yet, the most striking feature is ataxic impairment with cognitive defects while myoclonus is seldom observed. Spongiosis, astrocytic gliosis and neuronal loss are much like in subtype 1 only differing in the distribution within brain compartments. Contrary to the more common subtype 1, PrP^{Sc} aggregates reminiscent of plaques are visible and PrP^{Sc} immunostaining is mainly observed in the basal ganglia and thalamus.

Other sCJD subtypes observed in humans each constitute less than 10% of reported sCJD cases. Subtype 3 (sCJDMV2) shows considerable phenotypic similarity to subtype 2 regarding age of onset, clinical and histopathologic features. Nevertheless, duration is much longer than in subtype 2 sCJD (17 months with a range of 5-72 months). The predominant clinical symptom is ataxia but also cognitive and psychiatric impairment, myoclonus, aphasia and aphraxia, which sharply contrasts from sCJDVV2. Histopathologic differences are the presence of kuru plaques in the cerebellum and comparatively coarse spongiosis.

The dominant features of subtype 4 (sCJDMM2) are cognitive defects and, to a lesser extent, aphasia. Typically large vacuoles can be observed in the brain, which

are much larger than those seen in subtype 1 and often confluent. Severe astrogliosis is also apparent. Immunostaining is present in the rim of large vacuoles and in spot-like pattern where plaque-like regions are located.
Subtype 5 (sCJDVV1) can only be observed in 1% of sCJD cases. Its outstanding property is the comparatively early onset of disease, which is an average 39 years (range 24-49 years). Characteristic for this subtype is progressive dementia, later accompanied by myoclonus and pyramidal signs. Histopathologically, fine spongiosis, gliosis and, to a lesser extent, neuronal loss can be observed. The distribution of the lesions is considerably different from that exhibited by subtype 1 patients, with cerebellum and thalamus showing only minor spongiform changes.
The sFI subtype of sCJD is generally indistinguishable from fatal familial insomnia (FFI) and has been earlier described as thalamic sCJD. It corresponds to the MM genotype at codon 129 in connection with PrP^{Sc} type 2. Age at onset ranges from 36 to 72 years, with a median of 50 years and a rather long duration of 24 months (range 15-53 months). Ataxia, visual impairment and cognitive defects are among the common clinical symptoms. Furthermore, insomnia, dementia, myoclonus, tremor and dysarthria are apparent. Astrogliosis and neuronal loss concentrate on the thalamus while other brain compartments are much less affected. Spongiform changes are only moderately expressed. The amount of PrP^{Sc} found in sFI is considerably lower than in sCJDMM1. sFI PrP^{Sc} differs from that found in FFI with regard to the glycoform ratio. It more closely resembles PrP^{Sc} that is detected in sCJD subtypes.
It is important to note that both forms of PrP^{Sc} can be present in sCJD patients. In the organism they are either found collocated or separately (Puoti *et al.*, 1999). The phenotype of a patient with both PrP^{Sc} types is determined by the dominance of one of the PrP^{Sc} forms. In patients that are homozygous for methionine at codon 129 this seems to be type 1 while in VV patients type 2 determines the disease phenotype. Accordingly, MM1 and MM2 cases mostly exhibit the MM1 phenotype while VV1 and VV2 patients resemble sCJDVV2 phenotype. Immunostaining techniques are used in order to distinguish these cases of co-occurrence from each other.

Familial forms of human TSEs include Familial CJD (fCJD), GSS and FFI, which are predisposed by autosomal dominant mutations within the *PRNP* gene. Humans who possess a susceptible genotype are liable to disease (see Table 3).

Table 3: Variations in the human prion protein gene region (Kovacs *et al.*, 2002)

Polymorphism		Mutation		
Silent	Influential	Point		Insertional
P68P	M129V	P102L	T188A	27bp
A117A	N171S?	P105L	T188K	48bp
G124G	E219K?	A117V	**E196K**	96bp
V161V	24bp deletion?	G131V	F198S	120bp
N173N*		I138M*	**E200K**	144bp
H177H		G142S*	D202N	168bp
T188T*		Y145s	**V203I**	192bp
D202D		Q160s	**R208H**	216bp
Q212Q		D178N-129V	V210I	
R228R		D178N-129M	***E211Q***	
S230S		V180I	***Q212P***	
		V180I + M232R	*Q217R*	
		T183A	**M232R**	
		H187R	M232T	
		T188R	P238S	

Bold indicates CJD phenotype, underlined indicates GSS, *italics* indicate FFI. Others are not categorized, as the published data are insufficient, or findings are unusual to the known disease subtypes. *Referred from: http.//mad-cow-org/prion_point_mutations.html

Familial CJD

The first case of fCJD was reported as early on as 1924 but not until 1930 did Meggendorfer find out that the illness was inherited within families. A range of different polymorphisms in the human *PRNP* gene leads to fCJD. Like the sporadic form of CJD it occurs very seldom, i.e. in about 1 of 10 million humans (Lee *at al.*, 1999). Interestingly, the M/V polymorphism at codon 129 of the gene affects disease phenotype on the allele carrying the causative mutation. The same polymorphic site

on the other allele influences age at onset and duration of fCJD. Phenotypes can be differentiated by the codon 129 genotype in addition to the causative mutation. The denotation of the fCJD phenotype refers to the haplotype of the mutant allele (Gambetti *et al.*, 2003). The most common phenotypes will be characterised in the following.

The pathogenic mutation most often observed in fCJD cases consists of an E200K polymorphism combined with the encoding of methionine at codon 129 of the *PRNP* gene allele (haplotype $CJD^{E200K\text{-}129M}$). It shows a close similarity to sCJDMM1 with an age of onset between 33 and 84 years (mean age 58 years) while the duration ranges between 2 and 41 months (mean duration 6 months). The most striking clinical features are cognitive and mental impairment but also cerebellar and visual abnormalities, myoclonus etc. As the illness progresses, dementia, myoclonus, cerebellar impairment and seizures become apparent (Gambetti *et al.*, 2003). In contrast to sCJD victims, motor and sensory peripheral neuropathy is occurring. Histopathological implications are mainly spongiosis, astrocytic gliosis and neuronal loss. The latter two seem to depend on disease duration. Immunostaining in the brain can usually be seen in a synaptic pattern. The deposition pattern of PrP^{Sc} in the cerebellum appears to be controlled by the codon 129 polymorphism on the normal allele. Thus the synaptic pattern is limited to 129MM patients while plaque-like conformations are observed in 129MV cases (Gambetti *et al.*, 2003). PrP^{Sc} type 1 is found in $CJD^{E200K\text{-}129M}$ subjects.

The $CJD^{E200K\text{-}129V}$ phenotype is rarely observed. PrP^{Sc} in such patients is classified as type 2. This phenotype closely resembles that of sCJDVV2 with ataxia and later myoclonus being the rule. Plaque-like formation of PrP^{Sc} in the cerebellum is typical in $CJD^{E200K\text{-}129V}$ patients.

The haplotype of D178N-129V was confirmed in a number of patients, among them the persons who were originally diagnosed with fCJD at the beginning of the 20^{ieth} century (Gambetti *et al.*, 2003). This haplotype is of special importance as the same mutation is also observed in FFI with the difference that at codon 129 a methionine is encoded for. In $CJD^{D178\text{-}129V}$ patients cognitive impairment in the form of memory loss is pronounced but psychiatric symptoms e.g. depression have also been reported. At a later stage of disease ataxia, dysarthria and aphasia, tremor and myoclonus become apparent. Age of onset varies according to codon 129 polymorphism on the

non-mutant allele. In 129VV subjects the mean age at onset lies at 39 years (range 26-47 years) with mean duration of 14 months (range 9-18 months) compared to 49 years (range 45-56 years) and 27 months (Range 7-51 months) in 129VM subjects. Spongiform change and astrogliosis in the brain is commonly observed in association with neuronal loss. No PrP^{Sc} plaques are found in this phenotype but punctuate immunostaining patterns are predominant where type 1 PrP^{Sc} is present.
Another fCJD haplotype is $CJD^{V210I\text{-}129M}$ in which PrP^{Sc} type 1 is detected. In average it occurs at 59 years of age (range 46-80 years) and has a mean duration of 6 months (range 2 to 24 months). At first, clinical signs like memory and gait impairment combined with behavioural change present themselves, later leading to defects in sensory and motor function. Other symptoms such as myoclonus, dysarthria and cerebellar impairment follow. The main characteristic in histopathology is spongiosis and gliosis of the grey matter.
In addition to fCJD stemming from substitutions of single amino acids in PrP^{C} there are also forms that are linked to insertional repeat mutations in the *PRNP* gene (base pair inserts (BPI)). Those mutations can be associated both with 129V or 129M. Taken as a group, subjects show a very unspecific phenotype. Nevertheless they are more distinguishable when classified according to number of repeats. With four repeats or less a duration of an average of 6 months (range 2-14 months) with a mean age of onset of 62 years (52-82 years) can be observed though there are exceptions with a much longer duration of illness (Gambetti *et al.*, 2003). If the patient possesses five additional repeats or more, the average age of onset is lower (32 years with a range of 21 to 61 years) and a much longer duration of onset of 6 years has been reported (range 3 months to more than 19 years). In the first group of patients dementia is rapidly progressing and ataxia, visual impairment and myoclonus is present. In contrast, the second group exhibits a much slower progress of disease associated with mental impairment and cerebellar and pyramidal signs. BPI patients with up to four repeats show the same phenotype observed in classical CJD which contrasts with the second group of subjects in which a wide range of phenotypes is present, either similar to CJD, GSS or to neither (Gambetti *et al.*, 2003). Either type of PrP^{Sc} can be found in patients with such form of BPI. Furthermore, several other rare mutations led to fCJD forms that are hardly encountered. They can be distinguished by usage of clinical characteristics such as PrP^{Sc} type and immunohistopathologic features but will not be described in detail.

Fatal Familial Insomnia

The phenotype of FFI in humans is connected with a specific mutation of PrP^{C}, namely D178N-129M (Kovacs *et al.*, 2002). As mentioned above D178N can also be observed in fCJD cases. Autosomal dominant heritance of the polymorphism typically leads to the onset of the disease at a mean age of 49 years (range 20 to 72 years) (Gambetti *et al*., 2003). Survival time is at a mean of 11 months (±4 months) in 129MM patients and 23 months (±19 months) in 129MV individuals. The most striking clinical symptoms in FFI patients are a thoroughly disturbed circadian cycle including insomnia, sympathetic overactivity, endocrine abnormalities and attention deficits (Collins *et al.*, 2000). Patients may differ regarding these signs (Gambetti *et al.*, 2003). Thus insomnia, myoclonus and autonomic impairment seem to be more pronounced in 129MM patients in comparison to 129MV patients in whom more severe ataxia, dysarthria and seizures are present. In FFI cases prominent neuronal loss and astrogliosis can be seen in the thalamus (Montagna *et al.*, 2003). The presence of PrP^{Sc} deposits is seldom observed. If such are detected, they are located in the molecular layer of the cerebellum and in the subiculum-enthorinal region (Gambetti *et al.*, 2003). Furthermore, very little total PrP^{Sc} is deposited in FFI compared to sporadic CJD (Parchi *et al.*, 1995) and it is very far distributed throughout the brain. In FFI, type 2 PrP^{Sc} is observed exclusively, which is contrasting with the findings in fCJD with the D178N-129V haplotype in which type 1 PrP^{Sc} is present (Montagna *et al.*, 2003). Accordingly, the genetic polymorphism at codon 129 of *PRNP* determines the phenotype resulting from the D178N mutation on the same allele: FFI in case the amino acid is methionin and CJD if it is valine.

Gerstmann-Sträussler-Scheinker Syndrome

The phenotype of GSS is characterised by very slowly progressing cerebellar ataxia (Collins *et al.*, 2000) followed by dementia and spinal cord and tract involvement (Barbanti *et al.*, 1996). Taken as a whole, phenotypic variability is very pronounced, even among members of the same family. Phenotypes may resemble classical GSS, CJD or AD (Barbanti, 1996). The mean time of duration is more than 50 months (Kovacs *et al.*, 2002) with a mean age of onset of about 45 years. Humans succumbing to GSS always exhibit the widespread presence of multicentric amyloid

plaques in the brain (Collins *et al.*, 2000) while spongiosis is not present in all cases (Barbanti *et al.*, 1996). In the Austrian family in which GSS was first described by Gerstmann a polymorphism at codon 102 of the *PRNP* gene was associated with the illness, the same as in a range of other familiar cases (Barbanti *et al.*, 1996). Nevertheless there are a number of other point mutations (e.g. G131V) and an insert mutation of eight or nine extra octarepeats in the *PRNP* gene that can lead to the GSS phenotype in humans. Contrary to the situation in CJD, the codon 129 polymorphism does not seem to have a prominent influence on disease phenotype.

Acquired human TSEs are represented by the syndromes of kuru, iatrogenic CJD (iCJD) and (new) variant CJD (vCJD). Here, the polymorphism at codon 129 is involved in the liability to acquire kuru (Lee *et al.*, 2001), iatrogenic CJD (Collinge *et al.*, 1991) and variant CJD (Ironside, 1998). Humans homozygous for methionine appear to be more susceptible to these forms of prion disease.

Iatrogenic CJD

Iatrogenic CJD is caused through transmission of CJD, often through neurosurgery, corneal grafts and use of human dura mater and human pituitary growth hormone taken from corpses for therapeutic purposes. The first case of iCJD was reported in 1974 in a woman that had received a corneal transplant from a donor that developed CJD (Duffy *et al.*, 1974). In 1985 transmission through pituitary human growth hormone (hGH) was described and by 1988 infection through dura mater grafts had been observed (Thadani *et al*., 1988). Reports of iCJD arising from neurosurgical procedures can be traced as early as 1977 (Bernoulli *et al.*, 1977).
As to clinical symptoms there is evidence that disease phenotype is associated with the route of infection. While patients infected through contaminated corneal grafts, neurosurgery or dura mater transplants develop symptoms similar to those seen in sCJD (except for some atypical cases), transmission through hGH causes quite a different spectrum of clinical signs. Dementia is hardly seen in those cases and initial characteristics involve a so-called cerebellar syndrome (Will, 2003). It has also been suggested that infection via a peripheral route, e.g. through hGH injection, would cause longer incubation times than those infected by dura mater or corneal grafts. Yet there is some contradicting evidence as the longest iCJD incubation period has

been reported in a patient with corneal graft transmission (Lang *et al.*, 1998). Furthermore, iCJD patients that had received peripheral injection of dura mater particles showed no difference to those that had been treated by intracerebral inoculation of dura mater grafts (Lang *et al.*, 1998).

In 2000, Brown *et al.* examined 267 iCJD patients of whom 139 where infected by the donation of contaminated growth hormone and 114 by dura mater grafts. Other infections occurred through transplantation of corneal grafts (3 cases), neurosurgery (5 cases), gonadotropin donation (4 cases) and stereotactic EEG (2 cases). The range of iCJD incubation times was generally very broad, from 18 months to several decades (Will, 2003). As the risk of infection through dura mater grafts was considerably lowered by the invention of recombinant hormones for therapeutic purposes during the mid-1980ies, the majority of patients examined in the study had an incubation time of more than 10 years. The median incubation time among the hGH-infected cases was 12 years (range 5 to 30 years). This is probably dependent on the site of distribution of infectious agent and on the amount of proteinase-resistant PrP (PrP^{res}) transmitted. Low amounts and peripheral distribution, i.e. by subcutaneous injection, can lead to long delays until clinical symptoms can be observed in patients. The question whether codon 129 genotype has an influence on iCJD susceptibility or incubation time cannot be sufficiently answered. Homozygosity for methionine seems to be overrepresented among patients infected by dura mater grafts while among patients infected through hGH valine homozygosity is overproportional (Brown *et al*, 2000). The preference for one phenotype or the other nevertheless may depend on the genotype of the infected donor.

Valuable statistical results are scarce as the time of infection often had to be estimated, e.g. if a patient had received several injections of hGH, the mean of this timespan was declared as the point of infection. This, together with the relatively small size of samples, does not lead to assured statistical results concerning incubation times.

Kuru

Kuru is an acquired human TSE that was first described when an epidemic course of the disease was observed in the Fore tribe of Papua New Guinea. The Fore people that are situated in the Eastern Highlands numbered about 11000 in the 1950ies when the kuru phenomenon came to the attention of Western researchers (Gajdusek and Zigas, 1959). Kuru ("shaking" or "trembling" in the Fore language) was probably spread through endocannibalistic practices. At the death of a close relative a rite of mourning took place that involved the consumption of the deceased person's body parts. Women and children of both sexes mostly executed the ceremonies whereas male adults very seldom took part in the rituals. If they did so, they mainly consumed skeletal muscle, which was less infectious than neuronal tissue. In contrast, women and children consumed highly infectious tissues like the brain and other parts of the CNS. Regarded by the Fore as kind of sorcery, kuru was first thought to be a genetic disorder as it was confined to the area of the Fore people and some bordering tribes. The yearly incidence rate of the disease was about one percent of the population and up to ten percent in certain clans, which argued against a solely genetic cause of kuru. As the disease was of a fatal and very common kind the lethal genotype would soon have died out. Later on, kuru was regarded as some sort of slow virus with an extremely long incubation period (Gajdusek *et al.*, 1967).

It was possible to intracerebrally infect chimpanzees with kuru brain tissue with a resulting incubation time of 1.5 to 2.5 years while the clinical symptoms closely resembled those observed in human kuru victims. Further passage of the disease within the chimpanzee by inoculating animals with infected brain tissue also proved successful. Same-species transmission shortened incubation times considerable to approximately one year, which seemed to be an effect of the species barrier between humans and chimpanzees. The theory of a viral causative agent seemed valid at that time as other TSEs and scrapie were also regarded as disorders caused by a slow virus (Gajdusek *et al.*, 1967, Gajdusek, 1977).

The progress of kuru can be (according to Gajdusek) partitioned in three distinct stages: The ambulant stage, the sedentary stage and the terminal stage of disease. In the beginning the patient is still able to stand up and work. Then first signs of slight ataxia, postural instability and tremor especially in the limbs appear, along with dysarthria, impairment of speech and strabismus (Kompoliti *et al.*, 1999). In the

sedentary stage the kuru victim is no longer able to walk or stand independently. Tremors are more marked and severe while slight mental slowing can be observed. Psychiatric symptoms such as smiling, excessive laughter or crying and emotional instability is apparent. The phenomenon of uncontrollable laughter and euphoric behaviour led to the synonym “laughing sickness” in connection with kuru. In the last stage of the illness kuru victims are no longer able to sit up and exhibit increasing tremor, ataxia and dysarthria. Finally the patient develops complete incontinence and dysphagia from which results death because of the inability to drink and eat (Kompoliti *et al.*, 1999). The usual duration of the clinical course of disease lay between three and nine months (Gajdusek, 1977).

Examination of kuru brains revealed excessive neurodegeneration, together with astrocytic gliosis, myelin degeneration and spongiform vacuolisation (Gajdusek, 1977). Especially notable was the presence of plaques in the cerebellum. The characteristic “kuru-type” plaques measure approximately 30µ in diameter. The central core has a higher density than the surrounding fibrillar structure it is embedded in (Goldfarb, 2002). Microglial proliferation can be observed as well as severe loss and degeneration of Purkinje and granule cells in the cerebellar area.

A particularly specific feature of kuru is its age and sex distribution. The youngest patient examined in the study by Gajdusek and Zigas (1959) was aged between four and five while more than two thirds of the victims (76.5%) were adults. In terms of sex distribution one of the striking features was the predominance of kuru in women. This inequilibrium was not apparent in patients below the age of 20 years. In contrast, the male:female ratio for the age group between 20 and 29 was 1:7.8 . This effect was probably due to the predominance of women and children in cannibalistic ceremonies. Thus children of both sexes could infect themselves through consumption of contaminated food and often died before they reached adulthood. There was also the possibility of kuru inoculation through contact of infectious material with skin, conjunctiva or wounds. The adult participants in endocannibalism were female with a few exceptions. As the epidemic subsided after the official prohibition of cannibalistic rituals in the 1960s, less and less child patients could be observed while the age of onset continuously rose (Liberski and Gajdusek, 1997). Patients with kuru reported in latter years exhibited incubation times of several decades.

Apart from age and sex-dependent features genetic predisposition seems to play an important role in connection with kuru susceptibility. The above-mentioned polymorphism at codon 129 of the *PRNP* gene has a considerable effect. At the beginning of the epidemic, persons with the MM genotype and early contact with infectious tissues, i.e. young children, were the first to succumb to disease. At a later stage, heterozygous persons (MV) and those homozygous for valine (VV) developed kuru, depending on the amount of infectious agent they had consumed and also depending on age of exposure. The circumstances of the epidemic led to a distinct distribution of *PRNP* codon 129 polymorphisms among the Fore as no person with the MM genotype was among the survivors of the original epidemic and the majority (77%) of contemporary survivors (women older than 50 years) possess the heterozygous genotype (Collins *et al.*, 2000). Through this mechanism the MM genotype is nearly extinct in the surviving Fore because of the high incidence of kuru (Lee *et al.*, 2000). In a study by Cervenakova *et al.* (1998) it was shown that when a group <15 years of age was compared to an adult group >30 years, the difference in genotype frequency between codon 129 homozygous methionine kuru victims and heterozygous ones was significant. Homozygosity for methionine is moreover significantly linked to shorter duration of illness. It is also of interest that the neuropathology of heterozygous kuru patients slightly differs from that observed in homozygous patients. The development of the typical kuru amyloid plaques is obvious only in patients with at least one methionine allele at position 129 in contrast to patients homozygous for valine (Cervenakova *et al.*, 1998).

How kuru initially came into existence can be best explained by the occurrence of a case of fatal sCJD among the Fore and the subsequent consumption of infectious CNS tissue by family members (Gajdusek, 1977). Thus the illness would have spread among the tribe.

The insights won in analysing the kuru epidemic have gained a new importance since the emergence of vCJD. Beforehand, kuru had been the only human TSE in which the infectious agent had presumably been transmitted via the oral route. Like observed in kuru, there is also the common predisposition arising from the codon 129 genotype. It is therefore possible that the epidemiology of vCJD may take a similar course as compared to kuru, with more cases occurring as less susceptible genotypes are affected, though at the moment this is in no way predictable.

New Variant CJD

The first case of a new variant of CJD was reported in 1995, ten years after the first report of BSE in cattle (Beisel and Morens, 2004). Typical for the initial presentation of vCJD are psychiatric symptoms like anxiety, depression, behavioural change and memory loss that can be observed in more than half of the patients (60%). Thus psychiatric symptoms are very common in the early stages of vCJD. Other signs of the disease are headache, dizziness, memory loss and disturbances of gait, cognition and sight (Beisel and Morens, 2004). In the later stages neurological symptoms such as dementia, ataxia and dysarthria become predominant that gradually lead to delirium and terminal coma.

PrP plaques and florid plaques (plaques surrounded by vacuoles) can be observed in the brains of vCJD cases (Will *et al.*, 1996), a pattern unusual in sCJD but resembling plaque types seen in scrapie infection. Immunostaining for PrP^{Sc} also revealed an accumulation of small plaques in the brain (Will *et al.*, 1996).

In contrast to classical forms of CJD like sCJD, vCJD has a much earlier age of onset. The first 97 cases of confirmed and probable vCJD had a median age of onset of 28 years (Valleron *et al.*, 2001). Duration of the disease was rather longer than that usually observed in other CJD variants. Median duration ranged from 6 to 39 months (average 13 months) in the first hundred patients (Spencer *et al.*, 2002).

The new variant of CJD has up to now been observed mainly in patients homozygous for methionine at position 129 of the *PRNP* gene, which seems to be the major predisposing factor in vCJD (Will *et al.*, 2000). As seen in iCJD homozygosity itself (either MM or VV) might influence incubation times. The evidence gained from the kuru epidemic in New Guinea indicates that food-borne prion diseases can well lead to incubation periods of several decades. Accordingly it can be speculated that vCJD cases with either heterozygous (MV) or homozygous (VV) *PRNP* genotype will be observed in the future. The recent finding of preclinical vCJD in a *PRNP* codon 129 heterozygous patient that had been infected through blood transfusion suggests that this might be the case (Peden *et al.*, 2004). PrP^{res} was absent in the brain of the patient but was observed in the spleen. Thus vCJD infection, if only subclinical, does not seem to be limited to codon 129 homozygous humans.

As the first occurrence of vCJD in the United Kingdom followed an epidemic of the prion disease BSE in cattle it was soon speculated whether the disease might have crossed the species barrier. In support of this theory glycoform ratio patterns i.e. strain characteristics of vCJD and BSE are very similar to each other (Collinge *et al.*, 1996). Western blotting revealed clearly differing patterns of PK-resistant PrP. In sCJD and iCJD cases, type 1 and 2 have been observed. Type 3 occurred in patients who derived iCJD primarily via peripheral distribution of the infectious agent. This includes injection of contaminated hormones like hGH and gonadotropin. The band pattern of vCJD in Western blots resembles that of type 3 CJD but the band intensities are clearly distinguishable from the other PrP^{Sc} patterns.

There are additional implications in favour of a connection between BSE and vCJD raised by epidemiological factors. The number of sCJD cases diagnosed has only slightly risen over the last years, probably owing to better diagnostic measures and awareness. On the other hand, the number of reported vCJD cases in the UK has increased significantly since the disease was first discovered (Andrews *et al.*, 2000). In Andrew's study the number of vCJD onsets was estimated to increase by 23% per year between 1994 and 2000 and by 33% between 1995 and 2000 as far as probable and definite cases of vCJD were concerned. The cause of this rise in incidence was explainable when the temporal incidence of BSE and mortality of vCJD were compared. With the BSE epidemic seemingly declining, incidence of vCJD was increasing. Currently the number of deaths through vCJD is decreasing in the UK (Andrews *et al.*, 2003), so that a slowing of the trend is apparent. This nevertheless does not point at a future steady decline of vCJD cases because of the genetic implications described above. Since cases of vCJD in other codon 129 genotypes than the one hitherto observed might occur there could be another rise in the number of vCJD deaths. In connection with epidemiological issues it should also be noted that vCJD has hitherto only been diagnosed in countries in which BSE has occurred (Trevitt and Singh, 2003) while the incidence of sCJD shows a worldwide uniformity.

If vCJD was indeed caused by infection through BSE prions the infection would probably have taken place through oral infection with contaminated beef. As the example of kuru shows, such an infection route seems to be realistic. Proteinase-resistant PrP is mainly found in tissues of the central nervous system so that the greatest transmission risk appears to be related to processed meat (Trevitt and

Singh, 2003). Statistical examination revealed that there is a geographical difference in vCJD cases (1994-2000) in the UK. In the North of Great Britain the incidence of vCJD is higher than in the southern part. The rate ratio comparing the north with the south was 1.94 (confidence intervall: 1.27-2.98) for all 84 cases examined (Cousens *et al.*, 2001). It is yet unclear in which way consumption of meat or specific kinds of meat and meat products or slaughtering and cutting techniques have an influence on these numbers. Difficulties arise from evaluating the regional dietary intake of certain products and the possibility of cross-contamination of meat with CNS tissue. Rates of vCJD incidence could thus be correlated with meat consumption on the basis of data from the Household Food Consumption and Expenditure Survey (r=0,72). Correlation was not demonstrated for data recorded in a different survey (Dietary and Nutritional Survey of British Adults).

Apart from infection through infectious agent in meat and meat products it is necessary to address the question whether vCJD could be transmitted through blood transfusion or contamination of neurosurgical instruments. Llewelyn *et al.* (2004) investigated the case of an elderly blood recipient who developed vCJD 6.5 years after treatment. The red cells transmitted had come from a 24-year-old donor who, after 3 years and 4 months past the time of donating blood, succumbed to vCJD. Neuropathology in the recipient was characteristic for the changes seen in vCJD patients. Nevertheless, of the 48 recipients of blood components from different donors examined in the study only one developed vCJD. An alternative theory would be that the recipient was infected through consumption of contaminated meat though this is statistically unlikely (Llewelyn *et al.*, 2004). Experiments in rodents and sheep have furthermore produced evidence that infectivity can be transmitted by blood transfusion even before the donor had completed the incubation period (Houston *et al.*, 2000, Hunter *et al.*, 2002). Whether transmission through blood donation is possible in humans is unclear because little information exists as to the amount of infectivity in blood or blood compartments and to the dose necessary to infect a human being.

Neurosurgery may also bear the risk of transmission of infectious material, a possibility that was highlighted by the occurrence of iCJD after such invasive techniques. Here, as with blood donation, the amount of infectious agent transmitted and the mode of administration (peripheral or intracerebral) should play an important role. That neurosurgical procedures, which involve invasion into lymphoreticular

tissue, pose an increased risk as to vCJD infection has not yet been substantiated. Implicated in these considerations are the distribution and amount of host PrP^{C} throughout the body. In tissues that show high expression levels of PrP^{C} or in organs that are involved in prion propagation administration of infectious agent might lead to early onset of prion disease.

Distribution of PrP^{Sc} in Human Prion Diseases

The cellular form of the prion protein is mainly expressed in the brain and CNS, presumably in nerve endings. It seems to localise both on the presynaptic and postsynaptic side of neurons (Gohel *et al.*, 1999). As it was first detected in neuronal cells of the CNS, the expression of PrP^{C} was thought to be exclusively restricted to neuronal cells. Further investigations demonstrated that PrP^{C} was not only present in nerve cells of the CNS and peripheral nervous system but also in extraneuronal tissues and cells.

Immunogold labelling by Fournier *et al.* (1998) revealed that PrP^{C} is expressed in human lymph nodes, at the plasma membrane of tubule cells in the kidney and in cytoplasmic vesicles.

Like PrP^{C}, the infectious proteinase-resistant form PrP^{Sc} is localised in tissues other than those of the CNS. By analysing extraneural organs of 36 persons that had died of sporadic CJD Glatzel *et al.* (2003) demonstrated that PrP^{Sc} was present in spleen and muscle tissue in a number of patients. PrP^{Sc} amounts in those tissues were considerably lower than PrP^{Sc} levels found in the brain (by factor 1×10^{-4}). It was not possible to detect non-neural PrP^{Sc} by common Western blotting but differential precipitation of the protein was carried out in order to improve sensitivity of the analysis.

The presence of PrP^{Sc} in the sympathetic nervous system of vCJD victims was confirmed by Haik *et al.* (2003) who could detect PrP^{Sc} accumulation in neurons of gut-associated ganglia. Ganglia of the sympathetic nervous system did not harbour PrP^{Sc} in a sCJD case used as a control. These findings suggest involvement of sympathetic nerves in vCJD pathogenesis.

The results of the study also showed that the distribution of PK-resistant PrP^{Sc} is apparently determined by the CJD form. Contrary to the observations in sporadic or familial CJD, a high amount of PrP^{Sc} can be found in the lymphoreticular system in

vCJD. Head *et al.* (2004) were able to detect PrP^{Sc} in tonsils, spleen, lymph nodes and in the submucosa of the appendix in vCJD patients. The presence of extraneural PrP^{Sc} was limited to the tissues of these persons and was not observed in sCJD and iCJD cases. In this study, PrP^{Sc} was detected in adrenal, kidney, lung, heart or muscle, which finding was independent of CJD form. Hill *et al.* (1999) demonstrated that the accumulation of PrP^{Sc} in the lymphoreticular system is a feature characteristic of vCJD. Western blotting examination of tissue samples led to the result that PrP^{Sc} was present in all samples that were eligible for testing. Glycotyping of the PrP^{Sc} detected in the tonsils resulted in the discovery of a glycolysation pattern (type 4t) that was similar but clearly distinguishable from the one commonly found in vCJD-infected brains (type 4). This is probably due to the fact that glycosylation patterns generally vary between cells and tissues. In 2001 Wadsworth *et al.* found that PrP^{Sc} was located in the retina and proximal optic nerve of vCJD patients. Low concentration of the proteinase-resistant protein could be observed in rectum, adrenal gland and thymus of a single case among the four patients included in the study.

Taken together, these results give a hint as to the possible pathology of prion diseases. Peripheral and extraneural accumulation of PrP^{Sc} indicates which tissues might play a role in prion propagation and the mode of infection. Location in stomach and guts points to an oral uptake of the infectious agent. The presence of PrP^{Sc} in the lymphoreticular system seems to indicate peripheral propagation. Whether PrP^{Sc} found in muscle is the result of a spillover after the brain is infected or whether propagation in this tissue can take place at an earlier stage of the disease remains yet unclear.

Genetic implications in PrP^{Sc} distribution were observed in the study by Glatzel *et al*. (2003) relating to the human codon 129 polymorphism. Heterozygous and homozygous VV patients were tendentially overrepresented among patients with splenic PrP^{Sc}. In contrast, persons in whose muscle PrP^{Sc} was detected were in the majority homozygous for methionine. After analysis of PrP^{Sc} isoforms it was shown that the number of rare sCJD variants was above average among the patients examined in the study. The observation of splenic PrP^{Sc} was furthermore connected to a longer duration of the disease, which indicates a spillover of prions from the infected brain to non-neuronal tissues.

Genetic disposition i.e. genotype of the host seems to have an influence on disease pathogenesis resulting in various patterns of PrP^{Sc} distribution in the body. A crucial role has to be assigned to detection techniques for the protein in this context. PrP^{Sc} concentrations in specific tissues might have remained unobserved being present in amounts that lie below the limit of detection.

Mouse Models and Genetic Implications

Knockout Mouse Models

Mouse models are important to explore pathomechanisms of prion diseases. Knockout experiments in this species resulted in the identification of the prion as the probable causative agent of spongiform encephalopathies and of PrP^{C} as prerequisite of infection.
When the first knockout approaches were made, the occurrence of defective murine phenotypes was to be expected, as the unknown PrP^{C} function was expected to be impaired in the animals. Nevertheless, the knockout mice generated by Bueler *et al.* (1992) did not show any abnormalities although no PrP^{C} was detected in their bodies. In the so-called *Zrch* $Prnp^{0/0}$ mouse line a neomycin phosphotransferase (*neo*) gene cassette replaced 552 bp of the *Prnp* gene. In a successive study the same mouse line was challenged with mouse-adapted scrapie prions (Bueler et al., 1993). An intracerebral inoculum of the agent was used. Brains of knockout mice did not exhibit scrapie pathology nor were they differing from those of mice injected with normal brain homogenate. In contrast, wildtype mice presented typical characteristics of prion disease such as vacuolisation, neuronal loss and astrocytic gliosis especially in cortex, thalamus and hippocampus, beginning at 23 to 25 weeks p.i. Furthermore, heterozygous $Prnp^{+/-}$ mice, though contracting scrapie, showed considerably increased incubation times (more than 253 days compared to 180 days in wildtype mice). The impact of PrP^{C} on scrapie infection was mirrored in the duration of the disease: no heterozygous mice had died prior to 322 days post infection, while wildtype animals had died 13 days after the onset of clinical symptoms. In conclusion, the presence of PrP^{C} and the PrP^{C} amount in the body was crucial for prion infection and moreover associated with incubation times in mice.

In other mouse models the inactivation of the *Prnp* gene was accompanied by defects in the knockout mice lines (see Figure 5). Sakaguchi *et al.* (1995) had established a *Prnp*$^{-/-}$ mouse model that was resistant to mouse-adapted CJD infection. Infection of both wildtype and *Prnp* heterozygous mice was achieved and PrP^{res} was detected in their brains, contrary to $Prnp^{0/0}$ animals. Incubation times were ranging from 138 days in wildtype mice to 259 days in heterozygous animals. This mouse line, termed *Ngsk Prnp*$^{0/0}$, was generated by replacing the entire *Prnp* ORF by a resistance cassette. Ensuing observations of the mouse line revealed progressive gait impairment and hind limb ataxia in the animals (Sakaguchi *et al.*, 1996). Atrophy of the cerebellum and dramatic loss of Purkinje cells was observed in their brains. The number of GABA synapses was markedly reduced. Impairment of GABA-mediated synaptic function in *Prnp* knockout mice had already been described by Collinge *et al.* (1994). Changes in circadian rhythm were furthermore reported by Tobler *et al.* (1996). In knockout mice the periods of activity were significantly longer than in the wildtype control animals. Sleep regulation and sleep patterns were different between knockout and wildtype mice, e.g. sleep fragmentation was more distinct in the knockout model.

The question whether these defects were caused by the lack of PrP^{C} in the mice or were simply an artefact of knockout experiments was addressed by Moore *et al.* (1999). They reported the discovery of a protein that shares approximately 25% identity with PrP^{C}, which was termed doppel (Dpl). The encoding gene locus (*Prnd*) is located downstream of the *Prnp* locus. Interestingly, intergenic splicing of *Prnd* and *Prnp* generates chimeric mRNA transcripts. This pathway might be upregulated in *Prnp*$^{0/0}$ mouse lines (*Rcm0* and *Ngsk*) that exhibit progressive ataxia and loss of Purkinje cells. Moore *et al.* (1999) measured Dpl mRNA levels in the brains of different knockout mouse lines and compared the target alleles in the mice. According to this study, *Prnp*$^{0/0}$ mouse lines can be distinguished into two fractions. In the first group the targeted disruption is restricted to *Prnp* exon 3 and consequently no phenotypic abnormalities have been observed. In the other case an exon 3 splice acceptor site is removed together with a flanking region 5' to exon 3. As a result, more chimeric mRNA is produced by the animals of this genotype in comparison to wildtype mice. It was also noted that Dpl expression was considerably higher in defective mouse lines than in the *Zrch* mice. Thus the abnormalities observed in

Ngsk and other *Prnp*$^{0/0}$ mouse lines were apparently caused by Dpl upregulation rather than by absence of PrP^{C}.

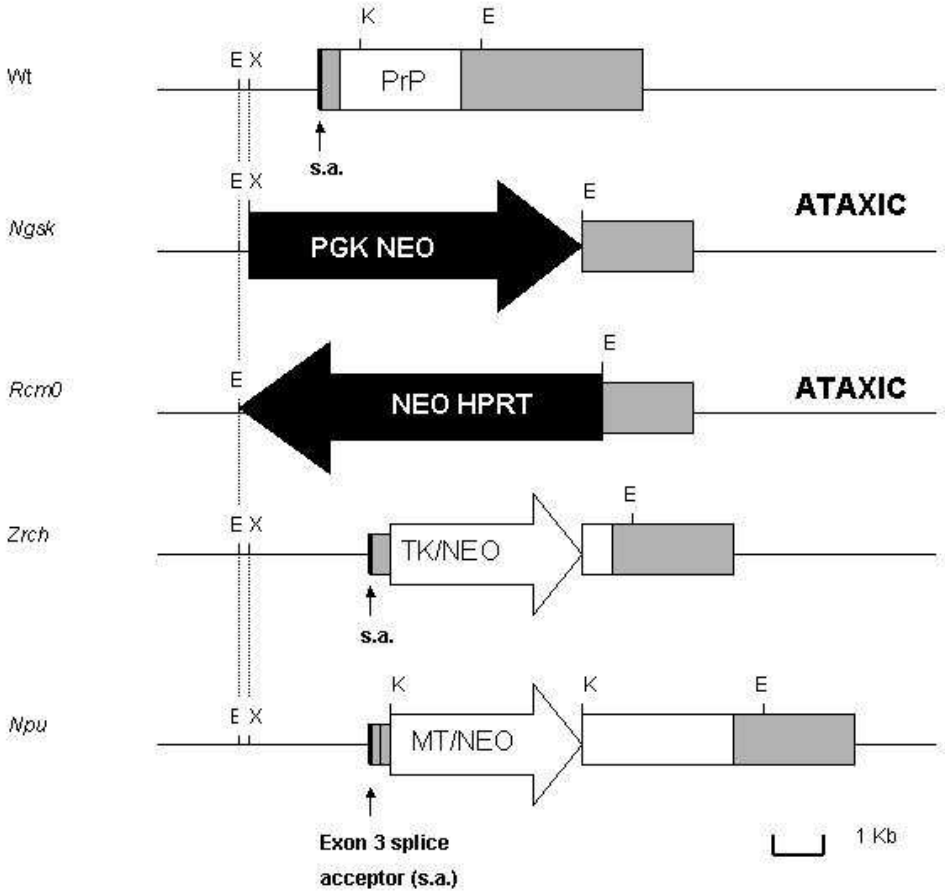

Figure 5: Structures of PrP gene disruptions in four lines of *Prnp*$^{0/0}$ mice (Moore *et al.,* 1999)

Alignment of published PrP knockout alleles showing the genomic region spanning PrP exon 3, the extent of PrP exon 3 deletion, the selectable marker used, and direction of selectable marker transcription (indicated by the large arrow). The structure of the wt *Prnp* allele is shown at the top. The large black arrows indicate the alleles that delete the exon 3 splice acceptor and are associated with the development of a late-onset ataxia. A small vertical arrow indicates the position of the exon 3 splice acceptor (s.a.) deleted in the *Ngsk Prnp*$^{0/0}$ and *Rcm0 Prnp*$^{0/0}$ alleles (Sakaguchi *et al*., 1995*; Moore et al.*, 1995)

Open box: PrP coding region; arrow,: selectable marker; grey box: PrP UTR; E: *Eco*RI; X,: *Xba*I; K,: *Kpn*I. TK: human HSV-1 thymidine kinase promoter; MT: mouse metallothionein promoter; PGK: mouse phosphoglycerate kinase promoter; Neo: neomycin phosphotransferase; HPRT: mouse hypoxanthine phosphoribosyltransferase.

However, a study by Wong *et al.* (2001) found changes in oxidative stress response of *Prnp*$^{0/0}$ knockout mice. The group measured a range of oxidative markers (ubiquitination, protein oxidation, lipid peroxidation and proteasome activity) in *Prnp*-deficient mice as compared to wildtype animals. These indicators of oxidative stress were elevated in brain lysates of the knockout animals. As implied by other experimental studies (Brown *et al.*, 1999, Wong *et al.*, 2001) the findings point to a role of PrP^{C} in cellular oxidative stress response *in vivo*. As a further approach Miele *et al.* (2002) investigated whether absence of PrP^{C} had an effect on gene expression in the brain. With the help of PCR techniques gene expression patterns were generated in *Prnp*-deficient and wildtype mice during brain development. For most genes expression levels were similar in both mouse lines although differences were detected for genes contributing to mitochondrial biogenesis and physiology. The findings were corroborated when mitochondrial morphology was examined by electron microscopy. The amount of mitochondria in hippocampal neuropil was markedly reduced in *Prnp*$^{0/0}$ mice (40%) compared to the wildtype controls. Not only was there a difference in the total number of mitochondria, there were also morphological changes. Significantly more of *Prnp*$^{0/0}$ mitochondria exhibited an unusual morphology with few and poorly outlined cristae, although the majority of mitochondria appeared to be normal. Significant discrepancy was also found in the diameters of the mitochondria, which were 21% larger in knockout mice than in the wildtype. Activity of mitochondrial manganese-dependent superoxide dismutase (MnSOD) was elevated in brains of *Prnp*-deficient mice, which was in accordance with the findings of Brown *et al.* (1998). This group had reported elevated MnSOD levels in the brain of knockout models. Miele *et al.* (2002) also noted that number of mitochondria and MnSOD activity was inversely correlated with PrP^{C} expression in various tissues. Taken together, an association between PrP^{C} expression and amount and physiology of mitochondria has been indicated. This mechanism could be involved in the pathogenesis of prion diseases. A role for PrP^{C} in oxidative stress response has been suggested as in scrapie infection SOD-like activity of PrP^{C} was considerably reduced in the brain (Wong *et al.*, 2001). This might have been a result of PrP^{C} loss of function.

Genetic Implications in Mice

Mouse PrP^C in its mature form contains 208 residues and shares the common characteristics of prion proteins (Riek *et al.*, 1996). It contains three α-helices, a short antiparallel β-sheet and the characteristic octapeptide repeat sequence. The *Prnp* gene encoding the protein comprises three exons with the ORF and two introns (Westaway *et al.*, 1994). It is located on the murine chromosome 2.
After inoculation with prions, incubation times differ dramatically in mice. These differences have been associated with polymorphisms in the murine prion protein gene (*Prnp*) (Carlson *et al.*, 1994, Moore *et al.*, 1998). A dimorphism at codon 108 and 189 of the *Prnp* gene determines whether the murine PrP^C is designated *Prnp-a* or *Prnp-b*. Mice that possess *Prnp-a* (with leucine at position 108, threonine at position 189) show considerably shorter incubation times than those with *Prnp-b* (with phenylalanine at position 108, valine at position 189) (Westaway *et al.*, 1987). Both isoforms also differ in the length of intron 2 (Westaway *et al.*, 1994). *Prnp-b* is characterised by a deletion of about 6 kb within the intron in comparison to *Prnp-a*. Nevertheless the size of intron 2 does not seem to have a considerable impact on experimental scrapie incubation times in mice (Carlson *et al.*, 1988).
In addition to these two alleles (*Prnp-a* and *Prnp-b*) Lloyd *et al.* (2003) identified another murine prion protein gene variant (Prnp-c). It encodes for phenylalanine at position 108 and threonine at residue 189. After inoculating *Prnp-c* mice with mouse-adapted scrapie prions intracerebrally, incubation times were considerably prolonged in the animals. Distinct neuropathologic features such as lesion profiles or pattern of PrP accumulation were not observed in the study.
Stephenson *et al.* (2000) searched for quantitative trait loci (QTLs) that might influence the development of prion disease in mice. Incubation times after inoculation with scrapie prions were analysed in animals carrying the *Prnp-a* allele. Marker analysis identified QTLs within chromosomes 9 and 11 that were significantly linked to scrapie incubation time in simple interval mapping. For both QTLs the interval of significance was found to be a rather broad one, which leaves the possibility that not one, but several influential polymorphisms can be found there. Successive composite interval analysis on the chromosomes indicated suggestive linkage for a possible QTL distal from the marker on chromosome 9 while on chromosome 11 no evidence for a marker outside the already determined interval was found. Notably, the laminin

receptor gene (*Lamr1*) is located on murine chromosome 9. The product, which is encoded by the gene, has been shown to interact with PrP^C (Rieger *et al.*, 1997). Heat-shock protein cognate 70 gene (Hsc70, also Hspa8) has also been mapped to chromosome 9 in the mouse. It is a putative candidate for involvement in scrapie pathogenesis because expression of heat-shock proteins is known to increase in scrapie-infected mice.

In a different approach Lloyd *et al.* (2001) examined *Prnp-a* mice that had been inoculated with scrapie prions and showed significantly different incubation times. Genomic screening by interval mapping identified three regions on chromosomes 2, 11 and 12, which might be linked to incubation time. Less significant linkage was confirmed for sections of chromosomes 6 and 7. Subsequent composite interval analysis by Lloyd *et al.* (2001) implied that multiple linked QTLs exist in these regions. For effects of three linked QTLs on murine chromosomes 2 and 12 only modest significance was calculated, whereas more than 45% of total variance observed in scrapie incubation times could be accounted for by two linked QTLs located on chromosome 11. Additional single nucleotide polymorphisms (SNPs) were identified in the promoter region of the *Prnp* gene though a possible effect on incubation times has yet to be examined. These findings indicate that polymorphisms other than the one already identified within the *Prnp* gene coding region may influence scrapie incubation times in mice. In a subsequent study mice were intracerebrally inoculated with a BSE strain instead of scrapie (Lloyd *et al.*, 2002). Once again regions on chromosomes 2 and 11 were found to be significantly associated with incubation times. Ensuing composite interval mapping moreover implied the existence of three linked QTLs on chromosome 2 like in accordance with the results of the previous study. It has not yet been determined whether these latter loci are the same. If so, their influence on incubation times in mice would be independent of the strain type. Incubation time variation was also observed in mice intercerebrally inoculated with BSE by Manolakou *et al.* (2001). Two mouse strains used in the study were similar with regard to the *Prnp* alleles. Nevertheless, a difference of 100 days in incubation time was seen in the animals. QTL mapping revealed loci on chromosomes 2, 4 and 8 that were highly significant. One QTL on chromosome 15 was significantly linked to incubation time in the mice. When effects of the single QTLs were measured independently from each other the phenotypic variance caused by one of them ranged between 4 and 7 %. Cumulative effects of all

QTL, when corrected for dominance, where adding to 50% of the difference in phenotype. Epistatic effects below the line of significance should be responsible for the remaining variance.

Not only can QTLs on different chromosomes play a role in controlling prion disease incubation times in mice, it is also possible that polymorphisms in functional regions of PrP^{C} led to alterations in their effects. The copper-binding octarepeat region of PrP^{C} has been an object of investigation because it is implicated in aspects of conversional transition of PrP^{C} to the infectious PrP^{Sc} isoform.

In order to elucidate these aspects Castilla *et al.* (2004) investigated the effect of an additional insertion in the octapeptide repeat sequence with regard to BSE infection. Mice transgenic for the bovine PrP^{C} (boTg mice) were intracerebrally inoculated with BSE agent. One of the mouse lines possessed six octarepeats (bo6ORTg mice) in the *PRNP* gene representing the bovine wildtype while the other mouse line had a sequence of seven repetitive octapeptides (bo7ORTg mice). Immunohistochemistry findings of the BSE-affected CNS were similar for animals of both lines. Incubation times clearly depended on the amount of PrP^{Sc} contained in the inoculum and on the expression levels of PrP^{C} in the animals. The more PrP^{Sc} was injected and the higher PrP^{C} expression in the mice the shorter were the incubation times measured. Nevertheless, incubation periods and survival times even varied between mouse lines with similar PrP^{C} expression, regardless of which inoculum was used for infection. The bo7ORTg mouse exhibited markedly decreased BSE incubation times and shorter lifespan. There also seemed to be an effect of number of octarepeats concerning disease susceptibility. Brain homogenate of bo6ORTg mice infected with BSE and killed at 120 and 150 days, respectively, was inoculated in both transgenic mouse lines. 33% and 50% of the inoculated bo7ORTg animals were tested positive for PrP^{Sc} Western blotting while in none of the inoculated bo6ORTg mice the proteinase-resistant protein was detected.

Differences in the number of octapeptide repeats of PrP^{C}, i.e. the insertion of additional repeats in the amino acid sequence, have been implicated in familial forms of human CJD. The neuropathologic syndromes might be due to the same mechanism. It was suggested that the presence of seven octarepeats in PrP^{C} influences the structure of PrP^{C}. In comparison to wildtype prion proteins such a structure could transformed into PrP^{Sc} with a greater efficiency. Propagation would be taking place much quicker than in the wildtype and the infectious isoform of PrP^{C}

would be present in the organism after a shorter lifespan following BSE infection. This idea was corroborated by the finding that inocula taken from asymptomatic transgenic mice were more efficient in infecting bo7ORTg animals than those possessing six octarepeats. These results imply a role of the octarepeats in prion propagation and disease susceptibility especially interesting with regard to the genetic situation in bovine BSE. Variation in the number of octapeptide repeats between five and seven has been observed in cattle and although the genotypes of five and six octarepeats seem to have no influence on BSE susceptibility these aspects have not been sufficiently investigated in the seven-octarepeat genotype.
It has been mentioned that additional octapeptides in the repeat region are associated with familial prion disease. Chiesa *et al.* (1998) generated transgenic Tg(PG14) mice with additional nine octarepeats, so that the animals possessed an overall number of 14 octapeptides. In transgenic mice exhibiting the highest expression levels a slowly progressive neurological syndrome was apparent. Initial symptoms consisted in ataxia, abnormal posture and clasping of the hindlimbs, when animals were held by the tail. In a later stage of the disease gait impairment became more pronounced, mice lost weight and neglected grooming of their coats. In the brain of the affected Tg(PG14) mice cerebellar atrophy and significant reduction in the number of granule cells and thickness of the molecular layer was observed, changes increasing with duration of disease. No signs of neuronal loss or spongiosis were apparent. Antibody staining revealed that gliosis and astrocytic hypertrophy were present mainly in the cerebellar cortex. Fine deposits of PrP were visibly immunostained in various regions of the brain. Further analysis demonstrated that part of PrP in the brains of the animals was detergent insoluble and resistant to low concentrations of proteinase K. PrP found in the Tg(PG14) animals also had a different cleavage pattern than that of wildtype mice implying conformational differences between the proteins. Cleavage resulted in a core fragment (PrP^{27-30}) that is also seen after cleavage of PrP^{Sc}. These results imply an association between the number of octarepeats in PrP^{C} and the liability of the protein to transition into a different conformational state with markedly distinct biochemical properties.
Experiments in transgenic mice have furthermore provided clues, which effect the octapeptide repeat region of PrP^{C} might have in modulating prion disease properties. Flechsig *et al.* (2000) introduced a truncated murine PrP transgene into PrP^{C} knockout mice. Codons 32 to 93 of the PrP transgene had been deleted, so that

transgenic mice were devoid of all five octarepeats. The transgenic animals were challenged with scrapie prions, which led to the development of scrapie-like syndromes that were similar to those observed in wildtype mice. Ataxia was pronounced but it was more apparent in the front legs while hind extremities were affected to a lesser extent than usual in mouse scrapie. The overall incubation times observed in the transgenic mice were longer than those seen in wildtype controls (31 to 45 weeks compared to 22.5 weeks). Furthermore, prion titers were found to be lower in the animals devoid of the octarepeat region. The alterations in disease characteristics were regarded as a direct result of the N-terminal deletion. Moreover, histopathological differences were detectable in brain and brainstem in which neither the typical lesions nor astrogliosis was observed. In contrast to this, no discrimination between wildtype and transgenic animals was possible in the histopathology of the spinal cord. The results of these mouse experiments implied that the octarepeat region of PrP^C is not essential for the occurrence of scrapie-like syndromes in the animals. The properties, i.e. incubation times, clinical presentation and histopathological aspects of such disorders are nonetheless affected by the murine genotype. The region comprising the octapeptide repeat sequence therefore efficiently modulates disease characteristics.

Scott *et al.* (1997) inoculated Tg(MBo2M) with BSE agent. MBo2M is a chimeric transgene consisting of bovine *PRNP* and murine *Prnp-a* sequences. Mice expressing the transgene were surprisingly resistant to BSE though *Prnp-a* mice are susceptible to the disease. The same technique was applied to mouse and human PrP genes in order to create a chimeric transgene (Telling *et al*., 1994). Inoculation with both human (CJD) prions and mouse prions resulted in disease. By comparing chimeric transgene and mouse PrP^C encoded by *Prnp-a* sequences differences in the amino acid chain were revealed. Bovine substitutions in the transgene sequence were located between residues 97 to 186 while human substitutions in the human transgene only extended from residues 97 to 167. As positions 184 and 186 are not homologous in bovine and murine PrP^C, amino acid substitutions at these residues might influence prion disease susceptibility in the chimeric mice. As an alternative amino acid residue 203 differs between PrP alleles (MoPrP and HuPrP: valine, BoPrP: isoleucine, MBo2M: valine) and thus might have modulating effects on TSE transmission.

Scrapie and Genetic Implications in Sheep

Scrapie is a slowly progressive disease of sheep and goats that belongs to the group of TSEs. It probably is the longest-known TSE in animals and has first been described in British sheep flocks in 1732. Scrapie occurs in European countries and has also spread to Canada and the United States while it could hitherto not be found in Australia and New Zealand.

The main route of transmission is thought to exist between ewe and offspring through contact with the placenta or placental fluids. Another source of transmission is supposed to be the consumption of infected placental tissues. Lateral transmission appears to be possible, as scrapie has been observed in mixed flocks of sheep and goats (Capucchio *et al.*, 2001). The scrapie agent seems to be able to survive for longer periods in the environment. Scrapie cases generally occur in single animals of a flock. Several cases of the disease can only be observed if the amount of infectious material circulating within the herd is immense, in which case scrapie incidence can be high.

Clinical symptoms of scrapie are seldom apparent in animals below an age of 12 months. Commonly first signs of the disease appear between 2 and 5 years of age. Scrapie disease duration ranges between 1 and 6 months. In the initial stages of the disease change of behaviour presents itself as infected sheep tend to separate themselves from the flock. There may also be increased excitability, nervousness, fear and increased sensitivity against noise and touch (Healy *et al*., 2003). Aggressive behaviour is known to occur sometimes. As the disease progresses, ataxia and incoordination in the hind limbs and the characteristic “bunny hop” gait can be seen. Tremor of the head or generalised tremors, grinding of teeth and vacant stare are other clinical symptoms. The infected animals are gradually deteriorating. While no loss of appetite is apparent, chronical severe weight loss is present. One of the most striking clinical features of scrapie is the continual scratching from which the disease derived its name. Affected sheep generally scratch flanks and rear quarters on objects or scratch their body parts with a hind foot. Nibbling or grinding of teeth often accompanies scratching. The constant scratching and rubbing leads to wool loss and skin damage in certain body regions. Rare symptoms observed in scrapie are apathy and drooling. The condition is invariably fatal.

As in other prion diseases vacuolisation and lesions in the brain, the loss of neurons and gliosis are observed in the brain of scrapie-affected sheep (Wood *et al.*, 1997). The pathogenesis of scrapie has been addressed in numerous studies. The oral route is held to be the main entrance of the infectious agent into the body. Van Keulen *et al.* (2002) determined the presence of PrP^{Sc} in the bodies of scrapie-infected sheep at various stages of disease. Lambs examined in the study were aged between 1 and 5 months. The same group also detected PrP^{Sc} in the later stages of the disease in lambs between 5 and 26 months of age (Van Keulen *et al.*, 2000). The findings indicate that in scrapie there are three different phases of prion pathology after probable infection via the oral route. Initially, the gut associated lymphoid system (GALT) is infected where PrP^{Sc} can be found in palatine tonsils, Peyer's patches and draining lymph nodes of the jejunum and ileum. Thus prions seem to be propagated in GALT areas in the early stages of scrapie. From there, infectious agent may gain access to other tissues, possibly via M-cells that are able to transport contents of the lumen to the mucosal immune system (Neutra *et al.*, 1996). As it has been observed for different types of pathogens (viruses and bacteria) this route could also be employed by prions. Van Keulen *et al.* (2002) furthermore determined the existence of PrP^{Sc} in GALT lymphoid follicles and as yet unidentified cells located beneath the surface epithelium. They proposed these cells to be either dendritic cells (DCs) or macrophages that might serve in the transport of infectious agent to germinal centres in lymphoid follicles. The death of DCs after interaction with lymphocytes supposedly releases their burden. Scrapie agent could then be phagocytised by lymphoid macrophages in the follicles. At a later stage of scrapie-infection PrP^{Sc} could already be found at follicular DCs, which might suggest a role for these cells as centres of PrP^{Sc} accumulation. Otherwise they could themselves be infected by the infectious agent. DCs and macrophages are thus candidates of transmission of the scrapie agent to GALT-draining lymph nodes. In such a manner the infectious agent would gain access to cortical and paracortical sinuses. PrP^{Sc} can be detected in cells in the sinuses, which might be the means of transportation of infectivity to lymph and to the blood system. This would represent the second stage of pathogenesis, namely the invasion of non-GALT-tissues. Neuroinvasion is the third phase of scrapie pathogenesis in sheep. According to Van Keulen's study, the connection between the enteric nervous system and the gut-related Peyer's patches could lead to the entry of infectivity into the neural tissue. After the enteric nervous

system (ENS) is infected, both sympathetic and parasympathetic neuronal pathways could lead the scrapie agent to brain and spinal cord via the N. vagus and the thorax-associated tissues. Both portals of entry are connected to neuronal cell bodies that innervate the abdominal viscera. Having reached the CNS the scrapie agent could spread further.

Beside this model of scrapie pathogenesis, there are other putative mechanisms which might be involved in pathogenesis, among them the invasion of the CNS through peripheral nerve endings in non-GALT lymphoid tissues or a sort of virus-like haematogenic spread. Such a phase of “viremia” could possibly occur during scrapie pathogenesis as PrP^{Sc} amyloid formations can be found in capillary endothelial cells of the hypothalamus at a stage of disease when PrP^{Sc} has only reached a limited region in brain and spinal cord (Van Keulen *et al.*, 2000).

From the results of various studies it seems to be plausible that scrapie is caused by the consumption of contaminated infectious feed. This is due to the presence of scrapie prions in the gut and gut-associated systems in the early stages of the disease. Other possible routes of infection are transmission via contact to wounds or body fluids (milk, blood etc.).

The ovine prion protein gene (*Prnp*) comprises three exons, 52, 98 and 4028 base pairs in length and two introns with a length of 2421 and 14031 base pairs respectively. It is located on chromosome 13 and characteristically possesses an extraordinary long 3’ untranslated region (3’UTR) of 3246 bp while the open reading frame is but 768 bp in length (Lee *et al.*, 1998). The exceptional length of the ovine PrP mRNA is caused by the insertion of three transposable elements in the 3’UTR that is supposedly ruminant-specific (Tranulis, 2002). The ovine PrP^{C} shares the common architecture of mammalian PrPs, which consist mainly of α-helices and two short antiparallel β-sheets.

Within the ovine *Prnp* gene, 23 polymorphisms have been described to date (Hills *et al.*, 2003). Among those are three polymorphisms at codons 136, 154 and 171 that cause amino acid exchange in PrP^{C} (see Table 4). A number of different PrP^{C} alleles can be distinguished by the amino acids encoded for at these residues. The PrP genotypes arising from the combination of the different alleles show diverse susceptibility to scrapie with codons 136 and 171 being the strongest determinants for this effect (Hunter, 1997, Tranulis, 2002).

Table 4: Sheep PrP gene – the three most important disease-related polymorphisms (Hunter, 1997)

Codon	Amino acid alternatives	Single-letter code
136	Valine	V_{136}
	Alanine	A_{136}
154	Arginine	R_{154}
	Histidine	H_{154}
171	Arginine	R_{171}
	Glutamine	Q_{171}
	Histidine	H_{171}

Among the so-called “valine” breeds (Cheviot, Swaledale etc.) that carry alleles with valine at codon 136, the VV_{136} RR_{154} QQ_{171} genotype is highly susceptible to natural scrapie and scrapie isolate SBBP/1 (Scrapie Sheep Brain Pool number 1) (Houston *et al.*, 2002). Incubation time is nevertheless also influenced by the polymorphic codons 154 and 171 (Elsen *et al.*, 1999). Within other breeds such as Suffolk, the V_{136} R_{154} Q_{171} allele is hardly encountered, so that the AA_{136} RR_{154} QQ_{171} genotype is most susceptible to scrapie (Hunter, 1997). This pattern can be partly explained by the existence of different scrapie strains. Thus SSBP/1 seems to target sheep encoding the V_{136} R_{154} Q_{171} allele, if present among the flock, while scrapie isolate CH1641 and BSE prions have been shown to target sheep according to codon 171 genotype, QQ_{171} animals being the most vulnerable (Goldmann *et al.*, 1994).
In addition to these “classical” allelic variants, two new *Prnp* forms have been recognised in Germany (Kutzer *et al.*, 2002), namely $A_{136}H_{154}R_{171}$ and $V_{136}R_{154}R_{171}$. Though present at low frequencies they have been found in common breeds such as Texel or Suffolk. It has to be taken into account that such unknown variants might have an influence on the evaluation of resistance grade of PrP genotypes.
Furthermore, scrapie susceptibility of sheep appears to be breed-specific, i.e.

genotypes known to be the most susceptible in one breed are not necessarily vulnerable in a different breed, also depending on the frequency of single alleles. Lühken *et al.* (2004) determined PrP genotypes in atypical scrapie cases in German Merinoland animals. All of these cases carried at least one AHQ allele, an allelic variant that has been suggested to be to a certain degree protective against scrapie. Elsen *et al.* (1999) investigated the relationship between PrP genotypes and scrapie susceptibility in a flock of Romanov sheep. In the study both the ARR and AHQ allelic variants inferred almost dominant resistance to the animals. ARQ and VRQ were found to cause susceptibility and behave in a codominant way. In the German study no such effect was observed, as the AHQ allele was not associated with higher resistance to scrapie. This might be due to the occurrence of high AHQ frequency together with low VRQ allelic frequency in German Merinoland. Consequently, relative resistance conferred by ovine PrP haplotypes and genotypes has to be viewed in the light of breed-specific and even flock-specific factors.

The A_{136} R_{154} R_{171} allele appears to have a protective effect against the occurrence of natural scrapie, AA_{136} RR_{154} RR_{171} homozygotes that develop scrapie are extremely rare (Ikeda *et al*., 1995) and were at first considered to be resistant against TSEs. In contrast to this opinion it could recently be shown that this genotype can be experimentally infected with cattle BSE prions by intracerebral inoculation (Houston *et al*., 2003). Thus no ovine PrP genotype seems to infer absolute genetic resistance to TSEs. In contrast, scrapie infection by the oral route has not yet been demonstrated in experimental studies. Nevertheless there is a possibility that ARR/ARR animals might be subclinically affected by the disease and therefore act as carriers of scrapie. No evidence of PrP^{Sc} presence has been found in lymphoid tissues of the animals successfully inoculated in Houston's study so that it is highly unlikely that they could transmit infectivity.

Caplazi *et al.* (2004) interpreted the high resistance level of the ARR/ARR as well as the ARR/ARQ PrP genotype in natural sheep scrapie as an indication of dominance of the ARR allele over the ARQ allele. In heterozygous (ARR/ARQ) sheep equivalent PrP expression was assessed followed by an analysis of allelic use. DNA clones containing the prion protein gene coding region were derived by RT-PCR and afterwards sequenced. The ratio of ARR to ARQ in the clones comparable to *Prnp* mRNA was similar, so that no difference in allelic use was detectable. Preferential

use of the ARR allele thus does not seem to overrule ARQ expression and therefore does not confer scrapie resistance in the heterozygous ovine genotype.
Remarkably, cells which express a susceptibility allele (V_{136} R_{154} Q_{171}) replicate PrP^{Sc} much more effectively than those expressing the A_{136} R_{154} R_{171} allele. The level of PrP^{C} expression in both cases was similar (Sabuncu *et al.*, 2003). This might point at the involvement of ovine PrP polymorphisms in prion propagation already at the cellular level.
It is noteworthy that apart from the polymorphisms already mentioned here there are a number of others, e.g. at codons 112 and 141 that show minor or no influence on scrapie susceptibility in sheep (Bossers *et al*., 2000). In a cell-free system, conversion efficiencies of nine different allelic variants of the ovine PrP were compared to each other. Efficiency was high for PrP^{136V}, PrP^{ARQ} and PrP^{141F} alleles. In contrast PrP^{154H}, PrP^{171R} and PrP^{112T} had low conversion efficiency. Differences in the liability of the alleles to convert to PrP^{Sc} thus seem to modulate susceptibility in sheep though it has yet to be shown that such experimental results also apply to the situation *in vivo*.
Furthermore, Seabury and Derr (2003) reported the discovery of another allelic variant of PrP^{C} ($P^{116}A^{136}R^{154}Q^{171}$) and the new genotypes PARQ/ARR and PARQ/ARQ) in hair sheep breeds. Albeit association with scrapie susceptibility has not been examined, such polymorphism could prove important for the understanding of PrP conversion.
As already mentioned above, genetic polymorphisms supposedly influence conformational properties of the PrP^{C}. This could either be due to an effect on the structural stability of the PrP^{C} or polymorphisms could hinder interaction and binding to chaperones. Impairment of PrP clearance would be another mechanism by which mutations might play a role. Rezaei *et al*. (2000), who also examined intermediates of ovine PrP^{C} variants, investigated the effects of polymorphisms on physio-chemical properties of PrP alleles. High-yield purification techniques were employed to produce recombinant sheep PrP in order to compare alleles with different amino acids as determined by codons 136 and 171 of *Prnp* (A136R171, V136Q171, A136Q171) that occur in natural scrapie and a further recombinant PrP allele that had been mutated (V136R171). At different pH levels standard free energy of unfolding was measured. As a result susceptibility variants with valine at position 136 exhibited higher energies than other variants. Furthermore, the natural scrapie

susceptibility allele VQ possessed an increased chemical and thermal stability. Accordingly there have to exist other factors except from intrinsic stability of the protein, which influence scrapie susceptibility. Possibly susceptibility diversity of ovine PrP^C alleles could be explained by the difference in potential interacting sites with chaperones e.g. access to functional sites could be blocked in PrP conformations determined by polymorphisms. Moreover, Rezaei *et al.* (2002) investigated whether differences in the proteolytic digestion of the alleles occurred. The susceptibility allele VQ showed increased proteinase-resistance at approximate neutral pH level. From these results it could be postulated that the VQ allele has a lower clearance rate than other variants of ovine PrP, which would consequently lead to a shortened scrapie incubation period in sheep of the VQ genotype.

Eghiaian *et al.* (2004) further investigated the implication of ovine PrP polymorphisms on PrP^C to PrP^{Sc} conversion. In the study the C-terminal domain of several PrP allelic variants was crystallised in order to determine the X-ray structure. Cocrystallisation with a Fab fragment provided additional information about the positions of PrP^C chains. The structures of the VRQ, ARQ and ARR PrP variants were afterwards determined and compared with each other. These PrP alleles are associated with high, medium and low scrapie susceptibility, respectively. The substitution of alanine by valine at residue 136 causes a rotation of a sidechain, which leads to the stabilisation of the VRQ allele through a hydrogen bond. Substitution of glutamine by arginine at position 171 displaces a sidechain thereby destabilising the protein by undoing a hydrogen bond. Thus a destabilisation of ARR and AHQ is apparent when compared to the susceptibility variants ARQ or VRQ. This seemingly contradicts the concept which proposes that destabilisation of PrP^C increases the probability of conversional transition. Yet stabilisation of the PrP^C leads to increased proteinase K resistance (Rezaei *et al.*, 2000). This would lead to a longer lifetime of the protein in the cell and the risk for the molecule to undergo aggregation or misfolding would be heightened. Furthermore, amyloidogenesis is slower for the resistance variant ARR than for ARQ (Rezaei *et al.*, 2002). Both properties of the resistance variant combined could reduce the sensitivity to prion disease. It is evident that substitutions of single amino acids in PrP^C lead to important implications for conformation aspects, though the mechanisms by which this accomplished are not yet clear.

The ability of PrP^C to bind copper has already been mentioned but there are also structural aspects to be considered. Wong *et al.* (2004) investigated the structural

features of two different recombinant ovine PrP alleles (PrP^{VRQ} and PrP^{ARR}). The melting curves of both alleles were compared, revealing that PrP^{VRQ} excels PrP^{ARR} in transition temperature (t_m). Thus the susceptibility allele seems to possess a higher thermodynamic stability than its resistant counterpart. N-terminally truncated forms of both alleles did not exhibit a different t_m as compared to the full-length proteins. This would indicate a crucial role for the core domain of PrP in terms of thermodynamic stability. Furthermore, the PrP alleles were incubated with copper in order to investigate the influence of the metal ions on PrP structural conformation. β-sheet content increased in PrP^{VRQ} while PrP^{ARR} did not show any considerable changes. In incubation at physiological temperature (37°C) this effect seems to depend on the presence of copper. The absence of copper ions increased β-sheet content in both PrP forms but the susceptibility allele maintained a higher amount of α-helical structures in comparison to PrP^{ARR}. The polymorphism at codon 171 could possibly determine the extent of β-sheet formation in the conversion process from PrP^{C} to PrP^{Sc}. Murine PrP, which closely resembles ovine PrP^{VRQ} and PrP^{ARQ}, carries a glutamine at a position that corresponds to codon 171 in the ovine PrP. In contrast, PrP^{ARR} contains arginine at residue 171 therefore this position might have a pivotal influence on the β-sheet content of PrP. These findings indicate that structural properties of the different ovine PrP alleles in connection with copper-binding faculties might be responsible for the modulation of the conversion to PrP^{Sc}. Thus differences in the rate of conformational shifting observed between ovine PrP forms might only occur in the presence of copper. In the study of Wong *et al.* (2004) both forms of ovine PrP exhibited proteinase-resistance after incubation with copper. Western blotting nevertheless showed that PK-resistant banding for PrP^{VRQ} and PrP^{ARR} differed from each other. As non-PK digested bands were found to be of equal intensity this might mirror quantitative differences as to β-sheet content, which causes distinguishable levels of PK-resistant matter. Unfortunately, PK-resistant fragments of recombinant PrP alleles differed from those suggested for similarly treated ovine PrP^{Sc} as regards molecular mass and, consequently, conformation, so that findings do not necessarily apply to the situation *in vivo*. Alternatively, recombinant PrP might constitute a structural intermediate of PrP^{Sc} formation. The finding that also ovine PrP^{ARR} might acquire proteinase resistance is in accordance with the results of Houston *et al.* (2003) who demonstrated the possibility of experimental scrapie inoculation in sheep carrying the genotype ARR/ARR. Copper

incubation did not lead to the same effects in the truncated form of ovine PrP^{VRQ} as compared to the full-length allele by maintaining a conformation dominated by α-helices. This points to the N-terminal domain as an important factor in connection with conversional change. Other metal binding sites in the C-terminus therefore are supposed to play a rather restricted role in structural conversion. Copper-related mechanisms might be involved in the function of PrP^{C}. If it indeed is responsible for the transport of copper changes between predominantly α-helical and increased β-sheet content might take place as a peculiar property of PrP^{C}. The presence of copper ions would then determine the extent of β-sheet structures and therefore convey PK-resistance to the protein. *In vivo* regulation of this mechanism might be achieved by chaperones and other ligands.

Apart from genetic disposition arising from polymorphisms in the PrP amino acid sequence and the ensuing effects on conformational change, it is possible that regulation of *Prnp* gene expression influences disease susceptibility (Hunter, 1997). The untranslated flanking regions of *Prnp* (3'UTR and 5'UTR) supposedly contain regulatory elements responsible for mRNA function (Goldmann *et al.*, 1999). Alternative polyadenylation of the prion protein gene generates two different variants of mRNA (4.6 kb and 2.1 kb). They both encode the same open reading frame. Experiments in cell models demonstrated that production of the ovine PrP^{C} can be influenced by sequences within the 3'UTR of the *Prnp* gene. Cellular transfection with constructs containing a short 3'UTR region resulted in varying expression of PrP^{C}. Cells transfected with full-length PrP 3'UTR (4.6 kb mRNA) expressed the protein at very low levels contrary to the situation *in vivo*. It has therefore been suggested that, *in vivo*, additional factors stabilise or activate the 4.6 kb mRNA. Polymorphisms of the 3'UTR may thus modulate the prion protein gene expression levels in sheep. However, a restriction fragment length polymorphism (RFLP) already identified within the 3'UTR by Hunter *et al.* (1991) showed no significant association with disease status in sheep.

BSE in Sheep?

Since the occurrence of BSE in the 1980ies it has been discussed whether the origin of the BSE agent might lie in the transmission of scrapie prions from sheep to cattle. Strain characteristics are distinct between scrapie and BSE agent but this might be due to cycling in the bovine species and the evolvement of the BSE strain. An alternative hypothesis is the original existence of a scrapie strain, exhibiting properties similar to those of the BSE agent, which had been transmitted to cattle but preserved its characteristic features.

Transmission of BSE to sheep via the oral route has already been demonstrated (Foster *et al*., 1993, 2000). Therefore a transmission of BSE from cattle to sheep through meat and bone meal (MBM)-containing fed components is not to be excluded. Notably, transmission of BSE to sheep has been accomplished also by blood transfusion (Houston *et al*., 2000). Whole blood was taken from an animal in the preclinical phase of BSE infection, i.e. from a sheep that had orally been challenged with BSE agent but did not yet show clinical signs of the disease. The recipient of the transfusion developed BSE symptoms after an incubation period comparable to that of the donor animal (610 and 629 days). This result has only been obtained in a single animal. It demonstrated that BSE infectivity is present in blood in the preclinical phase and that same-species transmission is possible via blood transfusion. This finding might lead to the establishment of sheep as animal models for diagnostic tests on blood samples and testing of leucodepletion efficiency.

The successful intracerebral transmission of BSE to sheep of the ARR/ARR PrP genotype has highlighted the need for BSE surveillance in the ovine species. Nevertheless the experimental infection of the relatively resistant ARR genotype does not significantly alter the risk of potential BSE transmission to sheep via the oral route which is supposed to be the principal route of infection in natural scrapie (Kao *et al*., 2003).

Natural cases of BSE in sheep have hitherto not been reported which does not exclude that such cases, possibly in a subclinical form, might exist. It is therefore important to establish a reliable system in order to distinguish between both TSEs. The search for such surveillance tools has led to the development of several approaches in the field of sheep strain typing.

In sheep approximately 20 scrapie strains have been identified. Scrapie strain differences are visible in the pattern of vacuolisation (lesion profile) in the brain of infected laboratory mice. PrP^{Sc} distribution and distinct scrapie incubation times in mice of different genotypes are also taken into consideration. A drawback to this method is the fact that a number of scrapie strains do not produce disease in mice. Ligios *et al.* (2002) attempted to create a method of lesion profiling in sheep naturally affected by scrapie strains. 69 cases of natural scrapie stemming from different flocks were used in the study. As PrP^{C} genotype is critical for scrapie susceptibility, sheep were genotyped. Variation was merely present at codon 136 of PrP^{C} (VV, AV or AA). Different regions of the brain were examined in order to assess severity and distribution of vacuoles in the brain. When grouped according to genotype, differences in lesion profiles were evident. As was to be expected, sheep of different genotype exhibited distinct profiles. Though variation was restricted to PrP^{C} codon 136, sheep homozygous for alanine showed more severe vacuolisation than those homozygous for valine, while heterozygous animals took an intermediate position. Furthermore, variation was also observed within sheep of the same codon 136 genotype. This is probably due to differences in overall genetic background, infection dose, disease duration, route of infection and combination of these factors. As these influences are hardly to be separated from each other, scrapie strains cannot be reliably distinguished by lesion profiles in natural scrapie cases.
These findings were corroborated in a study conducted by Begara-McGorum *et al.* (2002). Vacuolar lesion profiles of sheep infected with experimental (SSBP/1 scrapie source) or natural scrapie were compared to each other. The genotypes of the animals were VRQ/VRQ, VRQ/ARQ, VRQ/ARR and ARQ/ARQ respectively. Variation with regard to vacuolar lesion profiles of different brain regions was observed which were attributed to PrP genotype variation, breed-specific factors and scrapie agent. Furthermore, variation between the individual animals was visible. The study once again highlighted the multi-factorial influences on ovine scrapie lesion profiles, which prevent the usage of lesion profiling as a tool of strain discrimination. Furthermore, vacuolisation pattern can be an additional factor in the distinction of BSE from scrapie in sheep, together with determination of glycolysation pattern analysed by Western blotting after PK-digestion.
It has been suggested that physiochemical differences characteristic for scrapie strains are „translated“ into pathological features after infection, depending on the cell

type affected by the agent (Safar *et al.*, 1998). This might be due to PrP^C expression levels, accumulation rates, stability of aggregates and clearance of these structures. Consequently, cell-specific prion conformation and glycosylation might thus play a role in strain-specific prion propagation (Weissmann, 1991). Birkett *et al.* (2001) showed that isolated cell lines reproduce scrapie strain characteristics independently of host-specific factors. This points to high stability of strain specific features in scrapie.

Clinical signs in sheep affected by experimental BSE are not distinguishable from those seen in naturally occurring scrapie (Foster *et al*., 1993). For that reason it is indispensable to apply strain typing methods to allow differentiation between scrapie and putative BSE cases among sheep.

Bruce *et al.* (2002) employed serial passage of scrapie agent in mice in order to distinguish between strains. Transmission characteristics, i.e. lesion profiles and incubation times, varied considerable between the sources from which the material was derived, so that a number of strains were identified. A new strain was also distinguished that had not been observed beforehand. This might indicate that the strain spectrum might change over time, leading to new strains evolving. Strain identity with BSE was not observed in any of the animals used in the experiment, so that no evidence for the presence of BSE infection in the sample was found.

There has been experimental evidence that molecular mass of the non-glycosylated form of PrP^{Sc} is lower than in scrapie but there also have been contradicting results. Furthermore, there has been no agreement about glycoform ratio of the different strains. The high variability of scrapie strains represents an additional difficulty in this context, together with the genotypic variance caused by polymorphic residues in the ovine PrP gene. Nonno *et al.* (2003) investigated whether molecular strain typing could be a surveillance tool in order to differentiate strain types and PrP^{Sc} in sheep scrapie, BSE and ovine BSE. Glycoprofile and fragment size of PrP^{Sc} derived from natural scrapie cases was analysed. Sheep experimentally challenged with BSE were used to compare the results of the analysis. Scrapie and BSE cases were differing regarding glycoform ratio and molecular mass of the unglycosylated PrP^{Sc} form as was demonstrated by Western blotting of PK-treated material. A high proportion of diglycosylated protein and low molecular mass of the unglycosylated PrP^{Sc} form was observed in the ovine BSE cases when compared to scrapie-affected animals. This finding was pronounced in Cheviot sheep which points to breed-

specific or genetic influence on Western blotting characteristics. The monoglycolysated fraction in scrapie PrP^{Sc} was observed as a doublet band while in ovine BSE a single band was apparent. A higher proportion of fully glycosylated PrP^{Sc} might thus be present in BSE while variation may occur in scrapie cases. All scrapie-affected sheep in the study possessed the same PrP genotype (ARQ/ARQ) so that the impact of genetic factors on strain characteristics was not investigated. Sensitivity to PK-digestion was found to be distinct in BSE samples on the one hand and scrapie and ovine BSE on the other hand. This might be an adaptive effect leading to change of conformation and PK-sensitivity if the BSE agent is transmitted between species. Further adaptation could follow after additional same-species passages of the agent. Extensive strain typing appears to be a viable tool in distinguishing natural scrapie and BSE in sheep although there are some implications that differentiation might be impaired by the existence of scrapie strains, e.g. CH1641, that exhibit features similar to those seen in ovine BSE. Therefore, analysis of a greater number of natural scrapie samples and of BSE repeatedly passaged in sheep has to be carried out in order to improve the knowledge of PrP^{Sc} characteristics, also with regard to the different PrP genotypes present in sheep. Further attempts have been made to enable discrimination of BSE and scrapie in sheep. Thuring *et al.* (2004) employed glycosylation profiling of proteinase K-digested brain tissues by Western blotting in cases of experimental BSE and natural scrapie. Glycoform ratios were compared with help of antibody binding. Epitope mapping had revealed that the monoclonal antibody P4 used in the study bound to a specific N-terminal amino acid sequence in the PrP^{Sc} molecules (WGQGGSH). Binding of the P4 antibody was pronounced in ovine scrapie cases compared to that of the other antibody used in the study, 66.94b4. Therefore, the authors suggested the 66.94b4/P4 antibody binding ratio in Western blotting as an indicator of putative BSE infection in sheep, values above 1.5 indicating the presence of the bovine spongiform encephalopathy. The same tools for characterisation were used in a further study by Lezmi *et al.* (2004) together with an immunosorbent assay which resulted in the finding of increased PK-resistance in BSE-infected compared to scrapie-affected animals. Analysis of glycosylation pattern and antibody binding were similar to those described by Thuring *et al.* (2004). Moreover, immunohistochemical PrP labelling of brain and lymphoid tissues showed clearly distinct pattern for BSE and scrapie cases. An ensuing experimental design analysed features of both agents

when transmitted to ovine transgenic and wildtype mice (Baron *et al*., 2004) by Western blotting. Biochemical properties of both BSE and scrapie PrP^{Sc} already described were once again observed as well as the apparent similarity of the experimental scrapie isolate CH1641 with BSE. Brain samples of wildtype mice infected with scrapie strains or BSE were also inoculated into mice transgenic for ovine PrP^{C}. Resultant strain properties resembled those observed in wildtype mice as regards glycoform molecular masses and ratio which indicated the maintenance of strain-specific features on primary transmission.

Thus Western blotting of PK-digested material together with antibody binding and immunohistochemical tests seems to constitute potent tools to distinguish between BSE and scrapie in sheep. Stable and clearly distinct features of the different strains have been reported in a number of studies in several models. Despite of this observation, genetic implications present in sheep, i.e. PrP genotype, have not been sufficiently addressed. Animals used in the studies were predominantly of highly susceptible genotypes (ARQ/ARQ or AHQ/AHQ), which does not completely reflect the situation in sheep flocks. Furthermore, it has been mentioned that the genetic background is not restricted to the three main polymorphisms in the prion protein gene, so that the consistency of the analysed strain properties has to be investigated in a greater number of animals.

BSE and Genetic Implications in Cattle

The first case of BSE was reported in November 1986 in Great Britain (Donnelly *et al.*, 1997). By 1997 the number of confirmed BSE cases had risen to about 170 000. Supposedly the disease is caused by the consumption of meat and bone meal (MBM) contaminated with an infectious TSE agent. Whether this agent was originally derived from scrapie-affected sheep or from a spontaneously occurring BSE case is yet unclear. Indications that oral uptake of infectious material was the main cause for the BSE epidemic were brought forth by the drastic drop in reported BSE cases after a ban on MBM in cattle feed was introduced in the summer of 1988. Although BSE infections were still occurring in animals that were born after the ban, this probably was due to an incomplete enforcement. Moreover, experimental oral dosing of 30 Friesian/Holstein calves with BSE brain homogenate resulted in the transmission of the disease (Wells *et al.*, 1998). The calves originated from farms with no history of

BSE and were given pooled brain stems from BSE-infected cattle and then killed at different ages starting at two months after inoculation to an age of 44 months. Neuropathological features of the infected calves were then compared to those of controls. First signs of infection, i.e. presence of PrP^{Sc}, fibrils and infectivity were not detected until 32 months after inoculation. Clinical symptoms of the disease were observed 36 months after inoculation. Incubation periods in this experimental study were thus shorter than seen in natural BSE cases.
Incubation periods of BSE naturally occurring in cattle were estimated to be around five years as the majority of BSE cases were detected within the range of this age (Bradley, 1991). The possibility of maternal transmission has been addressed in several studies (Donnelly *et al.*, 1997, Ferguson *et al.*, 1997, Wilesmith *et al.*, 1997). Views on vertical transmission and interpretation of the enhanced risk for offspring of BSE-infected cows to develop BSE vary, though maternal transmission as a risk factor cannot be excluded. Stage of incubation of the dam when giving birth to a calf seems to play a critical role in this matter. In contrast, the transmission of BSE through embryo transfer is unlikely. Wrathall *et al.* (2002) found no signs of infection, neither in recipients of embryos derived from BSE-affected cows nor in the offspring.

The initial clinical presentation of BSE in cattle is limited to unspecific symptoms like decrease in milk yield, loss of weight despite uptake of feed and impairment of behaviour and gait (Braun, 2002). Behavioural changes are typical for BSE infection. They include nervousness, anxiety, aggressiveness and alarmed behaviour. Symptoms may not be apparent as long as the animal is not advanced upon or touched. Reactions to touch can be jerking, avoidance, licking of the muzzle and grinding of teeth. Often observed is tremor of lips, muzzle, throats, and parts of the body or generalised trembling. Symptoms can manifest themselves singly or in combination. There is an increased sensitiveness of BSE-affected animals to stimuli such as touch, noise and light which is typically not wearing away with continuation of the said stimuli and can be reproduced over and over again. Responsiveness to tactile stimuli is especially pronounced at the head and throat. In a later stage of the disease ataxia and impairment of balance appears to be more and more pronounced until the BSE-infected animal is no longer able to stand. The final stage of the disease is marked by terminal recumbence, coma and death.

The neuropathological changes seen in the brain of BSE-affected cattle characteristically consists of widely distributed vacuolisation of grey matter neuropil and neurons, gliosis and mild neuronal degeneration accompanied by PrP^{Sc} accumulation (Debeer *et al.*, 2003). Lesions can also be observed in the spinal cord. PrP^{Sc} accumulates in the form of abnormal brain fibrils that are termed scrapie-associated fibril (SAF) (Scott *et al.*, 1990). In comparison to scrapie where lesion profiles in the ovine brain mirror the strain type of the infectious agent, appearance of vacuolisation in the BSE brain is very uniform. This argued in favour of a single BSE strain responsible for BSE infections world-wide (Fatzer *et al.*, 1996, Orge *et al.*, 2000). Contrary to this, recent findings in French and Italian cattle have suggested the existence of more than one strain of the BSE agent in the bovine species (Biacabe *et al*., 2004, Casalone *et al*., 2004). To further elucidate the question whether several BSE strains can be detected, Lloyd *et al.* (2004) examined the properties of PrP^{Sc} in inbred mouse lines after primary passage of BSE agent. Comparison of neuropathological aspects, immunohistochemistry and Western blotting analysis in these mice resulted in the discrimination of two distinct BSE strains. On subpassage in mice, strain characteristics were stably reproduced. One strain, termed MRC1, caused a relatively short incubation time of 100±3 days and an overall diffuse PrP immunostaining pattern of the brain. The monoglycosylated form of PrP^{Sc} was predominant on Western blots of PK-digested brain homogenate samples in MRC1. Animals infected with the other putative BSE strain, called MRC2, exhibited a longer incubation time of 155±1days. Immunostaining of brain tissues showed distinct PrP deposits or plaques and neuronal loss. MRC2-derived PrP^{Sc} showed a diglycosylated-dominant pattern in immunostaining procedures. Strain characteristics determined by the available methods suggested that there might be a range of BSE strains in existence. It is nevertheless notable that strain characteristics varied with the genetic background of the inoculated mouse lines. Thus host genotype has to be taken into consideration when putative BSE strains are identified in inbred mouse lines.

The pathogenesis of BSE shares characteristics that are also observed in scrapie infection. In the rodent model PrP^{res} is first detected in Peyer's patches and mesenteric lymph nodes (Maignien *et al.*, 1999). Later on, the protein is found in spleen and axillary lymph nodes. The terminal stage of BSE infection involves the accumulation of PrP^{res} in the LRS organs and CNS. Comparing scrapie and BSE

infection it is notable that only in the former PrP^{res} can be detected in the region of the digestive tract between ileum and colon. Thus scrapie PrP^{res} is far wider distributed in this area than is BSE PrP^{res}. Findings in the rodent model nevertheless do not necessarily apply to the situation *in vivo*. In an experimental study in which calves were orally infected with BSE, infectivity was confined to CNS, peripheral nervous system (PNS) and ileum (Wells *et al.*, 1998).
The bovine prion protein gene *PRNP* extends over 20 kbp and is located on chromosome 13. It comprises three exons and two introns about 2.4 kbp and 14 kbp in length (Horiuchi *et al.*, 1998). Investigation of a recombinant bovine PrP of 217 residues (bPrP23-230) revealed the conformational structure that is common to mammalian prion proteins (Lopez Garcia *et al.*, 2000). The three-dimensional structure was found to be to a large extent similar to that of human and murine PrP^{C}. There were nevertheless local differences observed concerning backbone conformations and the regions of helix 1, helix 3 and a loop at residues 166-177. The apparent sequence identity in helix 1 and adjacent loop structures combined with the close resemblance of helix 1 backbone between human and bovine PrP^{C} might influence the height of the species barrier. In contrast, amino acid exchanges are seen in adjoining regions when bovine (or human) and murine PrP^{C} are compared. If such areas were indeed implicated in conformational change of PrP^{C}, these findings would indicate that the species barrier between humans and cattle is rather low. The barrier between cattle and humans on one hand and mice on the other hand would be far more effective. Protein surface charge was seen to vary between human PrP and bovine PrP, which might affect species barrier properties. Nevertheless, the relatively high similarity could argue for a transmission of BSE prions from cattle to human beings and the subsequent development of vCJD.

In cattle, an association between genotype and susceptibility to BSE remains yet to be established. In search for mutations in the bovine *PRNP* Hunter *et al.* (1994) investigated the coding region of the bovine *PRNP* gene by restriction fragment polymorphism (RFLP) and PCR analysis. Two polymorphisms were detected in the coding region of the gene. They consisted in a silent *Hin*dII RFLP and a variation in the number of octapeptides in a repeat sequence of the *PRNP* gene (see Figure 6). The number of these peptides was found to be either five or six. Comparison of genotypes in healthy and BSE-affected cattle did not suggest any influence of these

polymorphisms regarding BSE susceptibility. Also neither breed-specific differences nor differences at the age of onset of the disease were apparent. The genotype homozygous for five copies of the octarepeats seems to be rare in common cattle breeds and BSE-affected cows of this genotype were not included in Hunter's study.

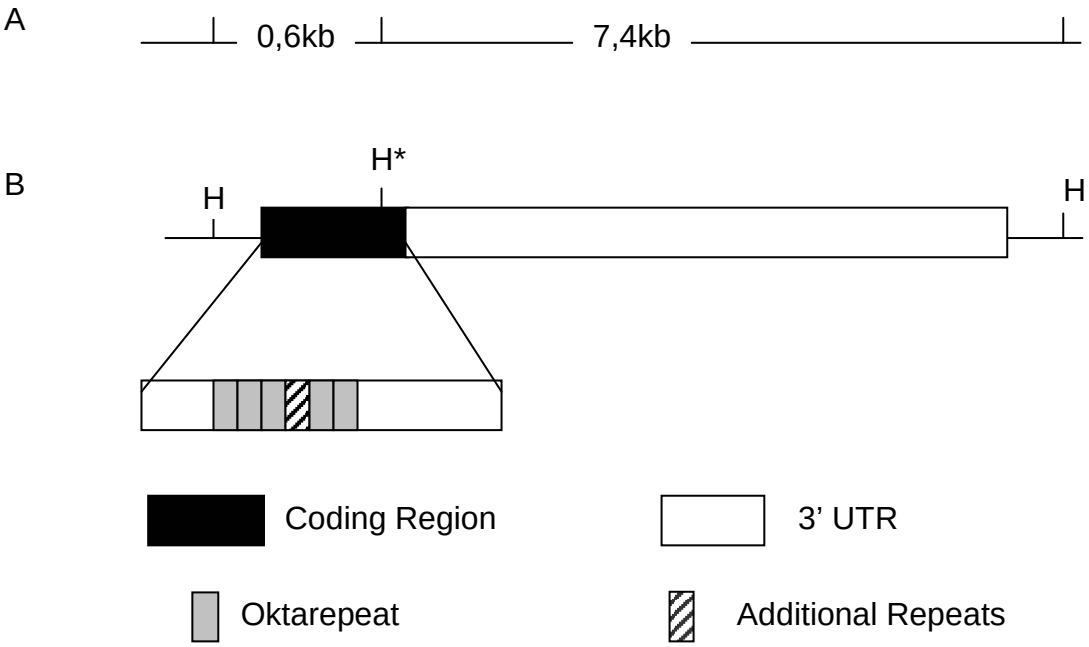

Figure 6: Model of the bovine *PRNP* gene and octarepeat region (Hunter *et al.,* 1994, modified)

(A) Distance of *Hin*dII-restriction sites (H)

(B) The ovine *PRNP* gene (open reading frame), untranslated region (UTR), polymorphic *Hin*dII-restriction site (H*) and octarepeats

In a further study, Neibergs *et al.* (1994) detected two amplified double-strand fragment length polymorphisms (AMFLPs) representing six and five octarepeats in BSE-cases and in a group of unaffected cattle. As in Hunters' investigation no difference in distribution of genotypes between both groups could be observed.

Though no effect of the polymorphism on disease susceptibility was established, distribution of octarepeat-genotypes seemed to vary between cattle breeds. Rare breeds and Brown Swiss cattle showed a higher frequency of the genotype homozygous for five octarepeats compared to more common breeds (Schlaepfer *et al.*, 1999, Premzl *et al.*, 2000). Schlaepfer *et al.* (1999) identified another genotype with seven octapeptide repeats in Brown Swiss cattle, a genotype hitherto also observed in the Italian cattle breed Bruna Alpina by Leone *et al.* (2002).
Hills *et al.* (2003) detected 51 polymorphisms (including the above mentioned) in the entire bovine *PRNP* gene, which consisted of 42 single nucleotide polymorphisms (SNPs) and 9 insertion deletion (indel) mutations (see Figure 7).

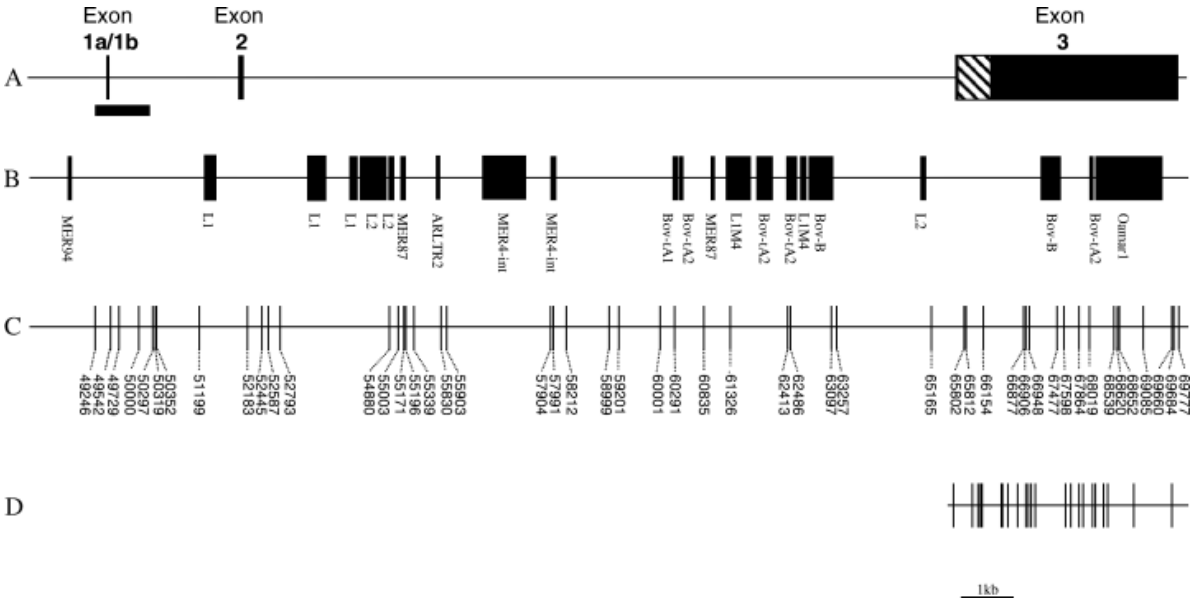

Figure 7: Diagram illustrating the genomic architecture of the bovine *PRNP* gene (Hills *et al.*, 2003).

In (A) the three exons are depicted by solid black boxes and the ORF is shown as a hatched box within exon 3. The solid line underneath exon 1 marks the promoter region identified by Inoue et al. (1997).

(B) Indicates location of repeat elements.

(C) Shows the positions of the variants identified here within GenBank Accession number AJ298878.

(D) Indicates the positions of the variants within the exon 3 region of ovine *PRNP* but the positions are not given for clarity.

Sequence variation within exon 3 of *PRNP* was observed to be even higher than variation throughout the whole gene sequence. Polymorphisms that have been identified in cattle and sheep are to a higher proportion indels as compared to polymorphism found in the human *PRNP* gene and are spread throughout the gene. In search for markers associated with BSE incidence in the bovine genome Hernández-Sánchez *et al.* (2002) performed a range of Transmission-Disequilibrium Tests (TDTs), which found significant segregation distortion for three marker loci on chromosomes 5, 10, and 20. After further analysis, only the marker on chromosome 10 showed significant segregation distortion. Analysis by flanking markers merely linked the marker on chromosome 5 significantly with BSE. Surprisingly, no markers on chromosome 13 which contains the *PRNP* gene sequence were connected to health status in the examined healthy and BSE- affected animals. This would argue for areas of the bovine genome other than the *PRNP* gene to be involved in putative genetic susceptibility. Another point mutation in the coding region, nevertheless a silent one, was detected at codon 192 of the bovine prion protein gene (Kuppinger *et al.* 2002). Association to differences in allelic frequencies could unfortunately not be examined because of lack of sample material. Takasuga *et al.* (2003) investigated the variability of the PrP^C sequence in eleven different cattle breeds among them Indonesian cattle in which two polymorphisms were found. One of these polymorphisms, a substitution of Asn-185 to Ser, had not been described before as Asn-185 is highly conserved among mammalian PrP sequences.

The overall findings of these studies indicated that in cattle contrary to the situation in humans or sheep polymorphism of the *PRNP* coding region do not seem to play a significant role in the genetic disposition to prion disease. The recent findings in mouse models that implicated the number of octarepeat region in the modulation of prion propagation and incubation times however shed a new light on this issue. It would therefore be necessary to investigate a putative connection between incubation periods and octapeptide number in cattle breeds e.g. Brown Swiss in which a seven-repeat genotype occurs.

Additionally, putative promoter regions in the 3' and 5' UTR areas of the *PRNP* gene have been searched for influential polymorphisms, as the regulation of PrP^C expression levels might be another mechanism by which BSE susceptibility could be determined. The UTRs of the gene is therefore an important focus of investigation and a number of polymorphisms have already been found in this region.

DNA sequencing by Humeny *et al.* (2002) led to the identification of further three polymorphisms in the coding region of the *PRNP* gene, two of them silent. Moreover, three novel polymorphic positions in the two 5' UTR exons of the *PRNP* gene were revealed whose significance regarding regulatory functions has yet to be investigated.

Sander *et al.* (2004) reported the discovery of 36 new polymorphisms in the *PRNP* gene of German cattle breeds. 48 healthy animals from six German cattle breeds and 43 BSE cases were included in the study in which the complete DNA sequence of the gene was investigated. None of the newly discovered polymorphisms led to a change in the PrP^C amino acid sequence. Nevertheless, a 32-bp insertion/deletion in the suggested promoter area in the 5'-flanking sequence of the *PRNP* gene was detected that might influence gene regulation. The 32-bp insertion is more often found in healthy animals than in BSE-affected ones, which led to a significant difference both in allele and genotype frequency between both groups of animals while a breed-specific effect was not observed.

The genomic DNA of the 5'-UTR region as well as exon 1, exon 2 and intron 1 of the bovine *PRNP* gene were cloned in order to disclose the regulation of PrP^C expression (Inoue *et al.*, 1997). Two regions of the promoter area showed expression activity. One of these comprises a potential binding site that could interact with Sp1 transcription factor (Sp1) in PrP^C expression, comparable to the situation in the rodent model. Sp1-sites are functional areas of promoters that are activated by transcription factors (Anderson *et al.* 1991). Transfection experiments in cells indicated that in order to generate sufficient promoter activity the existence of intron 1 is required, suggesting that a considerable influence of the intron on bovine *PRNP* gene expression exists (Inoue *et al*., 1997). Hills *et al.* (2003) observed a 12 bp-insertion/deletion polymorphism in intron 1 of the *PRNP* gene. When deletion was observed, the mutation caused the removal a putative Sp1 binding site in this area. Thus the indel polymorphism could cause differences in allele expression levels. Otherwise, the suggested Sp-1 deletion could lead to an alteration of the site of *PRNP* gene expression or both effects might occur in combination.

It is also possible that the mechanism of alternative splicing affects translational efficiency of the *PRNP* mRNA *in vivo*. By analysing the 5'UTR of the bovine *PRNP* mRNA, two distinguishable mRNA species were detected that differed from each other in length (Horiuchi *et al*., 1997). As usage of exon 2 and exon 3 did not vary

between both mRNA species, the difference in length was caused by alternative usage of a 5' splice site, so that the second fragment possessed a longer exon 1 (115 nt compared to 53 nt). PrP mRNA alternative splicing has as yet not been observed in sheep or any other species which points it out as a mechanism specific for cattle. Subsequent *in vitro* translation of the mRNAs did not lead to any considerable difference in translation efficiency between both. Thus it remains unclear whether alternative splicing is implicated in the pathogenesis of BSE *in vivo*. Taken together, the untranslated regions and especially the promoter area constitute elements in which factors that modulate genetic susceptibility to BSE might be found. Structural implications arising from amino acid exchange in the bovine PrP^{C} sequence seem to be of minor consequence for the development of BSE in cattle. Regulation of PrP^{C} expression is therefore increasing in importance, at least in BSE as a transmissible prion disease.

A Sporadic Form of BSE?

Only recently, there have been implications that a second form of BSE might be in existence. When brains of eight Italian BSE cases were examined there were neuropathological differences observed between two groups of animals (Casalone *et al.*, 2003). The first group resembled the typical phenotype seen in BSE, with granular, linear and glial PrP^{Sc} deposition while the second group exhibited plaque-like deposits, unicentric kuru-like plaques and granular deposits. The regional distribution was also distinguishable. While in the first group of animals the brainstem and thalamus were the main centres of PrP deposition and little involvement of olfactory bulb and cerebral cortexes was to be seen, in group 2 mainly thalamus, cerebral cortex and olfactory bulb were immunostained for PrP. In the cerebellum there were glial PrP deposits in group 1 and amyloid structures in group 2. Also PrP^{Sc} glycotype was differing considerably between the groups regarding molecular mass of the unglycosylated forms and ratio of glycosylated PrP in Western blotting. PK-treated brain homogenate from group 1 cattle presented the typical pattern with predominance of the high mass molecular glycoform, while group 2 individuals showed overrepresentation of low mass molecular glycoform and additionally, a PrP fragment with higher mobility in electrophoresis. These different PrP^{Sc} types seem to lead to divers PrP patterns and cerebral distribution. Casalone *et al.* (2003) also

pointed out that route of infection and prion propagation may have differed between both phenotypes. The genetic background of the animals was relatively similar, so that differences observed are probably not due to variation in genetic disposition though genetic factors hitherto unknown might also have played a role in disease pathogenesis. It is also notable that the animals showing the atypical phenotype were the oldest ones examined in the study. This should be taken into account as prolonged incubation times might influence PrP deposition in the body.
There were similarities observed between the properties of this apparently new form of BSE and sCJD as regards molecular properties of PrP^{Sc}. The presence of plaques was unusual for typical BSE but reminiscent of sCJDM/V2 albeit differences occurred in the amount of PrP distributed in specific areas of the brain i.e. cerebellum and thalamus. It is therefore not obvious whether the new BSE phenotype termed Bovine Amyloidotic Spongiform Encephalopathy (BASE) is a distinct disease form comparable to sCJD. Atypical cases of prion disease are also observed in humans (Kretzschmar *et al.*, 2003). Consequently, molecular properties of the PrP^{Sc} types have to be closely surveyed in the future, especially with regard to the so-called atypical phenotypes.

Prion Diseases in Other Species

Goat Scrapie and Genetic Implications

Cases of natural goat scrapie were first reported in 1942 (Cappucchio *et al.*, 2001). The incidence of this disease is very low and only a small number of scrapie cases has since been reported in goats.
The initial clinical signs are mostly behavioural changes occurring in irregular intervals. They include separation from the herd, resistance to milking and aggressive behaviour. The response to external stimuli like touch, noise and movements of people or animals is abnormally exaggerated. Grinding of teeth can be observed in this stage of the disease. With the progress of scrapie, nervousness and aggressiveness increase, leading to ongoing biting among the flock and attacks on other animals and humans. In the later stage of the disease, animals drift into a trance-like state while isolating themselves even more, finally falling into terminal recumbence. In the late stages cannibalism can be seen among scrapie-infected

goats, mainly executed on aborted foetuses. In the early stage ataxia in the back limbs is observed, impairing the balance of the animals. Muscular tremors in face and limbs are common signs, gradually increasing in frequency and finally being generalised. As in sheep scrapie, scratching and nibbling which results in skin lesions is apparent. The main difference between sheep and goat scrapie is that clinical symptoms appear earlier (between two and three months) in goats, probably due to a shorter incubation period, and that aggressive behaviour is more common and pronounced. In comparison to scrapie-affected sheep, goats refrain from rubbing themselves against objects but rather scratch themselves.

In goats several polymorphisms of the coding region of the prion protein gene can be found. Goldmann *et al.* (1996) observed three different goat specific PrP^{C} variants with amino acid exchanges encoded by codons 142, 143 and 240. Of these polymorphisms the dimorphism at codon 142 seemed to be associated with different incubation times of experimental BSE and scrapie strains. A fourth PrP allelotype was identical to the ARQ allele in sheep. Billinis *et al.* (2002) determined the genotypes of 51 goats, among them seven cases of clinical scrapie. In addition to the polymorphisms previously described (112, 136, 137, 138, 141, 142, 143, 151, 154, 171, 211, 240) eight additional polymorphisms, two of which were classified as silent mutations, were detected. Eleven different PrP genotypes were thus found. The majority of scrapie-infected goats which where screened for these polymorphisms possessed the H143H R154R (homozygous for histidine at codon 143, homozygous for arginine at codon 154) genotype. Animals with other genotypes did not show any clinical signs or histopathological characteristics of scrapie though protease-resistant PrP could be found in their brains. In animals carrying a different PrP genotype were seldom (7%) affected by scrapie. Consequently, these two polymorphisms of the *Prnp* gene seem to have an influence on scrapie susceptibility in goats. Considering the breed-specific effects of PrP alleles in sheep it is to be expected that liability to develop goat scrapie is additionally modulated by yet unidentified genes other than the *Prnp* gene.

CWD and Genetic Implications

Chronic Wasting Disease (CWD) is a fatal TSE hitherto only described in cervids, especially affecting elk and deer. The syndrome was first reported in 1967 in mule deer in Colorado and confirmed as a TSE in 1997. Today it is present in parts of North America that belong to the United States and Canada (see Figure 8).

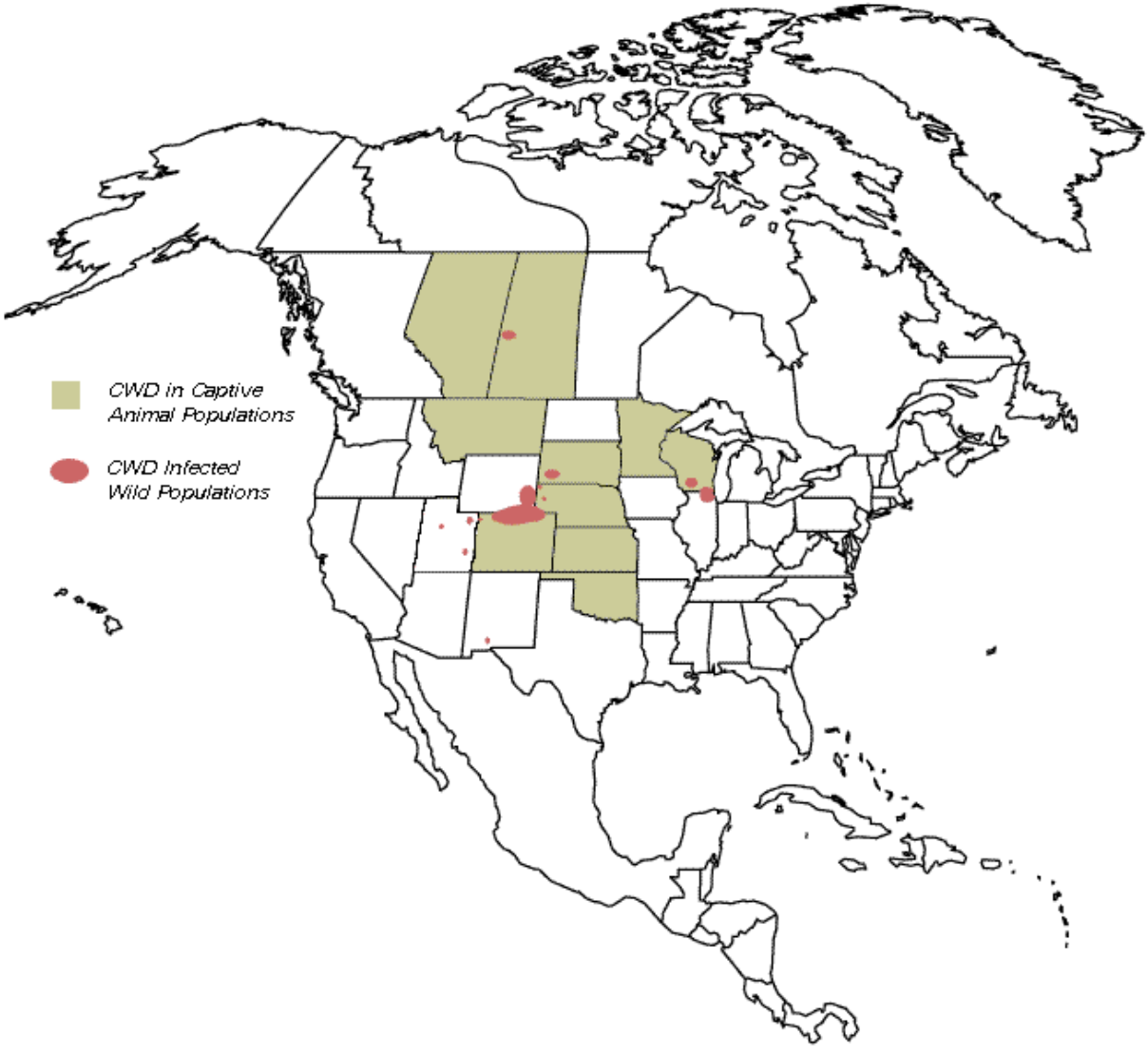

Figure 8: Distribution of CWD in North America
(National Wildlife Health Center, 2003)

The origin of CWD yet remains in the dark. There are several theories as to how the disease could have developed in North America. One of the possibilities is the transmission of sheep scrapie to deer (Salman, 2003). Outbreaks of scrapie have been recognised in the US, which would provide an argument in favour of this theory. Transmission of scrapie by intracerebral inoculation from sheep to elk has been successfully demonstrated (Hamir *et al.*, 2003). Of six elk inoculated with scrapie agent in the study only two animals succumbed to the disease, which might be the result of varying genetic disposition in elk. Another theory is that CWD is a genetic TSE occurring solely in deer or that it represents a yet unidentified TSE that could be present in other species. A sporadic case of CWD that was subsequently transmitted to other cervidae as the source of this TSE is also within the range of possibilities.
The clinical presentation of the disease is dominated by gradual weight loss, which is the most apparent and prevalent sign of CWD. Affected animals alter their behaviour, isolating themselves and falling into a state of lethargy with lowering of the head and blank stare. Free-ranging animals might show decreased fear of humans (Salman, 2003). Repeated walking in consistent patterns can furthermore be observed in CWD cases. Elk may also exhibit increased excitability and nervousness. Uptake of feed is not impaired though animals might loose interest in certain feedstuff such as hay. The uptake of fluids and urination is increased considerably. Excessive salivation and gnashing of teeth is another clinical sign of CWD (Williams and Young, 1992). The actual cause of death often appears to be pneumonia, which is commonly diagnosed in CWD animals.
CWD is primarily recognised in adult cervidae between three and five years of age though much older cases of the disease have been found (Salman, 2003). Duration of the clinical course approximates between several days up to a year.
The outstanding neuropathological signs of CWD are spongiform lesions in the grey matter of the CNS accompanied by intraneuronal vacuolisation, formation of amyloid plaques and astrocytosis. The lesions are found predominantly in olfactory tubercle, cortex, hypothalamus and vagal nucleus (Williams and Young, 1992). Minor differences occur between elk and deer concerning the location of the lesions. Inflammation is not present in these areas. Brain tissues are stained positively for the presence of disease-specific proteinase-resistant protein (PrP^{CWD}). Other tissues in which PrP antibody staining can be observed are tonsils, visceral and regional lymph

nodes, Peyer's patches and lymphoid tissue of the intestines and the spleen (Salman, 2003). This suggests that prion propagation in CWD is similar to that of scrapie in sheep or BSE in cattle.

Transmission routes of the infectious agent are largely unknown. CWD is seen both in free-ranging and farm animals. From this fact one can assume that contaminated feed is probably not the primary cause of CWD as the disease has also been observed in herds that were not fed with MBM. Additionally, the possibility of wild animals coming in contact was such feed components was estimated to be very small. Epidemiological data suggests that CWD is transmitted horizontally either directly or indirectly between animals (Miller and Wild, 2004). Oral inoculation of CWD has been demonstrated in mule deer and appears to be an efficient lateral route of transmission (Sigurdson *et al.*, 1999). The pathogenesis of CWD furthermore indicates that the infectious agent could be present in faeces or saliva, which might enable transmission of the disease to susceptible cervids through social contact. Therefore it is also possible that the infectious agent survives in the environment causing further infections in animals feeding from the contaminated soil. Cervids consume soil as a dietary mineral supplement and when feeding come in close contact to the soil (Bunk, 2004).

A connection between the consumption of venison potentially contaminated with CWD agent and the occurrence of CJD in humans has repeatedly been discussed. Belay *et al.* (2001) investigated three cases of CJD in unusually young patients but did not find a causal link to CJD. Patients were not genetically predisposed by homozygosity at codon 129 of the prion protein gene and the neuropathological presentation was not differing from that seen in classical CJD forms. Though transmission to humans appears to be unlikely, CJD surveillance including strain typing and molecular characterisation plays an important role in assessing the potential risk of exposure to CWD agent.

As observed in other TSEs e.g. scrapie in sheep genetic disposition might influence disease susceptibility to CWD in elk and deer which possibility was addressed in several studies of the elk and deer PrP gene. The cervid PrP gene consists of three exons with two intervening introns (O'Rourke, 2004). The complete sequence of the ORF, which encodes an octapeptide sequence comprising five repeats, is located in exon 3. O'Rourke *et al.* (1999) examined the sequence of the elk PrP gene and thereby revealed a polymorphism at cervid residue 132 (methionine to leucine) and a

silent polymorphism at codon 104. The polymorphic site at residue 132 corresponds to codon 129 in the human *PRNP* gene that is implicated in TSE susceptibility. Elk homozygous for methionine at codon 132 of the protein were overrepresented in CWD-affected animals when compared to healthy control animals (O'Rourke *et al.*, 1999). Consequently, homozygosity for methionine seems to predispose elk to CWD infection.

Brayton *et al.* (2003) investigated the structure of the mule deer PrP gene which led to the discovery of coding changes at residues 20 and 225 and silent mutations at codons 131, 146, 156, 202, 206. As up to four alleles differing at residue 138 (N/S) were seen, in a subsequent analysis of BAC clones a pseudogene (*PRNP*ψ) was detected that comprised the three exons of the functional *PRNP* gene but lacked introns. *PRNP*ψ encodes five or six octapeptide repeats, as a 24 bp repeat unit can be inserted after the second repeat. Both alleles of the pseudogene contained asparagine (N) at residue 138 which variation was used to distinguish it from the *PRNP* gene. Two coding changes were also seen in the pseudogene at codons 65 and 151.

In a group of 133 white-tailed deer O'Rourke *et al.* (2004) detected three polymorphism in the PrP gene, at codons 95 (Q to H), 96 (G to S), 116 (A to G) and three silent mutations at codons 51, 81 and 146 in order to analyse a possible association with CWD disease status. The polymorphisms, which caused amino acid substitution in the cervid PrP^{C}, had been reported before (Johnson *et al*., 2003, Heaton *et al*., 2003). Four different functional *PRNP* allelotypes (QGAS, QSAS, QGGS and HGAS) were found which all of them possessed serine (S) at residue 138. Furthermore, the influence of the *PRNP* pseudogene first recognised by Brayton *et al*. (2003) was analysed in deer. All *PRNP* genotypes were found in the animals but CWD-affected deer was more likely to posses the QGAS *PRNP* allele than the QSAS allele. This finding points to an involvement of residue 96 polymorphism in CWD susceptibility in the studied deer. The pseudogene *PRNP*ψ was not linked to CWD health status.

Taken together, polymorphisms of the PrP gene seem to influence CWD susceptibility in both deer and elk. It is nevertheless hard to say how the distribution of such genotypes looks like in free-ranging population of cervids as routes of transmission and exposure levels are still unclear and migration of animals into different areas is common.

Transmissible Mink Encephalopathy and Genetic Implications

Outbreaks of Transmissible Mink Encephalopathy (TME) have been reported in the United States in 1947 and later in Canada, Finland, Germany, Eastern Europe and other countries of the world.

Clinical signs of this TSE usually have durations between three days and six weeks. In the initial stages increased soiling of the nest and dispersal of faeces can be observed. Affected animals also step into their food and have difficulties in eating. Later in the course of TME, mink show unusual excitability and may arch their tails back comparable to a squirrel. Impairment of gait and ataxia of the hind limbs are present. In the final stages of the disease the animals circle rapidly, chew their tails and show increased jaw clenching. Seizures are detectable in single cases. Terminal recumbence and numbness invariably leads to death in TME-infected animals. Neuropathological changes such as spongiosis and astrocytosis are limited to the nervous system, i.e. cerebral cortex, hippocampus etc. (Robinson *et al.*, 1994).

TME is apparently differing from other TSEs, especially scrapie, as far as pathogenesis and propagation of the infectious agent are concerned. Hadlow *et al.* (1987) subcutaneously injected TME agent into royal pastel mink and investigated the distribution of PrP^{Sc}. Initially the agent was present in lymph nodes at the site of inoculation. As the disease progressed, PrP^{Sc} was found in the CNS although mink did not exhibit clinical symptoms yet. After spreading through the CNS the TME agent was detected in nonneuronal sites such as spleen, liver, kidney, intestine, lymph nodes and salivary gland. Thus, contrary to other TSEs, in TME there appears to be no pronounced lymphoreticular propagation of PrP^{Sc} prior to CNS invasion. In order to investigate the possible transmission of the TME agent between species Robinson *et al.* (1994) inoculated standard dark mink both intracerebrally and orally with brain homogenate of BSE-affected cows. Mink succumbed to a spongiform encephalopathy after a mean of 12 months in the group that had been treated intracerebrally and after 15 months if orally dosed with infectious agent. The clinical presentation was nevertheless differing from that commonly seen in TME. Animals showed decreased appetite, falling into a state of lethargy and pelvic ataxia of the limbs was observed. The neuropathological features were at variance as the encephalopathy induced by inoculation was milder in the cerebral cortex, with more marked changes in the caudal brainstem while the hippocampus was not affected.

Thus transmission of BSE to mink could be demonstrated which implies an association between the consumption of feedstuff contaminated with BSE agent and the outbreak of TME. Marsh *et al.* (1991) had already transmitted TME agent from mink to cattle. Intracerebral inoculation of two calves with TME mink brain led to spongiform encephalopathy in the animals at 18 and 19 months post inoculation. Furthermore, both cattle brains caused encephalopathy when inoculated back into mink both intracerebrally and orally. The farm, which provided the TME agent used in the study, had never employed feedstuff derived from sheep but a high amount of cattle-derived protein from fallen animals.

When the TME agent was first isolated from infected animals it was soon discovered that its biochemical characteristics resembled those of the scrapie agent (Marsh and Hanson, 1969). In TME, two strains can be distinguished by several properties transmitted to Syrian hamster. The drowsy (DY) strain leads to increasing lethargy in infected animals in contrast to the hyper (HY) strain that is characterised by cerebellar ataxia and increased excitability (Bessen and Marsh, 1992). Apart from clinical presentation, differing brain lesion profiles and brain titre of the proteinase-resistant PrP^{TME}, disease incubation times differ between the strains. The mean incubation time in DY-affected hamster is 168 days compared to 65 days in HY cases that latter show much more infectivity of brain tissue than observed in DY animals (100-fold). When passaged from hamsters into mink pathogenicity was only maintained by the DY strain. Thus the HY TME agent replicates faster in hamster brain as judged by titres but is not leading to infections in mink when repassaged. Biochemical properties of PrP^{TME} from both strains were closely characterised by several techniques (Bessen and Marsh, 1992). Due to differences in sedimentation in detergents, digestibility by proteinase K and electrophoresis pattern the HY and DY strain could be distinguished. PrP^{TME} of DY-affected brain was more readily proteolysed than was HY PrP^{TME}. Immunoreactivity to antibodies also differed for both strains in the N-terminal end of PrP^{TME} (residues 89 to 103). By sequencing of PK-digested PrP^{TME} the reason for the diverse migration patterns was discovered (Bessen and Marsh, 1994). The amino terminal end of PrP^{TME} starts at 10 amino acids or more prior to the HY strain in comparison to the DY strain. Degradation rates itself were found to be strain-specific as DY PrP^{TME} was more quickly hydrolysed. Strains produced different PrP^{TME} accumulation in brains of TME-infected hamsters, which implies specific mobility or aggregation processes of DY and HY. This might be

caused by variation in protein structure and conformations, interactions with chaperones and ligands or both. Bessen and Marsh (1994) additionally suggested that specific neuron populations could be targeted depending on strain type of the agent.

TME strain types are known to interfere with each other based on a study by Bartz *et al.* (2000) in which serial passage of TME agent in hamsters resulted in selection of one strain type. According to the presence of specific PrP^{TME} the DY strain was the major strain in the animals on first inoculation. When the TME agent was serially passaged in hamster, PrP^{TME} of the HY strain and conformation was detected while strain-specific phenotypes were confirmed. Only in case of a low-dose inoculum employed in the serial passage were DY clinical signs retained. To confirm the impression of interaction between both strain types, coinfection with hamster-adapted HY and DY strains followed. First passage could lead to DY phenotype and PrP^{TME} though further passage resulted in HY TME diagnosis. Consequently, strain adaptation appears to lead to selective determination for one PrP^{TME} conformation. Bartz *et al.* (2003) examined the competitive relationship between the two TME strains in hamsters. Cerebral PrP^{Sc} accumulation was apparent after DY TME inoculation into brain and sciatic nerve while no proteinase-resistant PrP could be found in tissues of the lymphoreticular system. Intraperitoneal injection of infectious agent was not successful in producing TME infection. Thus the DY strain pathogenesis does not seem to include replication processes in lymphoid organs. Nevertheless, when intraperitoneal superinfection of HY TME agent followed that of DY, animals exhibited TME infection. Clinical phenotype and strain properties were those of the HY TME strain though incubation times were longer than usually observed for this strain. Interestingly, this effect depended on the interval between both inoculations, as it was present within a 60 day-timespan but absent when 30 days lay between inoculations. Also there was no delay in incubation time when both inoculi were injected into sciatic nerve (DY strain) and intrasciatic nerve (HY strain). These findings indicate that DY TME infection can extend incubation times of the HY agent upon extraneuronal inoculation. Thus competition between both agents might take place at a replication site prior to infection of lymphoreticular and nervous system. The existence of distinct TME strains, which give risen to specific phenotypes, makes it difficult to determine whether genetic host factors might modify disease susceptibility and pathogenesis in mink. The mink PrP gene is located on

chromosome 11 (Khlebodarova *et al.*, 1995), consists of a 257 amino acid chain and shares up to 90% sequence homology to other mammalian PrPs (Kretzschmar *et al.*, 1992). It is yet unclear if there are influential polymorphisms to be found within the coding region of mink *Prnp* or in its UTRs.

Feline Spongiform Encephalopathy

Feline Spongiform Encephalopathy (FSE) is a TSE found in domestic cat and feline zoo animals. It was recognised for the first time in 1990 in domestic cats in the UK (Wyatt *et al.*, 1991). The clinical symptoms resemble those seen in other TSEs. Among the most common presentations are impairment of motoric functions, alteration of behaviour and sensorial disturbances. Ataxic and incoordinated gait, nervousness, anxiety or otherwise aggressiveness and increasing isolation are seen in affected cats (Wyatt *et al.*, 1991). Often there is also hypersensibility to touch and noise or hypersalivation present in FSE animals.
The neuropathological presentation is dominated by spongiform changes in the grey matter of the brain and broadly distributed vacuolisation accompanied by astrocytosis. Protein rods also seem to be a feature of FSE in cats (Wyatt *et al.*, 1991, Bratberg *et al.*, 1995, Leggett *et al.*, 1990). Pearson *et al.* (1992) examined the brains of 18 suspected FSE-cases. Of these, five were confirmed as FSE-affected presenting the typical lesions of the brain, presence of PrP^{Sc} and fibrils that consisted of the abnormal protein. The average age of the infected animals was 6.4 years, which is well in accordance with observations in other FSE-cases.
Ryder *et al.* (2001) investigated the distribution of PrP in extraneuronal tissues of FSE-affected cats. In most cases the lymphoid organs were free of PrP^{Sc} although in some cases PrP accumulation in the spleen and Peyer's patches was detectable and kidneys of all examined animals were found to be positive for PrP immunostaining. In a case of FSE that had been examined by Lezmi *et al.* (2003) the disease was recognised in a captive cheetah in France. Initial clinical signs were mainly progressive ataxia and weight loss despite of undiminished feed uptake. In the final stage hyperaesthesia became apparent while the ataxia had led to terminal recumbence. Duration of disease beginning at the occurrence of clinical signs was approximately eight weeks. The brain of the FSE-affected animal showed vacuolisation of the cerebellum accompanied by the presence of PrP^{Sc}. PrP^{Sc}

deposits were also observed in the cerebral cortex, grey matter of the brain stem and spinal cord. Additionally, retina and lymph nodes, kidney and adrenal gland were immunostained for PrP^{Sc}. In contrast, no PrP^{Sc} could be found in the enteric nervous system. This distribution of the abnormal PrP form shares characteristics with pattern of other TSEs both in humans (e. g. retinal accumulation of PrP^{Sc} in sCJD and vCJD) and animals (e.g. PrP^{Sc} in ovine scrapie cases).
Inoculating mice with FSE agent from brain homogenates of affected cats can distinguish FSE strains. Fraser *et al.* (1994) thus recorded incubation periods and patterns of brain lesions for FSE- and BSE-affected mice. A similarity could be observed between both TSEs, which suggests that both types of infectious agent might be of common origin. Cats could have been infected by feed containing BSE agent, which led to the development of FSE in the feline species.
To date, no genetic factors have been identified that might modulate FSE disease characteristics due to the low number of cases.

Spongiform Encephalopathy in Zoo Animals

TSEs do not only occur in free-ranging or farm animals, zoological gardens have also reported a number of cases among their inhabitants, both imported and born in captivity. Among the animals in which spongiform encephalopathies could be observed were feline, cheetah, puma, ocelot, tiger, and lion. Also kudu, oryx, nyala, eland, chamois and bison were affected.
FSE in captive animals has been recognised since the early 90ies. Willoughby *et al.* (1992) first reported the disease in a puma that was five years of age when clinical symptoms emerged. Progressive ataxia, gait disturbance and body tremor were seen in the puma born at an English zoo. Histopathology showed typical properties of spongiform encephalopathies such as brain lesions and gliosis. PrP immunostaining was positive in medulla and spinal cord. The animals had to be killed after disease duration of six weeks. Another exemplary case was that of an imported cheetah in an Australian zoo that showed locomotion impairment, imbalance and behavioural change (Peet and Curran, 1992). Grey matter in the corpus striatum, midbrain and thalamus was severely affected by spongiform lesion and the animal was killed at an age of five and a half years after a clinical course of four weeks. Other observations resemble these supposed cases of FSE in feline zoo animals. Common to these

incidences of FSE was the fact that the affected animals were fed with meat derived from culled cattle or other animals, either supplied from the zoo itself or from slaughterhouses. Thus it is very well possible that meat contaminated with BSE agent was fed to the animals who as a result succumbed to FSE. Recycling of the infectious agent within the zoo could have occurred.

A first case of spongiform encephalopathy was observed in a nyala that died in 1986 (Jeffrey and Wells, 1988). Ataxia of the hindlimbs and abnormal holding of the head finally led to its euthanisation after a 3-week-long duration of clinical signs. Histopathological findings resembled those seen in other TSEs and when mice were incubated with infectious material they succumbed with neuropathology comparable to that seen in BSE. It is notable that the nyala had been fed with MBM. More cases of spongiform encephalopathies were subsequently reported in eland (Fleetwood and Furley, 1990), oryx and kudu (Kirkwood *et al.*, 1990). Animals were mainly presenting progressive ataxia and gait impairment and TSE was diagnosed upon examination of the brains. Kirkwood *et al.* (1993) investigated TSE in kudu by several studies. Spongiform encephalopathy was detected in three kudus with no record of having been feed animal protein (Kirkwood *et al*., 1993). Only one among them had a clinical presentation of the disease although all three kudus were found to exhibit positive PrP immunostaining. This finding led to the suggestion that spongiform encephalopathy might be transmitted horizontally between animals. This view was supported by the case of another kudu that succumbed to disease but had never received ruminant-derived protein (Kirkwood *et al*., 1993). In this case both lateral and maternal transmission could have been the cause of TSE infection. A further possibility to study transmission dynamics was the introduction of a healthy kudu into a group of animals affected by the disease (Kirkwood *et al*., 1994). More than a year after the transfer the kudu showed clinical symptoms for eight weeks before it had to be killed. As a feed-borne infection of the animal was not likely according to the records both maternal and horizontal routes of transmission had to be taken into consideration.

From these case reports it can be concluded that feeding of contaminated feedstuff seems to be the major route in which the infectious agent was transmitted to zoo animals. In kudu, there is evidence for additional maternal or/and lateral transmission, which is difficult to distinguish from each other.

Cunningham *et al.* (2004) conducted mouse bioassay studies in order to investigate tissue distribution of infectious agent in kudu. BSE agent was found in skin, conjunctiva and salivary gland. This indicates the possibility of BSE transmission via a lateral route, possibly via saliva. Nevertheless, apart from lateral transmission, infection through feedstuff cross-contaminated with ruminant-derived protein cannot be excluded. Contamination in various stages of processing could have occurred even after the feed ban on potentially infectious material had been introduced in Great Britain in 1988.

Transmission of CJD to monkeys can lead to the development of a spongiform encephalopathy (Tateishi *et al.*, 1981). The disease has also been observed in rhesus monkeys (Bons *et al.*, 1996) and lemurs (Bons *et al.*, 1997) housed in zoos. After showing signs of a progressive neuropathological disorder tissues of these animals were examined by PrP immunohistochemistry. Monkeys exhibited vacuolisation of the neurons, astrogliosis and accumulation of abnormal PrP in the brain. The protein could also be detected in the gastrointestinal tract, tonsils and lymph vessels of the intestines in lemurs. All the animals had received animal-derived protein as part of their diet. To further investigate the pathogenesis of spongiform encephalopathy in zoo monkeys Bons *et al.* (1999) stained tissues of lemurs that had been orally inoculated with BSE agent for proteinase-resistant PrP. Although the infection was still subclinical in the animals, the protein could be found in tonsils, gastrointestinal tract and its lymphoid tissues and in the spleen. PrP^{Sc} was furthermore located in the CNS, namely the spinal cord and cerebral cortex. These findings coincided with those observed in zoo lemurs that had been fed with cattle-derived protein and later developed a neuropathological disorder. Neuropathology closely resembled the one present in the experimentally inoculated monkeys, even in subclinically infected animals. Consequently, more zoo animals than hitherto thought could have been infected by the BSE agent though clinical symptoms might have been absent.

Prion Disease in Pigs, Fish and Poultry?

As has been described a range of different species is liable to TSEs. Hitherto naturally occurring spongiform encephalopathy was not detected in the pig although animals were likely to be exposed to TSE agents stemming from scrapie-infected sheep or BSE-affected cattle. Successful transmission of BSE to the porcine species was only achieved through parenteral inoculation, i.e. simultaneous injection of BSE brain homogenate by three routes (intracranially, intravenously and intraperitoneally) (Dawson *et al.*, 1990, Wells *et al.*, 2003). Initial clinical signs of the disease consisted in change of behaviour, reduced appetite and slight ataxia, which were followed by increased confusion, impairment of gait and other locomotor functions. In the terminal stages tremor was apparent and animals were continuously recumbent. Incubation periods ranged between 74 and 163 weeks. BSE infection in pigs was characterised by severe vacuolisation of the brain neuropil in which changes in the forebrain were predominant (Ryder *et al.*, 2000). The extent of vacuolisation seems to depend on the progress of disease (Wells *et al.*, 2003). PrP accumulation patterns in the CNS were similar to those seen in spongiform encephalopathies of other species. PrP immunostaining was found in the neuropil, glial cells and neurons and increased in intensity during the terminal stages of infection. Though being easily distinguishable from BSE-affected animals, healthy control pigs exhibited vacuolisation of the neuropil and hypothalamus to a limited extent. In contrast to these experimental findings oral transmission of BSE by feeding of BSE brain homogenate to pigs proved unsuccessful (Dawson *et al.*, 1990, Wells *et al.*, 2003). Infectivity was not even detected in the digestive tract of the animals until seven years after threefold inoculation with infectious agent. Recently, transgenic mice have provided an opportunity to investigate the species barrier between cattle and pig. Castilla *et al.* (2004) inoculated porcine PrP transgenic mice (poTg mice) via the intracerebral route. Animals treated with a high dose of the agent exhibited clinical symptoms, vacuolisation and gliosis in the brain while in 14% of these animals PrP^{res} could be found. When inoculated with a reduced dose of BSE agent no clinical symptoms were observed nor could PrP^{res} be detected in the mice. Despite this, second passage of the agent was more successful. Brain homogenate of animals that had previously been classified as negative for the presence of PrP^{res} was inoculated in poTg mice. These latter animals exhibited neuropathology of prion disease and one

third of the mice were tested positive for PrP^{res} in Western blot analysis. Thus the initial inoculation of poTg mice seems to have led to subclinically infected mice whose brain homogenates were sufficiently infectious to cause spongiform encephalopathy in other poTg mice. Neuropathology in the latter animals was similar to the one observed for the first passage.

As other non-ruminant species such as lemurs are susceptible to BSE infection, the question remains as to why pigs are not. Oral infection has been found to be less efficient compared to intracerebral inoculation in other species such as sheep. Thus, the species barrier between cattle and pig seems to be high enough to prevent pigs from contracting natural TSE while being exposed to the BSE agent through MBM feeding. There is still no explanation as to why BSE incubation times in pigs vary widely between animals. This may be an effect of the species barrier, which is influenced by the strain of the infectious agent and the host genotype. A comparison of PrP gene sequences shows that although a similarity exists there are single polymorphic residues that may account for the species barrier. As the BSE agent is exceptionally invariable during transmission, incubation time variance could be due to this genetic disposition in the recipient. Martin *et al.* (1995) sequenced the coding region of the PrP gene in six pigs. No differences were apparent between the sequences that encode for a 257 amino acid protein. In a further approach a total number of 66 pigs stemming from 12 different breeds were examined for PrP gene heterogeneity (Lipp *et al.*, 2004). The prion protein gene was amplified from genomic DNA and sequenced. No variation could be observed in the sequence and the results were identical to the porcine sequence that had been published beforehand (Martin *et al.*, 1995). The pig breeds included common breeds e.g. Piétrain but also Chinese Meishan and wild boar. From these results it can be suggested that the porcine PrP gene is, contrary to what is observed in other species such as sheep or cattle, homogeneous to a high extent. This together with the relative resistance of pigs to TSE infection implies that the risk of transmission of TSEs to this species is unlikely.

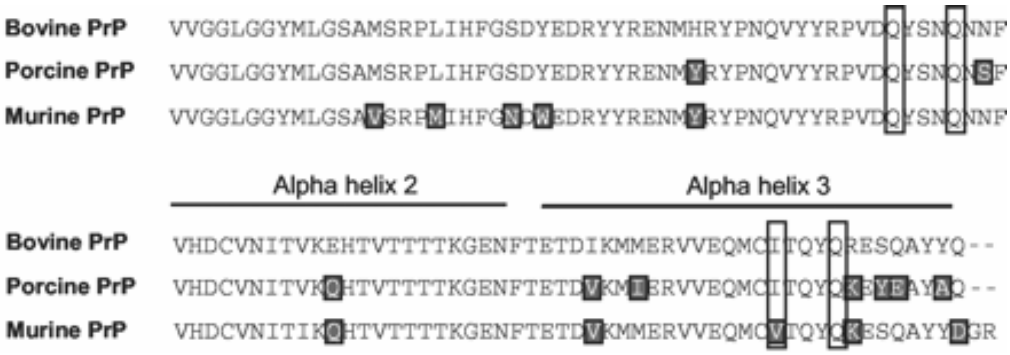

Figure 9: Alignments of amino acid sequences of bovine, porcine, and murine PrPs. (Castilla *et al.*, 2004)

White letters with grey shading represent amino acid changes with respect to the bovine sequence. Vertical boxes indicate the protein X epitope.

Other species that are kept for food production have been fed with feed components, which contained ruminant-derived protein, namely poultry and fish. Theoretically, they might develop spongiform encephalopathies.

Homologues of the tetrapod prion proteins have been demonstrated to exist in Atlantic salmon and pufferfish (Oidtmann *et al.*, 2003). The genes exhibit sequence homology and common structural features to PrP^C-encoding genes. Fish prion mRNA was also identified by Rivera-Milla *et al.* (2003) but differed widely from the one seen in mammals (see Figure 9). It is thus unlikely that fish could contract prion diseases from contaminated feed. Among other factors, the species barrier is influenced by the degree of homology in the PrP^C amino acid sequence that two species bear to each other. However, the understanding of the species barrier is still far from complete, so fish prion-like proteins and their association to their mammalian counterparts remain to be investigated.

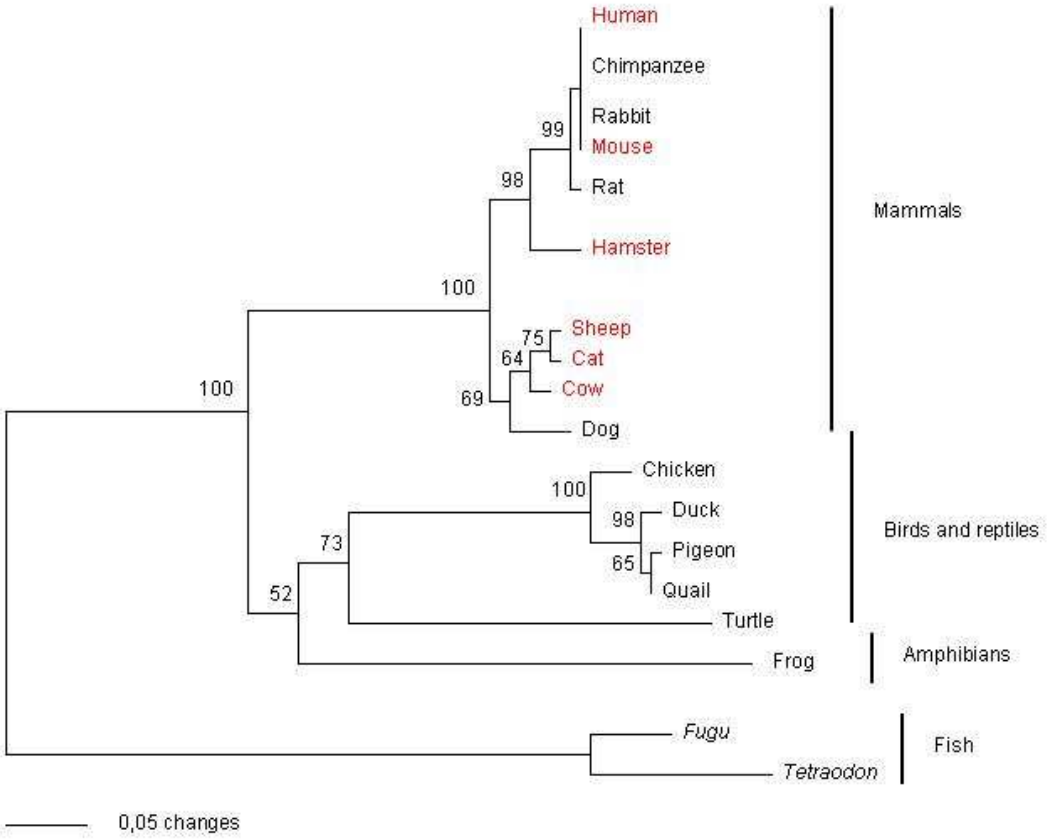

Figure 10: Phylogenetic relationships among vertebrate prion proteins (Rivera-Milla et al., 2003)

The neighbour-joining tree is based on genetic distances between amino acid sequences of globular domains. Numbers at the internodes are bootstrap confidence values (1000 replications). The horizontal scale bar indicates genetic distance. Taxa known to develop prion disease are shown in red.

In chicken a prion-like protein was identified by Harris *et al.* (1991). The sequence identity to mammalian prion proteins such as mouse PrP^{C} is rather low (33%) but structural compartments are similar. The newly-discovered protein comprises a repeat sequence of hexapeptides and hydrophobic regions. It was termed ch-PrPLP (chicken prion protein-like protein) and also possesses a GPI anchor with which it is attached to the cell surface. Its expression is limited to spinal cord and brain. A further examination of the ch-PrPLP gene revealed that a single-copy gene encodes the protein and that its ORF is confined to a single exon (Gabriel *et al.*, 1992). Leaving aside variation in the N-terminal repeat region and signal sequences, ch-

PrPLP is approximately 55% homologous compared to mammalian prion protein. The mRNA localization was investigated by in situ hybridisation, which revealed the presence of ch-PrPLP in neurons of the CNS. It is distributed throughout the brain and spinal cord in adult animals, in embryos it can additionally be detected in nonneuronal tissues such as intestine and heart (Harris *et al.*, 1993). In order to clarify the function of the suggested chPrP, copper binding ability of the N-terminal repeats was tested (Hornshaw *et al.*, 1995). Synthetic peptides of the hexarepeats were found to bind copper ions. This was interpreted as a hint at a possible function of chPrP in copper metabolism, which is also suggested for mammalian proteins. However, mass spectrometry and dichronism studies of chPrP recombinant cDNA showed that this does not seem to be true for the mature chPrP. No copper-binding ability was observed though the presence of Cu^{2+} ions destabilised the protein. According to these results, the functions of chicken PrP^{C} could be different from those of mammalian prion proteins, possibly in intercellular processes or embryonic development. Taken together, the existence of a chicken PrP^{C} might raise concerns regarding spongiform encephalopathies in poultry. Based on the low degree of homology between chPrP and mammalian PrPs a feed-borne infection by BSE or scrapie agent seems rather unlikely.

Candidate Genes in Prion Diseases

Genetic dispositions to prion diseases have been already found in a range of species. Polymorphisms that increase or decrease susceptibility have first been described within the coding region of the PrP gene. Consequently, this area provided the main focus of experimental studies. Recent experiments have raised the question as to the role of regulatory factors in the untranslated regions of the *PRNP* gene. Polymorphisms in these regions could have a considerable influence on genetic liability to disease through varying regulation of PrP gene expression. Furthermore, there are a number of proteins that could be involved in the overall context of prion diseases and accordingly are considered as candidate genes for susceptibility.
It has already been mentioned that chaperones (e.g. protein X) might aid the conformational conversion of PrP^{C}. If this is true, variability in the genes encoding such chaperones could affect the rate of transition or the amount of PrP^{Sc} produced

during pathogenesis. Specific agents can not only influence the conversion, but also the metabolism of PrP^{C} and PrP^{Sc}.

The Laminin Receptor

In a yeast two-hybrid system, interaction between the laminin receptor precursor (LRP) and PrP^{C} was identified (Rieger *et al*., 1997). By mapping analysis a common binding domain of PrP^{C} and laminin was localised between amino acids 161 to 180 of LRP. In insect and mammalian cells the same interaction was observed *in vivo*. In murine scrapie–infected neuroblastoma cells (N2a cells), LRP concentration was considerably increased, as were LRP levels in brain and spleen of scrapie-infected mice. In contrast, after inoculation with BSE, mice showed no alteration in LRP levels in these organs, contrary to findings in a hamster model in which a dramatic elevation of LRP expression in brain was confirmed. LRP levels were found to be correlated with PrP^{Sc} accumulation in rodent models. These results implicated a role of the cell-surface laminin receptor precursor in PrP interaction, possibly as a receptor or co-receptor. Evidence for such a relationship was found by Gauczynski *et al.* (2001). They were able to confirm localisation of the 37 k-Da LRP on the cell surface of N2a cells. It has been demonstrated that the mature form of the protein, the 67 k-Da laminin receptor (LR), is present on the cell surface (Gauczynski *et al.*, 2001). This was also the case in the former study. Consequently, the 37 k-Da LRP/67 k-Da LR system was supposed to constitute a receptor for PrP^{C}. The C-terminal domain of the laminin receptor precursor was apparently directed to the extracellular space. Thus PrP^{C} would be allowed to interact with binding domains of the precursor molecule. In order to identify these specific binding domains for PrP^{C}, Hundt *et al.* (2001) employed a yeast two-hybrid system and PrP-binding assays in both neuronal and non-neuronal cells. Yeast two-hybrid analysis revealed that the region between amino acid residues 144 and 179 of PrP^{C} is a direct LRP-binding domain, termed PrPLRPbd1 which results were corroborated by cell-binding assays. In these assays the presence of a second interaction domain (PrPLRPbd2) was indicated which is localised between amino acid positions 53 and 93 of PrP^{C}. Moreover, the experimental assays demonstrated that the latter binding domain was heparan sulfate proteoglycan (HSPG)-dependent. Binding domains on the LRP were also identified in the study. The direct PrP interaction was localised between residues 161

and 179 of the precursor while there was evidence of another HSPG-dependent binding domain either in the area between amino acid position 101 and 160 or 181 and 285 of the precursor molecule. The authors proposed a model of LRP-LR function as a PrP^C receptor. In this model binding takes place directly via PrPLRPbd1 and in a HSPG-dependent fashion via PrPLRPbd2. While simultaneous binding to both interaction domains of the receptor the binding complex with PrP^C would be significantly stabilised (see Figure 10). This model suggests a mechanism in which interaction between the PrP^C and the LRP would faciliate possible functions of the protein, among them cell to cell interaction.

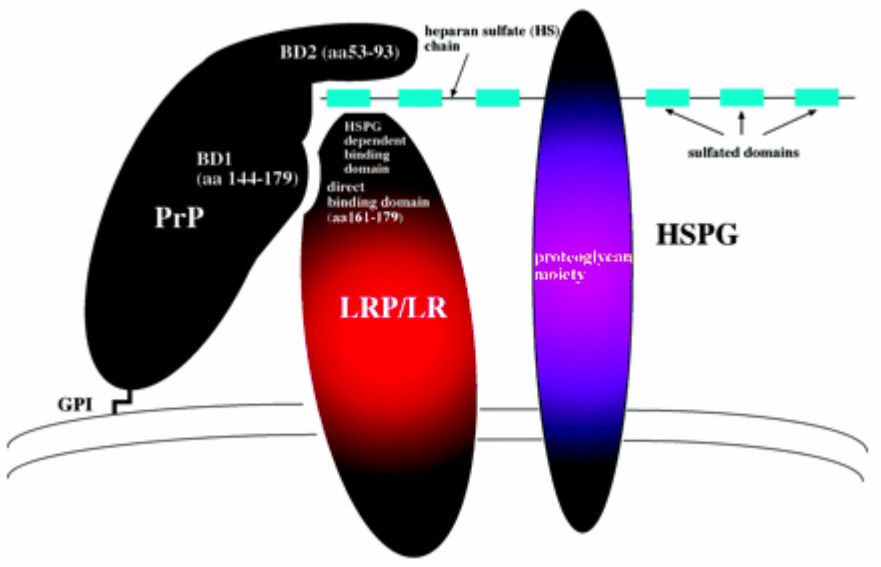

Figure 11: Model for the function of LRP-LR as receptor for PrP (Hundt *et al.*, 2001)

The PrP molecule binds to LRP-LR via PrPLRPbd1 and PrPLRPbd2. PrPLRPbd2 (aa 53-93) is dependent on the presence of a heparan sulfate arm of a HSPG molecule whereas PrPLRPbd1 (aa 144-179) interacts directly with LRP-LR.

Leucht *et al.* (2003) further examined the role of the 37 k-Da/67 k-Da LR in PrP^{Sc} propagation in a neuronal cell model. The accumulation of the PK-resistant protein was prevented in scrapie-infected cells by three different approaches. Cells were either transfected with antisense LRP-RNA-expression plasmid, LRP mRNA-specific siRNAs or incubated with anti-LRP/LR-antibodies. The first two knockdown approaches were efficient in reducing the expression of the targeted receptor thereby preventing accumulation of PrP^{Sc} in the cells. An additional effect was the reduction

of PrP^C levels. After a seven-day time span the same effect was observed in the approach employing antibodies.

Taken together, by knocking down LRP/LR on the surface of cells or blocking the binding sites to interaction, prion propagation in cells can be abolished. This might be due to a blockage of the PrP^C internalisation process although the cell could express a low amount of PrP^C. The negative effect of laminin receptor inhibition on PrP^{Sc} accumulation and propagation in neuronal cell cultures could be due to lack of PrP^C in the endocytic pathway of the cell. Otherwise, the LRP/LR system on the cell surface itself could be involved in PrP^{Sc} formation so that blockage of receptor expression would prevent the conversion process.

Shmakov *et al.* (2000) tried to localise the 67 k-Da LR in human small intestinal mucosa. Immunohistochemical proceedures and a monoclonal antibody were used to determine LR expression in duodenal and jejunal biopsy samples. Two patterns of LR expression were observed in this study. The first of them consisted of LR expression in the brush border of intestinal epithelial cells, in the Golgi apparatus of enterocytes and in secretory granules of Paneth cells. The other pattern consisted in an immunostained endothelium while the epithelium was found to be negative for the presence of the receptor. The mechanisms of this differential LR expression in the brush border have yet to be determined. Nevertheless, secretory and endocytic functions are implied by LR expression in the brush border of epithelial cells and in secretory granules. There may also be a connection of the expression patterns with sensitivity to prion disease infection via the oral route. Individuals possessing LR expression in the brush border of intestine cells might be more liable to such an infectious challenge as binding sites for pathogens i.e. prions would be provided to a greater extent.

The fact that a number of isoforms of the LR exist has been an object of speculation in connection with PrP^C binding. Simoneau *et al.* (2003) addressed the question which of those isoforms can interact with the receptor. Purification of LR-rich mouse brain fraction and ensuing overlay assays identified such LR forms of 44, 60, 67 and 220 kDa. The isoforms were corresponding to distinct maturation states of the LR molecule. All of these receptor variants were detected in the mouse brain samples and in addition to this all of them were found to bind PrP^C. These findings highlight the importance of the LR as a PrP^C interaction partner in the brain and its involvement in putative physiological functions of PrP^C.

In conclusion, the LRP/LR system appears to play a significant role in interaction with PrP^{C} and its metabolism. Impairment of receptor function has been shown to inhibit PrP^{Sc} propagation in cell culture, so that LRP/LR seems to be involved in mechanisms of prion disease pathology. Differential expression of the receptor on the surface of intestine epithelial cells may also affect the susceptibility of individuals to prion infection. These implications qualify LRP/LR gene as a candidate in connection with the susceptibility to prion diseases.

The Neurotrophin Receptor

Another receptor, the low affinity neurotrophin receptor ($p75^{NTR}$), was shown to mediate the effects of a neurotoxic PrP^{C} fragment (PrP(106-126)) (Della-Bianca *et al.*, 2001). In presence or absence of the receptor human neuroblastoma cells were challenged with the peptide and cell damage following this treatment was analysed. PrP(106-126) toxicity induced cell death in presence of $p75^{NTR}$ already at low concentration while no negative effect was visible when the receptor was absent. Thus $p75^{NTR}$ might modulate prion neurotoxicity in cells. In further experiments, binding of PrP^{C} to the receptor was demonstrated as well as the involvement of both the extracellular and intracellular $p75^{NTR}$ domain in the cytotoxic effects of PrP(106-126). Activation of the receptor was therefore necessary to elicit neurotoxicity. If the same was true for PrP^{Sc} $p75^{NTR}$ would represent a molecule involved in mediation of neurotoxic effects in prion diseases.

The Doppel Protein

It has been mentioned above that a homologue of PrP^{C} designated doppel (Dpl) is encoded by *Prnd*, downstream of the prion protein gene. Dpl exhibits 25% identity of amino acids when compared to PrP^{C}. Resemblance to PrP^{C} is also apparent regarding structural properties. Dpl formation is mainly α-helical and intra-molecular disulfide bonds stabilise its conformational state. Like PrP^{C}, Dpl is attached to the surface of cells by a GPI anchor. In contrast to the ubiquitous expression of PrP^{C}, Dpl expression is limited to the reproductive system. Similar functions for PrP^{C} and Dpl have nevertheless been suggested because of the similarities between both proteins.

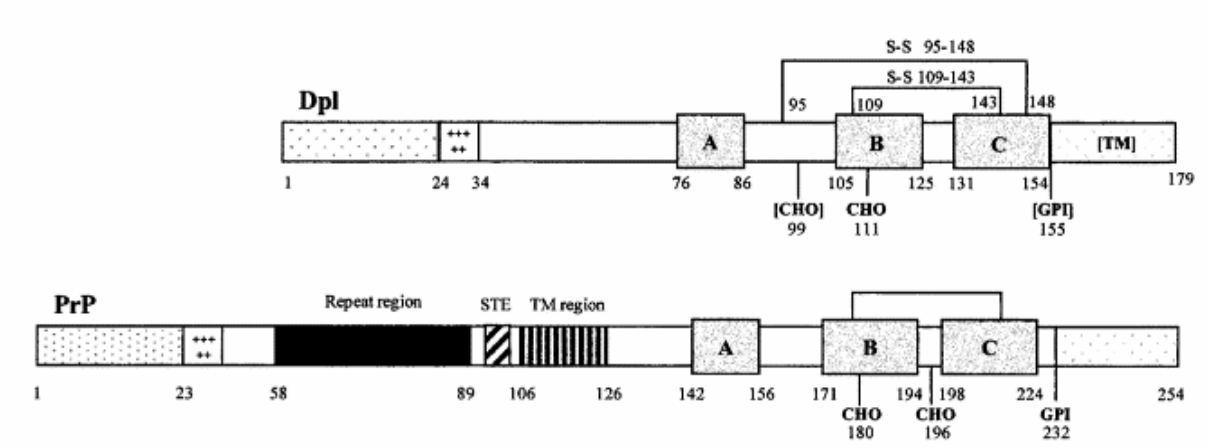

Figure 12: Sequences of (murine) Dpl and PrP showing the relative positions of key structural features (Whyte *et al.,* 2003)

Grey boxes: α-helices A, B and C; dotted boxes: N-terminal and C-terminal cleavable signal peptides (predicted N-terminal signal peptide cleavage site at Dpl residues 24 or 27, C-terminal cleavage and GPI addition site predicted at codon 155); CHO: Asn-linked glycosylation sites; GPI: glycosylphosphatidylinositol addition sites. Disulfide bonds are as indicated.
Black boxes: octa-repeat region; TM region: transmembrane region containing the palindromic sequence (112–119); STE: stop transfer effector region; box with vertical stripes: transmembrane region (TM); white box with the + symbols: cluster of basic residues.
The numbering of both sequences is that of the mouse.

It has recently been reported that, like PrP^C, Dpl can interact with the 37 k-Da LRP (Yin *et al*., 2004). In a yeast two-hybrid system, Dpl bound to the LRP which was afterwards confirmed in transfected cell lines and in tissue extracts. Mapping approaches showed that the interaction site of Dpl with LRP was located between amino acids 100 and 220, in the middle segment of LRP. Moreover, interaction between Dpl and the receptor was inhibited by the deletion of an N-terminal region of 54 amino acids in LRP. It has been suggested that the N-terminal deletion might lead to shielding of residues 100-220 necessary for proper receptor interaction with Dpl. Interaction between PrP^C and Dpl has been suggested since the negative effects of Dpl in certain knockout mouse lines described above had been reported. Massimino *et al.* (2004) examined the interactions of human Dpl and PrP^C in neuroblastoma cells. Analysis of the distribution of both proteins in single and double transfected cells showed that Dpl and PrP^C co-localise on the plasma membrane to a

considerable extent. In transfected cells expressing both Dpl and PrP^C, immunofluorescent labelling signals indicated that the proteins share microdomains of the plasma membrane. This implies that both proteins can interact with each other and are apparently endocytosed together. Additionally, the Dpl glycosylation pattern was determined and compared to that of PrP^C. As the mature Dpl protein is shorter in length than PrP^C and a range of glycoforms comparable to those seen in PrP^C was observed, glycosylation of human Dpl appears to be more complex and highly heterogeneous. Nevertheless, the obvious similarities which both proteins exhibit in their properties and their physiological features implicated them in common functions in the organism.

One of the functions suggested for PrP^C is the binding of copper in synaptic regions. The major site of copper binding in PrP^C is the octapeptide repeat region. As the Dpl protein lacks such an octarepeat region it was to be expected that copper binding would not be detected. Despite of this Qin *et al.* (2003) investigated such a putative ability in a rodent Dpl peptide (101-145) by MALDI mass spectronomy. Further analyses revealed that copper ions were indeed bound selectively to an α-helical region of the peptide. Possibly additional copper binding sites are present in the Dpl protein. Like in PrP^C, the copper binding ability of Dpl might have implications for the neurotoxic effects of Dpl. Cereghetti *et al.* (2004) further clarified copper binding properties of Dpl. Analysis of *in vitro* copper(II) binding of human recombinant Dpl demonstrated that binding took place in a different pattern as compared to PrP^C. In addition to two affinity sites at physiological pH, two other copper binding sites were observed at lower pH levels. Electron paramagnetic resonance analyses revealed that the complexes composed of Dpl protein and copper ions possessed a different number and co-ordination sphere than PrP^C-copper complexes. Consequently, there might be distinct structural and functional effects of Dpl copper binding which might be an explanation why both Dpl and PrP^C are located in different tissues in an organism and why a Dpl isoform comparable to PrP^{Sc} has not been found.

It has been previously described that Dpl overexpression causes Purkinje cell loss and ataxia in *Prnp* knockout mice. Furthermore, overexpression of N-terminally truncated PrP^C in Purkinje cells of transgenic mice causes a similar cerebellar syndrome in the animals (Flechsig *et al.*, 2003). Co-expression of the complete PrP^C could abolish the negative effects of the transgene. As mentioned above the same is true for neurological syndromes caused by Dpl overexpression. A common

pathologic mechanism of both the truncated PrP^{C} and Dpl might be responsible for this effect. Impairment of a cell signalling pathway might be such a mechanism. Anderson *et al.* (2004) therefore generated transgenic mice that selectively expressed the Dpl protein in Purkinje cells. Ataxia and Purkinje cell loss were afterwards apparent, similar to the phenotypes observed in N-terminal truncated PrP transgenic mice. The dose-dependent ataxia caused by moderate Dpl expression in Purkinje cells was counterbalanced by the presence of two *Prnp* alleles in the mice. These results indicate a common mechanism by which negative cellular effects of Dpl and N-terminally truncated PrP^{C} are mediated, though the exact nature of this process is yet unclear. The impact of deletion of the PrP^{C} N-terminal domain points to a critical involvement of this region. Intracellular trafficking or the physiological function of PrP^{C} might be altered by the deletion, which might be also true for the structurally similar Dpl protein.
Taken together, expression of Dpl and N-terminally truncated PrP^{C} elicit considerably similar phenotypes arising through Purkinje cell death. This implies common mechanisms of Dpl and PrP^{C} by which these changes are caused and highlights the physiological relationship between both proteins. In a further approach by Yamaguchi *et al.* (2004) the background of Zrch I *Prnp* knockout transgenic mice that are non-ataxic was used to study the effect of Dpl expression. The animals expressed the Dpl protein either in neurons or specifically in Purkinje cells. The knockout mice developed ataxia and Purkinje cell loss in contrast to $Prnp^{+/+}$ animals used as controls. Thus Dpl itself appeared to be toxic in this cell type while the presence of PrP^{C} rescued these effects. Functional implications of Dpl and N-terminally truncated PrP^{C} were once again highlighted by the development of comparable neuropathological phenotypes in the transgenic animals.

Plasminogen

In several species including human, sheep and cattle, plasminogen is able to form a complex with PrP^{Sc} (Maissen *et al.*, 2001). Plasminogen selectively precipitates with the infectious PrP^{Sc} from TSE-infected mouse brains. The same is true for PrP^{Sc} and human plasminogen in brain homogenate of sCJD patients, PrP^{Sc} and bovine plasminogen in BSE-infected cattle and PrP^{Sc} and ovine plasminogen in scrapie-

affected sheep of both the VRQ/AHQ and VRQ/ARR PrP genotype. The exact function which plasminogen fulfils in the complex has yet to be determined.
An important finding was made by Ellis *et al.* (2002) who investigated the effect of PrP^C on plasminogen activation. Tests were conducted in the presence or absence of copper. A PrP^C mutant lacking the octapeptide repeat region did not activate plasminogen, contrary to wildtype, which stimulated activation of the molecule. Interaction of plasminogen and PrP^C is apparently depending on copper levels and might be involved in prion disease pathogenesis. As has been mentioned above, activation of plasminogen by PrP^C in the CNS can furthermore be accomplished by t-PA-mediation (Epple *at al.*, 2004).
Kornblatt *et al.* (2003) demonstrated that the human plasminogen molecule binds to the ovine PrP^C. PrP^C was afterwards degraded to form a single fragment that contained the core structure of the protein, an effect also elicited by plasmin. Theoretically, this mechanism could constitute a protective process by which prion propagation could be prevented.

Protein X

Chaperones that have protective or enhancing functions on protein formation appear to be important binding partners of PrP^C. A molecule designated protein X has been implicated in the conversion process of PrP^C to PrP^{Sc}. Its existence had been suggested by experimental studies in transgenic mice. Telling *et al.* (1994) constructed mouse lines, termed Tg(HuPrP), which expressed the human PrP^C at much higher levels (4- to 8-fold) compared to endogenous mouse PrP^C. Inoculation of the transgenic animals with human prions less frequently led to infection than of wildtype controls. In order to further investigate this effect transgenic mice expressing a chimeric transgene of human and murine PrP^C (MHu2M mice) were generated by Telling *et al.* (1995). It was known that such chimeric transgenes led to transmission of either one of the corresponding prions, in this case either human or murine prions. MHu2M animals were highly susceptible to infection by human prions. In order to explain this apparent difference to Tg(HuPrP) mice, these latter animals were crossed with *Prnp* knockout mice. The animals resulting from this cross were susceptible to human prions, an effect which was only observed to a limited extent in offspring from crosses of Tg(MHu2M) with knockout mice. Thus the murine PrP^C

seemed to inhibit propagation of human prions except when the murine *Prnp* gene was ablated. The species barrier caused by amino acid difference in the PrP^{C} sequence between human and murine PrP^{C} is mainly determined by a central domain between residues 96 and 167 with which the proteins are thought to bind to PrP^{Sc}. It has been suggested that a different, non-central domain of PrP^{C} binds to a macromolecule designated protein X. The affinity of this binding appeared to be dependent on species-specific factors, i.e. it would be highest when prion protein and protein X stem from the same species. As even low levels of murine PrP^{C} in transgenic HuPrP mice abolished the conversional transition of PrP^{C} to the infectious isoform, the murine protein was proposed to have a higher affinity to protein X in comparison to human PrP^{C}. N-terminally truncated PrP^{C} elicited PrP^{Sc} formation, so that the PrP^{C} binding site of protein X might lie at the C-terminus (amino acid residues 215 and 230), a region differing in five positions between human and murine PrP^{C}. It is yet unclear whether protein X is a folding chaperone of PrP^{C}, comparable to heat-shock proteins, or otherwise chemically modifies PrP^{C} so as to convert to the PrP^{Sc} isoform. An alternative hypothesis is that protein X might aid in the interaction between PrP^{C} and PrP^{Sc}.

Kaneko *et al.* (1997) examined the putative protein X binding more closely in scrapie-infected murine N2a cells. In the transgenic mouse/human chimeric cell line amino acids at specific residues of the murine C-terminal PrP^{C} domain were replaced by those found in the human PrP^{C}. Changes at positions 214 or 218 inhibited the formation of PrP^{Sc} from murine PrP^{C}. Binding of protein X was suggested to take place through specific side chains of the residues that are part of the same surface in the C-terminal α-helix of PrP^{C}. The side chains themselves appear to form a discontinuous epitope with two residues of a neighbouring loop of the amino acid chain. PrP^{Sc} formation was also abolished by the substitution of a basic residue at positions 167, 171 and 218, which mutations seem to cause a dominant negative inhibition. They might bind to protein X and consequently would keep it from fulfilling its role in the conversion process of PrP^{C} to PrP^{Sc}.

The results of these studies suggest that a factor, probably a protein, may influence transition of PrP^{C} by an unknown mechanism and that putative binding sites have been identified that modulate these functions.

Heat Shock Proteins

Heat shock proteins (Hsp) are also implicated in prion propagation. Hsp70 expression was found to be increased in mice terminally ill with scrapie (Kenward *et al.*, 1994). It is not clear whether this is solely due to cell stress response. Hsps are chaperone molecules that can prevent the aggregation of prion proteins. Hsps therefore might be part of a protective system against detrimental effects of certain protein structures that have negative effects on cells. They bind and release hydrophobic regions of proteins which stops proteins from aggregating. This process also enables the proteins to refold into an innocuous conformational state. The finding that not only Hsp70 genes but also polyubiquitin C were increasingly expressed in murine scrapie infection supported the hypothesis of Hsps as chaperone molecules (Kenward *et al.*, 1994). Polyubiquitinisation is one of the mechanisms that can protect cells against negative effects of misfolded or aggregated proteins. Increased expression of both chaperones implies that both act as protectors against these unwanted consequences.

Notably, when human NT-2 cells were treated with heat shock stress not only the mRNA levels of the Hsp70 gene were increased but also those of the PrP gene (Shyu *et al*., 2002). This simultaneous increase of gene expression was also demonstrated for resulting protein levels. The rat PrP^{C} promoter used in the study was found to contain two heat-shock elements that interact with a heat-shock transcription factor. Thus, cellular stress apparently upregulated expression of both genes and synthesis of the proteins.

Contrary to Hsp70 in mammalian cells, in yeast the expression of Hsp104 is required for the propagation of infectious protein forms, e.g. [PSI^{+}] that are comparable to prions (Jung *et al*., 2002). This is probably due to the modifying effects of heat-shock proteins on prion formation. Curing of yeast prions can be accomplished by adding guanidine to the yeast culture. Notably, a mutation in the amino acid sequence of Hsp104 impaired the effects of guanidine in curing yeast prion. Theses findings suggest that mutations in heat-shock proteins may modulate prion propagation. The importance of heat-shock proteins in aggregation processes of yeast prions was further highlighted Krobitsch *et al.* (2000). The deletion of the gene encoding for Hsp104 stopped aggregation of fusion proteins. This result corroborates the suggestion that heat-shock proteins can be crucial for protein conversion processes.

Wegrzyn *et al*. (2001) further investigated yeast prion loss after Hsp inactivation. Three approaches were employed to secure the ablation of the Hsp: deletion of the Hsp104 gene, modification of the gene promoter leading to merely low levels of expression and overexpression of an inactive mutant of Hsp104. In each case loss of [*PSI*$^{+}$] prions was afterwards observed. When Hsp104 levels were reduced, prion aggregates decreased in number but increased in size. From these results it was concluded that Hsp 104 caused large prion aggregates to break up and favoured the formation of small aggregate structures or seeds that might lead to further prion propagation.

Hsp70, another member of the heat-shock family, might also be involved in prion maintenance. Recently, Roberts *et al.* (2004) investigated the propagation of yeast prions designated URE3 under the influence of cytosolic Hsp70 and a mutant form of the heat-shock protein. This latter form of Hsp70 contained an amino acid substitution in the peptide-binding domain of the molecule. In yeast, Hsp70, together with Hsp40 and Hsp140 forms can convert denatured proteins back to their normal form. It was surprising that URE3 was maintained in the cells under influence of Hsp70 but abolished when the heat-shock protein was mutated. Roberts et al. (2004) proposed a model in which Hsp70 continues to bind and released a URE3 prion binding domain. Through this mechanism, the protein becomes ever more unstructured. This situation changes in the presence of aggregated prions. Then, the Hsp70 can only interact with URE3 as long as the prion domain is exposed. In this case, while Hsp70 acts in order to refold proteins in a non-aggregated state, it might favour prion formation.

Studies of heat-shock proteins in yeast and mammalian cells have implied that this group of proteins plays an important role in the conversion process to prion-like states and aggregation of prions. The mechanisms by which this is achieved are yet not clear and there seems to be a range of factors that influence Hsp action, e.g. the aggregation state of the protein substrate. Nevertheless, Hsps are valid candidates for influencing the susceptibility to prion disease because of the pivotal effects their expression has on prion propagation.

Squalestatin and Clusterin

PrP^{Sc} formation leading to neurotoxicity in cells can be influenced by other molecules such as clusterin and squalestatin. Both proteins can prevent PrP aggregation in cells. Bate *et al.* (2004) investigated the effects of squalestatin, a protein involved in cholesterol metabolism of cells, on prion propagation. Three prion-infected cells lines (N2a, SMB and ScGT1 cells) were treated with squalestatin. The accumulation of PrP^{Sc} in these cells was reduced in a dose-dependent fashion following the treatment. Neuronal cells that were treated with squalestatin survived significantly longer after incubation with prions than did untreated cells. It has been suggested that squalestatin could have an effect on the trafficking of PrP^{C} in the cell. PrP^{C} thus could be prevented from interacting with other molecules, also with such necessary for conformational conversion to PrP^{Sc}. Cholesterol-sensitive processes have been implicated in modification of prion propagation and PrP^{Sc}-induced cell death, so that squalestatin might be a modulator of such pathogenic processes.

Clusterin, also designated apolipoprotein J, is a multifunctional heterodimeric glycoprotein. Clusterin mRNA is primarily expressed in the brain, ovary, testis and liver but also in other tissues such as heart, spleen or lung (DeSilva *et al.*, 1990). An impact of the protein on prion disease pathogenesis was first revealed when interaction between clusterin and soluble AD β-amyloid (sβA) was affirmed (Matsubara *et al.*, 1995). The mechanism was characterised with the help of a synthetic peptide ($A\beta_{1\text{-}40}$). The formation of complexes was specifically inhibited by clusterin at physiologic pH. These findings were furthermore corroborated by the experimental results of McHattie *et al.*(1999). The neurotoxic peptide PrP106-126 can spontaneously form fibrillar structures *in vitro* and therefore was used as a model for PrP^{C}. Clusterin was found to prevent maximal aggregation of the peptide, an effect was dose-dependent. The inhibition was reversed by the application of an antibody that binds to clusterin. This indicated that clusterin might interact with PrP^{Sc} in prion diseases thereby impairing the aggregation process. In a further approach the role of clusterin in BSE infection was investigated. Clusterin mRNA levels were significantly elevated in spinal cord of BSE field cases (McHattie *et al.*, 1999). Sites of increase in clusterin mRNA were neuroglia including the astrocytes. The amount of total mRNA did not differ between BSE-affected cattle and clinically normal control

animals, so that the differences were supposedly elicited as a reaction to BSE infection.

The localisation of clusterin with reference to PrP^{Sc} disposition was examined in other TSEs, namely in human prion diseases (GSS, CJD forms, FFI) and in a mouse model of human TSE (Sasaki *et al.*, 2002). Clusterin expression rose with duration of the disease. Co-localisation of clusterin with PrP^{Sc} deposits was observed and seemed to be dependent on the type of disposition. It was more pronounced in kuru plaques than in punctuate or synaptic PrP^{Sc} aggregates. In the mouse model an induction of clusterin in astrocytes was observed that was linked to PrP^{Sc} disposition. Why clusterin expression should be dependent on plaque type and PrP^{Sc} accumulation patterns could be explained in several ways. Dependency might be caused by the localisation site (extracellular or intracellular) of the protein and PrP^{Sc} aggregates. Intracellular PrP^{Sc} plaques of the synaptic type would be less accessibly to the extracellulary expressed clusterin molecules. Another possibility is an association with deposition properties of PrP^{Sc}, which would allow compact aggregates to interact more efficiently with clusterin. Increased astrocytic clusterin expression in this mouse model furthermore confirmed the findings of McHattie *et al.* (1999). In the mice, weak immunostaining for PrP^{C} was combined with enhanced presence of clusterin in later stages of the disease. This moreover indicates that clusterin might inhibit PrP^{Sc} aggregation. In fact, a recent report suggests that the effects of clusterin on prion disease pathology should not be underestimated. Clusterin knockout mice were inoculated with BSE prions and compared to wildtype controls by Kempster *et al.* (2004). Deletion of clusterin led to significantly increased incubation times (mean incubation time 386 days compared to 346 days in the wildtype). Astrocytosis was apparent in both groups of mice though more pronounced in the knockout animals. Furthermore, there was an increased number of diffuse proteinase-resistant PrP^{BSE} detected in the latter group. From these results one could conclude that clusterin may bind to PrP^{BSE} in order to sequester the protein. This mechanism might induce neurons to produce further PrP^{C}, which may serve as a substrate for prion propagation and neurotoxic aggregation. This feed back effect may in turn lower the expression of PrP^{C} if the misfolded protein remains unsequestered and decrease the growth in number of neurotoxic aggregates. At the same time PrP^{BSE}, which is not bound by clusterin, may stimulate astrocytosis. It is

nevertheless possible that other proteins interact with PrP^{BSE} in the absence of clusterin thus accelerating BSE incubation times in the knockout mice.
Although it seems likely that clusterin is involved in the aggregation process of misfolded proteins and in the pathogenesis of prion diseases, the exact mechanisms by which it exerts its impact are not yet clear.

Other Candidates

The relevance of other genes or gene products in connection with prion disease remains yet to be determined. Miele *et al.* (2001) employed a differential display reverse-transcriptase PCR (DDRT-PCR) approach in order to investigate differential expression of genes following TSE infection. Expression profiles of genetic transcripts in the spleens of TSE-inoculated laboratory mice were compared to those of wildtype controls. Increase of expression levels in spleen was delectable for a transcript exhibiting 100% homology to the erythroid differentiation-related factor (EDRF, also erythroid associated factor eraf). In the terminal stages of prion disease EDRF expression levels were increased in scrapie-infected mice. Northern blotting analysis of *EDRF* gene expression indicated that the distribution of the protein was limited to spleen, bone marrow and blood in mice and blood and bone marrow in humans. As EDRF seems to be associated with hematopoeitic activity, expression levels of the differentiation factor were examined in naturally BSE-infected and scrapie-infected sheep. Expression was restricted to bone marrow and spleen tissues in cattle and blood in sheep. The level of expression was significantly reduced in bone marrow in the initial clinical stages of BSE-infection and also in blood taken from sheep in the clinical stage of scrapie. A TSE infection influences the expression of EDRF in erythroid cells although the mechanism by which this effect is produced is not known. It can therefore not be excluded that EDRF may play a role in prion propagation or otherwise TSE pathogenesis.
Glycosylation differences between PrP^{C} and PrP^{Sc} could be caused by decreased activity of *N*-acetylglucosaminyltransferase III (*GnTIII*) in cells that produce PrP^{Sc} (Rudd *et al.*, 1999). Some cells forming PrP^{Sc} apparently show a diminished function of the enzyme although the cause and impact thereof needs to be clarified.

Taken together, a range of molecules appears to interact with PrP^{C} and/or PrP^{Sc} in different ways. The mechanism by which this takes place is not yet clear in many cases. Special emphasis has to be put on the relationship between PrP^{C} and Dpl, as the interaction between both proteins and studies of the Dpl protein itself provide important information about PrP^{C} metabolism and function. The LR is another important interaction partner of PrP^{C}. It appears to be involved not only in PrP^{C} life cycle within the cell but might modulate its action in the organism. Other molecules such as the heat-shock protein family may be directly involved in prion protein structural conversion and aggregation as well as cell mechanisms protecting against protein misfolding. For some substances e.g. clusterin, an association with PrP^{Sc} propagation is apparent though the definite mechanisms of this relationship are unclear.

Deletion of genes encoding for such interaction partners and also the introduction of mutations in the DNA sequence has been employed in order to study their effects on prion protein and prions. Specific regions of the amino acid sequence have been found to be necessary to maintain their functions. Thus mutations in the encoding genes may be of considerable importance for the modulation of prion disease pathogenesis and susceptibility. Consequently, they should be regarded as candidate genes for involvement in prion diseases. A better understanding of genetic disposition of an individual at such loci might provide an improved insight into prion disease-associated factors.

Genetic Implications in Prion Diseases

Many questions concerning the cause and course of prion diseases remain to be answered. Disease susceptibility and pathogenesis are two major factors that are influenced by genetic determinants of the host individual. Together with properties of the infectious agent such as strain type and prion structure, apparently host genetic dispositions are associated with distinct phenotypic disease characteristics. This also includes transmissibility of the agent between species, incubation times, pathological changes and clinical presentation.

There are several properties of PrP^{C} that are controlled by genetic factors. Its amino acid sequence is determined by the coding region of the PrP gene sequence. The sequence of the amino acids has implications for the protein conformation as well as

for the stability of such structures. Hydrogen bonding or salt bridges between certain amino acids can prevent α-helices from being destabilised. Mutational substitution of single amino acids can disturb this protective mechanism against misfolding. Moreover, polymorphisms in protein areas important for structural properties e.g. the octapeptide repeat region may modulate the conversion process. Therefore polymorphisms of the coding *Prnp* gene region appear to influence the susceptibility of PrP^{C} to shift to the infectious isoform PrP^{Sc}. Glycosylation differences also seem to be associated with structural conversion. How far they are determined by host genetic disposition is yet unclear.

The propensity of proteins to aggregate is not only dependent on the conformational stability of the protein that serves as a substrate. The higher the amount of protein present, the higher the risk that accumulation and, finally, aggregation of stable isoforms, may take place. Conversional transition might therefore be enhanced by the synthesis of high titers of PrP^{C} in cells. Expression levels of the PrP gene can thus indirectly influence prion propagation. As gene expression is regulated by the promoter area in the non-coding sequence of the gene, polymorphisms in this region may modulate PrP^{C} expression. A high amount of PrP^{C} could thus lead to faster aggregation and shorter incubation times in prion diseases. Apart from disease-associated factors that are determined by protein sequence, structure and expression, a number of factors has been identified that interact with the prion protein and/or its proteinase-resistant isoform.

Receptors probably mediate effects of PrP^{C} so that its functions might by impaired or modulated by mutations in genes encoding for such receptors. Other molecules are apparently involved in the formation of aggregates, which implies a role in structural conversion process. The Dpl protein has a special status in this context because of its similarity to PrP^{C}.

Genetic host factors also influence the susceptibility of one species to be infected with agent derived from another. The height of the species barrier is supposed to be dependent on the degree of homology between amino acid sequence of the prion agent and host PrP^{C}. Furthermore, variation in glycosylation of host proteins seems to influence transmission. Thus prion diseases should be more easily transmitted between species with a similar primary amino acid structure.

In human prion diseases implications of genetic disposition are relatively well known. A range of polymorphisms in the human *PRNP* gene has been defined and disease

phenotypes are classified by several characteristics. In the ovine species the association between distinct PrP^C alleles and disease susceptibility is especially pronounced. Although there may be additional genetic host factors and breed-specific differences the observed risk of scrapie infection is mainly dependent on three known polymorphic sites in the coding region of the PrP gene. In several other species such as mouse, goat and mink, polymorphisms in the coding region are known to affect disease characteristics.

In cattle, the genetic disposition in connection with BSE is less clear. Polymorphisms located in the coding region of the *PRNP* gene did not seem to have any effect on disease susceptibility. Nevertheless experiments in mice have highlighted the importance of the number of octapeptides in the PrP^C amino acid sequence. Therefore it might be possible that the allele carrying seven repeats elicits shorter incubation times in certain breeds, e.g. Brown Swiss, which has not yet been thoroughly investigated. A polymorphism in the promoter region has been described which is supposed to influence BSE susceptibility by modulating expression of the *PRNP* gene.

In species used for food production, genetic background may be of crucial importance. In the United Kingdom, a sheep breeding program already exists with the aim to eradicate genotypes susceptible to scrapie from the flock and favour breeding of less susceptible animals. It has been a matter of discussion whether such a program is appropriate considering the breed-specific differences in genotype susceptibility. As the infection of sheep with BSE prions cannot be excluded it is moreover necessary to develop a reliable surveillance system in order to identify putative ovine BSE cases. The role of genetic disposition in cattle has hitherto not been determined, so that breeding programs to this effect cannot yet be established. Other species such as poultry or fish seem to have a very low risk to be affected by prion diseases though they should be surveyed for the possible occurrence of spongiform encephalopathies.

The genetic background of humans and animals apparently constitutes an important influence both on the transmission between species and the readiness with which subjects succumb to disease. It is therefore necessary to further explore which structural properties of PrP^C can predispose for prion infection and what role differential expression of its gene may play in pathogenesis. Candidate genes, i.e. genes that either encode for proteins that interact with prions or PrP^C or exhibit

similar characteristics, have to be closely examined with regard to a possible effect on TSEs.

References

Aguzzi,A., Heppner,F.L., Heikenwalder,M., Prinz,M., Mertz,K., Seeger,H., and Glatzel,M. (2003). Immune system and peripheral nerves in propagation of prions to CNS. Br. Med. Bull. *66*, 141-159.

Alonso,D.O., DeArmond,S.J., Cohen,F.E., and Daggett,V. (2001). Mapping the early steps in the pH-induced conformational conversion of the prion protein. Proc. Natl. Acad. Sci. U. S. A *98*, 2985-2989.

Anderson,G.M. and Freytag,S.O. (1991). Synergistic activation of a human promoter in vivo by transcription factor Sp1. Mol. Cell Biol. *11*, 1935-1943.

Anderson,L., Rossi,D., Linehan,J., Brandner,S., and Weissmann,C. (2004). Transgene-driven expression of the Doppel protein in Purkinje cells causes Purkinje cell degeneration and motor impairment. Proc. Natl. Acad. Sci. U. S. A *101*, 3644-3649.

Andrews,N.J., Farrington,C.P., Cousens,S.N., Smith,P.G., Ward,H., Knight,R.S., Ironside,J.W., and Will,R.G. (2000). Incidence of variant Creutzfeldt-Jakob disease in the UK. Lancet *356*, 481-482.

Andrews,N.J., Farrington,C.P., Ward,H.J., Cousens,S.N., Smith,P.G., Molesworth,A.M., Knight,R.S., Ironside,J.W., and Will,R.G. (2003). Deaths from variant Creutzfeldt-Jakob disease in the UK. Lancet *361*, 751-752.

Antzutkin,O.N., Balbach,J.J., Leapman,R.D., Rizzo,N.W., Reed,J., and Tycko,R. (2000). Multiple quantum solid-state NMR indicates a parallel, not antiparallel,

organization of beta-sheets in Alzheimer's beta-amyloid fibrils. Proc. Natl. Acad. Sci. U. S. A *97*, 13045-13050.

Askanas,V., Bilak,M., Engel,W.K., Leclerc,A., and Tome,F. (1993). Prion protein is strongly immunolocalized at the postsynaptic domain of human normal neuromuscular junctions. Neurosci. Lett. *159*, 111-114.

Barbanti,P., Fabbrini,G., Salvatore,M., Petraroli,R., Cardone,F., Maras,B., Equestre,M., Macchi,G., Lenzi,G.L., and Pocchiari,M. (1996). Polymorphism at codon 129 or codon 219 of PRNP and clinical heterogeneity in a previously unreported family with Gerstmann-Straussler-Scheinker disease (PrP-P102L mutation). Neurology *47*, 734-741.

Baron,T., Crozet,C., Biacabe,A.G., Philippe,S., Verchere,J., Bencsik,A., Madec,J.Y., Calavas,D., and Samarut,J. (2004). Molecular analysis of the protease-resistant prion protein in scrapie and bovine spongiform encephalopathy transmitted to ovine transgenic and wild-type mice. J Virol. *78*, 6243-6251.

Bartz,J.C., Bessen,R.A., McKenzie,D., Marsh,R.F., and Aiken,J.M. (2000). Adaptation and selection of prion protein strain conformations following interspecies transmission of transmissible mink encephalopathy. J Virol. *74*, 5542-5547.

Begara-McGorum,I., Gonzalez,L., Simmons,M., Hunter,N., Houston,F., and Jeffrey,M. (2002). Vacuolar lesion profile in sheep scrapie: factors influencing its variation and relationship to disease-specific PrP accumulation. J Comp Pathol. *127*, 59-68.

Beisel,C.E. and Morens,D.M. (2004). Variant Creutzfeldt-Jakob disease and the acquired and transmissible spongiform encephalopathies. Clin. Infect. Dis. *38*, 697-704.

Belay,E.D. (1999). Transmissible spongiform encephalopathies in humans. Annu. Rev. Microbiol. *53*, 283-314.

Belay,E.D., Gambetti,P., Schonberger,L.B., Parchi,P., Lyon,D.R., Capellari,S., McQuiston,J.H., Bradley,K., Dowdle,G., Crutcher,J.M., and Nichols,C.R. (2001). Creutzfeldt-Jakob disease in unusually young patients who consumed venison. Arch. Neurol. *58*, 1673-1678.

Bendheim,P.E., Brown,H.R., Rudelli,R.D., Scala,L.J., Goller,N.L., Wen,G.Y., Kascsak,R.J., Cashman,N.R., and Bolton,D.C. (1992). Nearly ubiquitous tissue distribution of the scrapie agent precursor protein. Neurology *42*, 149-156.

Bernoulli,C., Siegfried,J., Baumgartner,G., Regli,F., Rabinowicz,T., Gajdusek,D.C., and Gibbs,C.J., Jr. (1977). Danger of accidental person-to-person transmission of Creutzfeldt-Jakob disease by surgery. Lancet *1*, 478-479.

Bessen,R.A. and Marsh,R.F. (1992). Identification of two biologically distinct strains of transmissible mink encephalopathy in hamsters. J Gen. Virol. *73 (Pt 2)*, 329-334.

Bessen,R.A. and Marsh,R.F. (1992). Biochemical and physical properties of the prion protein from two strains of the transmissible mink encephalopathy agent. J Virol. *66*, 2096-2101.

Bessen,R.A. and Marsh,R.F. (1994). Distinct PrP properties suggest the molecular basis of strain variation in transmissible mink encephalopathy. J Virol. *68*, 7859-7868.

Biacabe,A.G., Laplanche,J.L., Ryder,S., and Baron,T. (2004). Distinct molecular phenotypes in bovine prion diseases. EMBO Rep. *5*, 110-115.

Billinis,C., Panagiotidis,C.H., Psychas,V., Argyroudis,S., Nicolaou,A., Leontides,S., Papadopoulos,O., and Sklaviadis,T. (2002). Prion protein gene polymorphisms in natural goat scrapie. J Gen. Virol. *83*, 713-721.

Birkett,C.R., Hennion,R.M., Bembridge,D.A., Clarke,M.C., Chree,A., Bruce,M.E., and Bostock,C.J. (2001). Scrapie strains maintain biological phenotypes on propagation in a cell line in culture. EMBO J *20*, 3351-3358.

Bons,N., Mestre-Frances,N., Charnay,Y., Salmona,M., and Tagliavini,F. (1996). Spontaneous spongiform encephalopathy in a young adult rhesus monkey. C. R. Acad. Sci. III *319*, 733-736.

Bons,N., Mestre-Frances,N., Guiraud,I., and Charnay,Y. (1997). Prion immunoreactivity in brain, tonsil, gastrointestinal epithelial cells, and blood and lymph vessels in lemurian zoo primates with spongiform encephalopathy. C. R. Acad. Sci. III *320*, 971-979.

Bons,N., Mestre-Frances,N., Belli,P., Cathala,F., Gajdusek,D.C., and Brown,P. (1999). Natural and experimental oral infection of nonhuman primates by bovine spongiform encephalopathy agents. Proc. Natl. Acad. Sci. U. S. A *96*, 4046-4051.

Borchelt,D.R., Scott,M., Taraboulos,A., Stahl,N., and Prusiner,S.B. (1990). Scrapie and cellular prion proteins differ in their kinetics of synthesis and topology in cultured cells. J Cell Biol. *110*, 743-752.

Bosques,C.J. and Imperiali,B. (2003). The interplay of glycosylation and disulfide formation influences fibrillization in a prion protein fragment. Proc. Natl. Acad. Sci. U. S. A *100*, 7593-7598.

Bossers,A., de Vries,R., and Smits,M.A. (2000). Susceptibility of sheep for scrapie as assessed by in vitro conversion of nine naturally occurring variants of PrP. J Virol. *74*, 1407-1414.

Bradley,R. (1991). Bovine spongiform encephalopathy (BSE): the current situation and research. Eur. J Epidemiol. *7*, 532-544.

Brandner,S., Raeber,A., Sailer,A., Blattler,T., Fischer,M., Weissmann,C., and Aguzzi,A. (1996). Normal host prion protein (PrPC) is required for scrapie spread within the central nervous system. Proc. Natl. Acad. Sci. U. S. A *93*, 13148-13151.

Bratberg,B., Ueland,K., and Wells,G.A. (1995). Feline spongiform encephalopathy in a cat in Norway. Vet. Rec. *136*, 444.

Braun,U. (2002). [Clinical signs and diagnosis of BSE]. Schweiz. Arch. Tierheilkd. *144*, 645-652.

Brayton,K.A., O'Rourke,K.I., Lyda,A.K., Miller,M.W., and Knowles,D.P. (2004). A processed pseudogene contributes to apparent mule deer prion gene heterogeneity. Gene *326*, 167-173.

Brown,D.R. and Besinger,A. (1998). Prion protein expression and superoxide dismutase activity. Biochem. J *334 (Pt 2)*, 423-429.

Brown,D.R., Wong,B.S., Hafiz,F., Clive,C., Haswell,S.J., and Jones,I.M. (1999). Normal prion protein has an activity like that of superoxide dismutase. Biochem. J *344 Pt 1*, 1-5.

Brown,D.R., Clive,C., and Haswell,S.J. (2001). Antioxidant activity related to copper binding of native prion protein. J Neurochem. *76*, 69-76.

Brown,P., Goldfarb,L.G., and Gajdusek,D.C. (1991). The new biology of spongiform encephalopathy: infectious amyloidoses with a genetic twist. Lancet *337*, 1019-1022.

Brown,P., Preece,M., Brandel,J.P., Sato,T., McShane,L., Zerr,I., Fletcher,A., Will,R.G., Pocchiari,M., Cashman,N.R., d'Aignaux,J.H., Cervenakova,L., Fradkin,J., Schonberger,L.B., and Collins,S.J. (2000). Iatrogenic Creutzfeldt-Jakob disease at the millennium. Neurology *55*, 1075-1081.

Bruce,M., Chree,A., McConnell,I., Foster,J., Pearson,G., and Fraser,H. (1994). Transmission of bovine spongiform encephalopathy and scrapie to mice: strain variation and the species barrier. Philos. Trans. R. Soc. Lond B Biol. Sci. *343*, 405-411.

Bruce,M.E., McConnell,I., Fraser,H., and Dickinson,A.G. (1991). The disease characteristics of different strains of scrapie in Sinc congenic mouse lines: implications for the nature of the agent and host control of pathogenesis. J Gen. Virol. *72 (Pt 3)*, 595-603.

Bruce,M.E., Will,R.G., Ironside,J.W., McConnell,I., Drummond,D., Suttie,A., McCardle,L., Chree,A., Hope,J., Birkett,C., Cousens,S., Fraser,H., and Bostock,C.J. (1997). Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. Nature *389*, 498-501.

Bruce,M.E., Boyle,A., Cousens,S., McConnell,I., Foster,J., Goldmann,W., and Fraser,H. (2002). Strain characterization of natural sheep scrapie and comparison with BSE. J Gen. Virol. *83*, 695-704.

Bucciantini,M., Giannoni,E., Chiti,F., Baroni,F., Formigli,L., Zurdo,J., Taddei,N., Ramponi,G., Dobson,C.M., and Stefani,M. (2002). Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. Nature *416*, 507-511.

Bueler,H., Fischer,M., Lang,Y., Bluethmann,H., Lipp,H.P., DeArmond,S.J., Prusiner,S.B., Aguet,M., and Weissmann,C. (1992). Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. Nature *356*, 577-582.

Bueler,H., Aguzzi,A., Sailer,A., Greiner,R.A., Autenried,P., Aguet,M., and Weissmann,C. (1993). Mice devoid of PrP are resistant to scrapie. Cell *73*, 1339-1347.

Bunk,S. (2004). Chronic wasting disease-prion disease in the wild. PLoS. Biol. *2*, E121.

Calzolai,L., Lysek,D.A., Guntert,P., von Schroetter,C., Riek,R., Zahn,R., and Wuthrich,K. (2000). NMR structures of three single-residue variants of the human prion protein. Proc. Natl. Acad. Sci. U. S. A *97*, 8340-8345.

Caplazi,P.A., O'Rourke,K.I., and Baszler,T.V. (2004). Resistance to scrapie in PrP ARR/ARQ heterozygous sheep is not caused by preferential allelic use. J Clin. Pathol. *57*, 647-650.

Capucchio,M.T., Guarda,F., Pozzato,N., Coppolino,S., Caracappa,S., and Di,M., V (2001). Clinical signs and diagnosis of scrapie in Italy: a comparative study in sheep and goats. J Vet. Med. A Physiol Pathol. Clin. Med. *48*, 23-31.

Carlson,G.A., Goodman,P.A., Lovett,M., Taylor,B.A., Marshall,S.T., Peterson-Torchia,M., Westaway,D., and Prusiner,S.B. (1988). Genetics and polymorphism of the mouse prion gene complex: control of scrapie incubation time. Mol. Cell Biol. *8*, 5528-5540.

Carlson,G.A., DeArmond,S.J., Torchia,M., Westaway,D., and Prusiner,S.B. (1994). Genetics of prion diseases and prion diversity in mice. Philos. Trans. R. Soc. Lond B Biol. Sci. *343*, 363-369.

Casalone,C., Zanusso,G., Acutis,P., Ferrari,S., Capucci,L., Tagliavini,F., Monaco,S., and Caramelli,M. (2004). Identification of a second bovine amyloidotic spongiform encephalopathy: molecular similarities with sporadic Creutzfeldt-Jakob disease. Proc. Natl. Acad. Sci. U. S. A *101*, 3065-3070.

Castilla,J., Gutierrez-Adan,A., Brun,A., Pintado,B., Parra,B., Ramirez,M.A., Salguero,F.J., Diaz,S.S., Rabano,A., Cano,M.J., and Torres,J.M. (2004). Different behavior toward bovine spongiform encephalopathy infection of bovine prion protein transgenic mice with one extra repeat octapeptide insert mutation. J. Neurosci. *24*, 2156-2164.

Castilla,J., Gutierrez-Adan,A., Brun,A., Doyle,D., Pintado,B., Ramirez,M.A., Salguero,F.J., Parra,B., Diaz,S.S., Sanchez-Vizcaino,J.M., Rogers,M., and Torres,J.M. (2004). Subclinical bovine spongiform encephalopathy infection in transgenic mice expressing porcine prion protein. J Neurosci. *24*, 5063-5069.

Caughey,B., Raymond,G.J., and Bessen,R.A. (1998). Strain-dependent differences in beta-sheet conformations of abnormal prion protein. J Biol. Chem. *273*, 32230-32235.

Cereghetti,G.M., Negro,A., Vinck,E., Massimino,M.L., Sorgato,M.C., and Van Doorslaer,S. (2004). Copper(II) binding to the human Doppel protein may mark its functional diversity from the prion protein. J Biol. Chem. *279*, 36497-36503.

Cervenakova,L., Goldfarb,L.G., Garruto,R., Lee,H.S., Gajdusek,D.C., and Brown,P. (1998). Phenotype-genotype studies in kuru: implications for new variant Creutzfeldt-Jakob disease. Proc. Natl. Acad. Sci. U. S. A *95*, 13239-13241.

Chiesa,R., Piccardo,P., Ghetti,B., and Harris,D.A. (1998). Neurological illness in transgenic mice expressing a prion protein with an insertional mutation. Neuron *21*, 1339-1351.

Chiti,F., Taddei,N., White,P.M., Bucciantini,M., Magherini,F., Stefani,M., and Dobson,C.M. (1999). Mutational analysis of acylphosphatase suggests the importance of topology and contact order in protein folding. Nat. Struct. Biol. *6*, 1005-1009.

Chiti,F., Calamai,M., Taddei,N., Stefani,M., Ramponi,G., and Dobson,C.M. (2002). Studies of the aggregation of mutant proteins in vitro provide insights into the

genetics of amyloid diseases. Proc. Natl. Acad. Sci. U. S. A *99 Suppl 4*, 16419-16426.

Collinge,J., Palmer,M.S., and Dryden,A.J. (1991). Genetic predisposition to iatrogenic Creutzfeldt-Jakob disease. Lancet *337* , 1441-1442.

Collinge,J., Whittington,M.A., Sidle,K.C., Smith,C.J., Palmer,M.S., Clarke,A.R., and Jefferys,J.G. (1994). Prion protein is necessary for normal synaptic function. Nature *370*, 295-297.

Collinge,J., Sidle,K.C., Meads,J., Ironside,J., and Hill,A.F. (1996). Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. Nature *383*, 685-690.

Collinge,J. (2001). Prion diseases of humans and animals: their causes and molecular basis. Annu. Rev. Neurosci. *24*, 519-550.

Collins,S., McLean,C.A., and Masters,C.L. (2001). Gerstmann-Straussler-Scheinker syndrome,fatal familial insomnia, and kuru: a review of these less common human transmissible spongiform encephalopathies. J Clin. Neurosci. *8*, 387-397.

Collins,S.J., Lawson,V.A., and Masters,C.L. (2004). Transmissible spongiform encephalopathies. Lancet *363*, 51-61.

Cordeiro,Y., Machado,F., Juliano,L., Juliano,M.A., Brentani,R.R., Foguel,D., and Silva,J.L. (2001). DNA converts cellular prion protein into the beta-sheet conformation and inhibits prion peptide aggregation. J Biol. Chem. *276*, 49400-49409.

Cousens,S., Smith,P.G., Ward,H., Everington,D., Knight,R.S., Zeidler,M., Stewart,G., Smith-Bathgate,E.A., Macleod,M.A., Mackenzie,J., and Will,R.G. (2001). Geographical distribution of variant Creutzfeldt-Jakob disease in Great Britain, 1994-2000. Lancet *357*, 1002-1007.

Cunningham,A.A., Kirkwood,J.K., Dawson,M., Spencer,Y.I., Green,R.B., and Wells,G.A. (2004). Bovine spongiform encephalopathy infectivity in greater kudu (Tragelaphus strepsiceros). Emerg. Infect. Dis. *10*, 1044-1049.

Dawson,M., Wells,G.A., Parker,B.N., and Scott,A.C. (1990). Primary parenteral transmission of bovine spongiform encephalopathy to the pig. Vet. Rec. *127*, 338.

de Silva,H.V., Harmony,J.A., Stuart,W.D., Gil,C.M., and Robbins,J. (1990). Apolipoprotein J: structure and tissue distribution. Biochemistry *29*, 5380-5389.

DeArmond,S.J., Sanchez,H., Yehiely,F., Qiu,Y., Ninchak-Casey,A., Daggett,V., Camerino,A.P., Cayetano,J., Rogers,M., Groth,D., Torchia,M., Tremblay,P., Scott,M.R., Cohen,F.E., and Prusiner,S.B. (1997). Selective neuronal targeting in prion disease. Neuron *19*, 1337-1348.

DeArmond,S.J. and Bouzamondo,E. (2002). Fundamentals of prion biology and diseases. Toxicology *181-182*, 9-16.

Debeer,S., Baron,T., and Bencsik,A. (2003). Neuropathological characterisation of French bovine spongiform encephalopathy cases. Histochem. Cell Biol. *120*, 513-521.

Deleault,N.R., Lucassen,R.W., and Supattapone,S. (2003). RNA molecules stimulate prion protein conversion. Nature *425*, 717-720.

Della-Bianca,V., Rossi,F., Armato,U., Dal Pra,I., Costantini,C., Perini,G., Politi,V., and Della,V.G. (2001). Neurotrophin p75 receptor is involved in neuronal damage by prion peptide-(106-126). J Biol. Chem. *276*, 38929-38933.

DeMarco,M.L. and Daggett,V. (2004). From conversion to aggregation: protofibril formation of the prion protein. Proc. Natl. Acad. Sci. U. S. A *101*, 2293-2298.

Dobson,C.M. (2001). Protein folding and its links with human disease. Biochem. Soc. Symp. 1-26.

Donnelly,C.A., Ghani,A.C., Ferguson,N.M., and Anderson,R.M. (1997). Recent trends in the BSE epidemic. Nature *389*, 903.

Duffy,P., Wolf,J., Collins,G., DeVoe,A.G., Streeten,B., and Cowen,D. (1974). Letter: Possible person-to-person transmission of Creutzfeldt-Jakob disease. N. Engl. J Med. *290*, 692-693.

Eghiaian,F., Grosclaude,J., Lesceu,S., Debey,P., Doublet,B., Treguer,E., Rezaei,H., and Knossow,M. (2004). Insight into the PrPC-->PrPSc conversion from the structures of antibody-bound ovine prion scrapie-susceptibility variants. Proc. Natl. Acad. Sci. U. S. A *101*, 10254-10259.

Ellis,V., Daniels,M., Misra,R., and Brown,D.R. (2002). Plasminogen activation is stimulated by prion protein and regulated in a copper-dependent manner. Biochemistry *41*, 6891-6896.

Elsen,J.M., Amigues,Y., Schelcher,F., Ducrocq,V., Andreoletti,O., Eychenne,F., Khang,J.V., Poivey,J.P., Lantier,F., and Laplanche,J.L. (1999). Genetic susceptibility and transmission factors in scrapie: detailed analysis of an epidemic in a closed flock of Romanov. Arch. Virol. *144*, 431-445.

Epple,G., Schleunig,W.-D., Kettelgerdes,G., Köttgen,E., Geßner,R., and Praus,M. (2004). Prion protein stimulates tissue-type plasminogen activator-mediated plasmin generation via a lysine-binding site on kringle 2. J Thromb Haemost *2*, 962-968.

Fatzer,R., Graber,H.U., Meyer,R.K., Cardozo,C., Vandevelde,M., and Zurbriggen,A. (1996). Neuronal degeneration in brain stem nuclei in bovine spongiform encephalopathy. Zentralbl. Veterinarmed. A *43*, 23-29.

Ferguson,N.M., Donnelly,C.A., Woolhouse,M.E., and Anderson,R.M. (1997). A genetic interpretation of heightened risk of BSE in offspring of affected dams. Proc. R. Soc. Lond B Biol. Sci. *264*, 1445-1455.

Flechsig,E., Shmerling,D., Hegyi,I., Raeber,A.J., Fischer,M., Cozzio,A., von Mering,C., Aguzzi,A., and Weissmann,C. (2000). Prion protein devoid of the octapeptide repeat region restores susceptibility to scrapie in PrP knockout mice. Neuron *27*, 399-408.

Flechsig,E., Hegyi,I., Leimeroth,R., Zuniga,A., Rossi,D., Cozzio,A., Schwarz,P., Rulicke,T., Gotz,J., Aguzzi,A., and Weissmann,C. (2003). Expression of truncated PrP targeted to Purkinje cells of PrP knockout mice causes Purkinje cell death and ataxia. EMBO J *22*, 3095-3101.

Fleetwood,A.J. and Furley,C.W. (1990). Spongiform encephalopathy in an eland. Vet. Rec. *126*, 408-409.

Foster,J., Goldmann,W., Parnham,D., Chong,A., and Hunter,N. (2001). Partial dissociation of PrP(Sc) deposition and vacuolation in the brains of scrapie and BSE experimentally affected goats. J Gen. Virol. *82*, 267-273.

Foster,J.D., Hope,J., and Fraser,H. (1993). Transmission of bovine spongiform encephalopathy to sheep and goats. Vet. Rec. *133*, 339-341.

Fournier,J.G., Escaig-Haye,F., Billette,d., V, and Robain,O. (1995). Ultrastructural localization of cellular prion protein (PrPc) in synaptic boutons of normal hamster hippocampus. C. R. Acad. Sci. III *318*, 339-344.

Fournier,J.G., Escaig-Haye,F., Billette,d., V, Robain,O., Lasmezas,C.I., Deslys,J.P., Dormont,D., and Brown,P. (1998). Distribution and submicroscopic immunogold localization of cellular prion protein (PrPc) in extracerebral tissues. Cell Tissue Res. *292*, 77-84.

Fournier,J.G., Escaig-Haye,F., and Grigoriev,V. (2000). Ultrastructural localization of prion proteins: physiological and pathological implications. Microsc. Res. Tech. *50*, 76-88.

Fournier,J.G. and Grigoriev,B. (2001). Prion diseases: contribution of high-resolution immunomorphology. J Cell Mol. Med. *5*, 367-377.

Fraser,H. and Dickinson,A.G. (1968). The sequential development of the brain lesion of scrapie in three strains of mice. J Comp Pathol. *78*, 301-311.

Fraser,H., Pearson,G.R., McConnell,I., Bruce,M.E., Wyatt,J.M., and Gruffydd-Jones,T.J. (1994). Transmission of feline spongiform encephalopathy to mice. Vet. Rec. *134*, 449.

Gabriel,J.M., Oesch,B., Kretzschmar,H., Scott,M., and Prusiner,S.B. (1992). Molecular cloning of a candidate chicken prion protein. Proc. Natl. Acad. Sci. U. S. A *89*, 9097-9101.

Gajdusek,C., Gibbs,C.J., and Alpers,M. (1967). Slow-acting virus implicated in kuru. JAMA *199*, 34.

Gajdusek,D.C. and ZIGAS,V. (1959). Kuru; clinical, pathological and epidemiological study of an acute progressive degenerative disease of the central nervous system among natives of the Eastern Highlands of New Guinea. Am. J Med. *26*, 442-469.

Gajdusek,D.C., Gibbs,C.J., and Alpers,M. (1966). Experimental transmission of a Kuru-like syndrome to chimpanzees. Nature *209*, 794-796.

Gajdusek,D.C. (1977). Unconventional viruses and the origin and disappearance of kuru. Science *197*, 943-960.

Gambetti,P., Kong,Q., Zou,W., Parchi,P., and Chen,S.G. (2003). Sporadic and familial CJD: classification and characterisation. Br. Med. Bull. *66*, 213-239.

Gauczynski,S., Peyrin,J.M., Haik,S., Leucht,C., Hundt,C., Rieger,R., Krasemann,S., Deslys,J.P., Dormont,D., Lasmezas,C.I., and Weiss,S. (2001). The 37-kDa/67-kDa laminin receptor acts as the cell-surface receptor for the cellular prion protein. EMBO J *20*, 5863-5875.

Glatzel,M., Abela,E., Maissen,M., and Aguzzi,A. (2003). Extraneural pathologic prion protein in sporadic Creutzfeldt-Jakob disease. N. Engl. J Med. *349*, 1812-1820.

Gohel,C., Grigoriev,V., Escaig-Haye,F., Lasmezas,C.I., Deslys,J.P., Langeveld,J., Akaaboune,M., Hantai,D., and Fournier,J.G. (1999). Ultrastructural localization of cellular prion protein (PrPc) at the neuromuscular junction. J Neurosci. Res. *55*, 261-267.

Goldfarb,L.G., Brown,P., Cervenakova,L., and Gajdusek,D.C. (1994). Molecular genetic studies of Creutzfeldt-Jakob disease. Mol. Neurobiol. *8*, 89-97.

Goldfarb,L.G. (2002). Kuru: the old epidemic in a new mirror. Microbes. Infect. *4*, 875-882.

Goldmann,W., Hunter,N., Smith,G., Foster,J., and Hope,J. (1994). PrP genotype and agent effects in scrapie: change in allelic interaction with different isolates of agent in sheep, a natural host of scrapie. J. Gen. Virol. *75 (Pt 5)*, 989-995.

Goldmann,W., Martin,T., Foster,J., Hughes,S., Smith,G., Hughes,K., Dawson,M., and Hunter,N. (1996). Novel polymorphisms in the caprine PrP gene: a codon 142 mutation associated with scrapie incubation period. J Gen. Virol. *77 (Pt 11)*, 2885-2891.

Goldmann,W., O'Neill,G., Cheung,F., Charleson,F., Ford,P., and Hunter,N. (1999). PrP (prion) gene expression in sheep may be modulated by alternative polyadenylation of its messenger RNA. J. Gen. Virol. *80 (Pt 8)*, 2275-2283.

Guijarro,J.I., Sunde,M., Jones,J.A., Campbell,I.D., and Dobson,C.M. (1998). Amyloid fibril formation by an SH3 domain. Proc. Natl. Acad. Sci. U. S. A *95*, 4224-4228.

Hadlow,W.J., Race,R.E., and Kennedy,R.C. (1987). Temporal distribution of transmissible mink encephalopathy virus in mink inoculated subcutaneously. J Virol. *61*, 3235-3240.

Haeberle,A.M., Ribaut-Barassin,C., Bombarde,G., Mariani,J., Hunsmann,G., Grassi,J., and Bailly,Y. (2000). Synaptic prion protein immuno-reactivity in the rodent cerebellum. Microsc. Res. Tech. *50*, 66-75.

Haik,S., Faucheux,B.A., Sazdovitch,V., Privat,N., Kemeny,J.L., Perret-Liaudet,A., and Hauw,J.J. (2003). The sympathetic nervous system is involved in variant Creutzfeldt-Jakob disease. Nat. Med. *9*, 1121-1123.

Hamir,A.N., Miller,J.M., Cutlip,R.C., Stack,M.J., Chaplin,M.J., and Jenny,A.L. (2003). Preliminary observations on the experimental transmission of scrapie to elk (Cervus elaphus nelsoni) by intracerebral inoculation. Vet. Pathol. *40*, 81-85.

Harris,D.A., Falls,D.L., Johnson,F.A., and Fischbach,G.D. (1991). A prion-like protein from chicken brain copurifies with an acetylcholine receptor-inducing activity. Proc. Natl. Acad. Sci. U. S. A *88*, 7664-7668.

Harris,D.A., Lele,P., and Snider,W.D. (1993). Localization of the mRNA for a chicken prion protein by in situ hybridization. Proc. Natl. Acad. Sci. U. S. A *90*, 4309-4313.

Head,M.W., Ritchie,D., Smith,N., McLoughlin,V., Nailon,W., Samad,S., Masson,S., Bishop,M., McCardle,L., and Ironside,J.W. (2004). Peripheral tissue involvement in

sporadic, iatrogenic, and variant Creutzfeldt-Jakob disease: an immunohistochemical, quantitative, and biochemical study. Am. J Pathol. *164*, 143-153.

Healy,A.M., Weavers,E., McElroy,M., Gomez-Parada,M., Collins,J.D., O'Doherty,E., Sweeney,T., and Doherty,M.L. (2003). The clinical neurology of scrapie in Irish sheep. J Vet. Intern. Med. *17*, 908-916.

Heaton,M.P., Leymaster,K.A., Freking,B.A., Hawk,D.A., Smith,T.P., Keele,J.W., Snelling,W.M., Fox,J.M., Chitko-McKown,C.G., and Laegreid,W.W. (2003). Prion gene sequence variation within diverse groups of U.S. sheep, beef cattle, and deer. Mamm. Genome *14*, 765-777.

Hernandez-Sanchez,J., Waddington,D., Wiener,P., Haley,C.S., and Williams,J.L. (2002). Genome-wide search for markers associated with bovine spongiform encephalopathy. Mamm. Genome *13*, 164-168.

Hill,A.F., Butterworth,R.J., Joiner,S., Jackson,G., Rossor,M.N., Thomas,D.J., Frosh,A., Tolley,N., Bell,J.E., Spencer,M., King,A., Al Sarraj,S., Ironside,J.W., Lantos,P.L., and Collinge,J. (1999). Investigation of variant Creutzfeldt-Jakob disease and other human prion diseases with tonsil biopsy samples. Lancet *353*, 183-189.

Hills,D., Schlaepfer,J., Comincini,S., MacLean,I., Dolf,G., Ferretti,L., Olsaker,I., and Williams,J.L. (2003). Sequence variation in the bovine and ovine PRNP genes. Anim Genet. *34*, 183-190.

Hirakura,Y. and Kagan,B.L. (2001). Pore formation by beta-2-microglobulin: a mechanism for the pathogenesis of dialysis associated amyloidosis. Amyloid. *8*, 94-100.

Horiuchi,M., Ishiguro,N., Nagasawa,H., Toyoda,Y., and Shinagawa,M. (1997). Alternative usage of exon 1 of bovine PrP mRNA. Biochem. Biophys. Res. Commun. *233*, 650-654.

Horiuchi,M., Ishiguro,N., Nagasawa,H., Toyoda,Y., and Shinagawa,M. (1998). Genomic structure of the bovine PrP gene and complete nucleotide sequence of bovine PrP cDNA. Anim Genet *29*, 37-40.

Hornshaw,M.P., McDermott,J.R., Candy,J.M., and Lakey,J.H. (1995). Copper binding to the N-terminal tandem repeat region of mammalian and avian prion protein: structural studies using synthetic peptides. Biochem. Biophys. Res. Commun. *214*, 993-999.

Hornshaw,M.P., McDermott,J.R., and Candy,J.M. (1995). Copper binding to the N-terminal tandem repeat regions of mammalian and avian prion protein. Biochem. Biophys. Res. Commun. *207*, 621-629.

Houston,E.F., Halliday,S.I., Jeffrey,M., Goldmann,W., and Hunter,N. (2002). New Zealand sheep with scrapie-susceptible PrP genotypes succumb to experimental challenge with a sheep-passaged scrapie isolate (SSBP/1). J. Gen. Virol. *83*, 1247-1250.

Houston,F., Foster,J.D., Chong,A., Hunter,N., and Bostock,C.J. (2000). Transmission of BSE by blood transfusion in sheep. Lancet *356*, 999-1000.

Houston,F., Goldmann,W., Chong,A., Jeffrey,M., Gonzalez,L., Foster,J., Parnham,D., and Hunter,N. (2003). Prion diseases: BSE in sheep bred for resistance to infection. Nature *423*, 498.

Humeny,A., Schiebel,K., Seeber,S., and Becker,C.M. (2002). Identification of polymorphisms within the bovine prion protein gene (Prnp) by DNA sequencing and genotyping by MALDI-TOF-MS. Neurogenetics. *4*, 59-60.

Hundt,C., Peyrin,J.M., Haik,S., Gauczynski,S., Leucht,C., Rieger,R., Riley,M.L., Deslys,J.P., Dormont,D., Lasmezas,C.I., and Weiss,S. (2001). Identification of interaction domains of the prion protein with its 37-kDa/67-kDa laminin receptor. EMBO J *20*, 5876-5886.

Hunter,N., Foster,J.D., Benson,G., and Hope,J. (1991). Restriction fragment length polymorphisms of the scrapie-associated fibril protein (PrP) gene and their association with susceptibility to natural scrapie in British sheep. J Gen. Virol. *72 (Pt 6)*, 1287-1292.

Hunter,N., Goldmann,W., Smith,G., and Hope,J. (1994). Frequencies of PrP gene variants in healthy cattle and cattle with BSE in Scotland. Vet. Rec. *135*, 400-403.

Hunter,N. (1997). PrP genetics in sheep and the applications for scrapie and BSE. Trends Microbiol. *5*, 331-334.

Hunter,N., Foster,J., Chong,A., McCutcheon,S., Parnham,D., Eaton,S., MacKenzie,C., and Houston,F. (2002). Transmission of prion diseases by blood transfusion. J Gen. Virol. *83*, 2897-2905.

Ikeda,K., Kawada,N., Wang,Y.Q., Kadoya,H., Nakatani,K., Sato,M., and Kaneda,K. (1998). Expression of cellular prion protein in activated hepatic stellate cells. Am. J Pathol. *153*, 1695-1700.

Ikeda,T., Horiuchi,M., Ishiguro,N., Muramatsu,Y., Kai-Uwe,G.D., and Shinagawa,M. (1995). Amino acid polymorphisms of PrP with reference to onset of scrapie in Suffolk and Corriedale sheep in Japan. J Gen. Virol. *76 (Pt 10)*, 2577-2581.

Ilangumaran,S., Robinson,P.J., and Hoessli,D.C. (1996). Transfer of exogenous glycosylphos-phatidylinositol (GPI)-linked molecules to plasma membranes. Trends Cell Biol. *6*, 163-167.

Inoue,S., Tanaka,M., Horiuchi,M., Ishiguro,N., and Shinagawa,M. (1997). Characterization of the bovine prion protein gene: the expression requires interaction between the promoter and intron. J. Vet. Med. Sci. *59*, 175-183.

Ironside,J.W. (1998). Prion diseases in man. J Pathol. *186*, 227-234.

Jackson,G.S., Murray,I., Hosszu,L.L., Gibbs,N., Waltho,J.P., Clarke,A.R., and Collinge,J. (2001). Location and properties of metal-binding sites on the human prion protein. Proc. Natl. Acad. Sci. U. S. A *98*, 8531-8535.

Jeffrey,M. and Wells,G.A. (1988). Spongiform encephalopathy in a nyala (Tragelaphus angasi). Vet. Pathol. *25*, 398-399.

Johnson,C., Johnson,J., Clayton,M., McKenzie,D., and Aiken,J. (2003). Prion protein gene heterogeneity in free-ranging white-tailed deer within the chronic wasting disease affected region of Wisconsin. J Wildl. Dis. *39*, 576-581.

Jung,G., Jones,G., and Masison,D.C. (2002). Amino acid residue 184 of yeast Hsp104 chaperone is critical for prion-curing by guanidine, prion propagation, and thermotolerance. Proc. Natl. Acad. Sci. U. S. A *99*, 9936-9941.

Kaneko,K., Zulianello,L., Scott,M., Cooper,C.M., Wallace,A.C., James,T.L., Cohen,F.E., and Prusiner,S.B. (1997). Evidence for protein X binding to a discontinuous epitope on the cellular prion protein during scrapie prion propagation. Proc. Natl. Acad. Sci. U. S. A *94*, 10069-10074.

Kao,R.R., Houston,F., Baylis,M., Chihota,C.M., Goldmann,W., Gravenor,M.B., Hunter,N., and McLean,A.R. (2003). Epidemiological implications of the susceptibility to BSE of putatively resistant sheep. J Gen. Virol. *84*, 3503-3512.

Kempster,S., Collins,M.E., Aronow,B.J., Simmons,M., Green,R.B., and Edington,N. (2004). Clusterin shortens the incubation and alters the histopathology of bovine spongiform encephalopathy in mice. Neuroreport *15*, 1735-1738.

Kenward,N., Hope,J., Landon,M., and Mayer,R.J. (1994). Expression of polyubiquitin and heat-shock protein 70 genes increases in the later stages of disease progression in scrapie-infected mouse brain. J Neurochem. *62*, 1870-1877.

Khlebodarova,T.M., Malchenko,S.N., Matveeva,N.M., Pack,S.D., Sokolova,O.V., Alabiev,B.Y., Belousov,E.S., Peremislov,V.V., Nayakshin,A.M., and Brusgaard,K. (1995). Chromosomal and regional localization of the loci for IGKC, IGGC, ALDB, HOXB, GPT, and PRNP in the American mink (Mustela vison): comparisons with human and mouse. Mamm. Genome *6*, 705-709.

Kim,B.H., Lee,H.G., Choi,J.K., Kim,J.I., Choi,E.K., Carp,R.I., and Kim,Y.S. (2004). The cellular prion protein (PrPC) prevents apoptotic neuronal cell death and mitochondrial dysfunction induced by serum deprivation. Brain Res. Mol. Brain Res. *124*, 40-50.

Kirkwood,J.K., Wells,G.A., Wilesmith,J.W., Cunningham,A.A., and Jackson,S.I. (1990). Spongiform encephalopathy in an arabian oryx (Oryx leucoryx) and a greater kudu (Tragelaphus strepsiceros). Vet. Rec. *127*, 418-420.

Kirkwood,J.K., Cunningham,A.A., Wells,G.A., Wilesmith,J.W., and Barnett,J.E. (1993). Spongiform encephalopathy in a herd of greater kudu (Tragelaphus strepsiceros): epidemiological observations. Vet. Rec. *133*, 360-364.

Kirkwood,J.K. and Cunningham,A.A. (1994). Epidemiological observations on spongiform encephalopathies in captive wild animals in the British Isles. Vet. Rec. *135*, 296-303.

Kirkwood,J.K., Cunningham,A.A., Austin,A.R., Wells,G.A., and Sainsbury,A.W. (1994). Spongiform encephalopathy in a greater kudu (Tragelaphus strepsiceros) introduced into an affected group. Vet. Rec. *134*, 167-168.

Kitamoto,T., Ogomori,K., Tateishi,J., and Prusiner,S.B. (1987). Formic acid pretreatment enhances immunostaining of cerebral and systemic amyloids. Lab Invest *57*, 230-236.

Kocisko,D.A., Come,J.H., Priola,S.A., Chesebro,B., Raymond,G.J., Lansbury,P.T., and Caughey,B. (1994). Cell-free formation of protease-resistant prion protein. Nature *370*, 471-474.

Kompoliti,K., Goetz,C.G., Gajdusek,D.C., and Cubo,E. (1999). Movement disorders in Kuru. Mov Disord. *14*, 800-804.

Kornblatt,J.A., Marchal,S., Rezaei,H., Kornblatt,M.J., Balny,C., Lange,R., Debey,M.P., Hui Bon,H.G., Marden,M.C., and Grosclaude,J. (2003). The fate of the prion protein in the prion/plasminogen complex. Biochem. Biophys. Res. Commun. *305*, 518-522.

Kourie,J.I. and Henry,C.L. (2002). Ion channel formation and membrane-linked pathologies of misfolded hydrophobic proteins: the role of dangerous unchaperoned molecules. Clin. Exp. Pharmacol. Physiol *29*, 741-753.

Kovacs,G.G., Trabattoni,G., Hainfellner,J.A., Ironside,J.W., Knight,R.S., and Budka,H. (2002). Mutations of the prion protein gene phenotypic spectrum. J Neurol. *249*, 1567-1582.

Kretzschmar,H.A., Neumann,M., Riethmuller,G., and Prusiner,S.B. (1992). Molecular cloning of a mink prion protein gene. J Gen. Virol. *73 (Pt 10)*, 2757-2761.

Kretzschmar,H.A., Sethi,S., Foldvari,Z., Windl,O., Querner,V., Zerr,I., and Poser,S. (2003). Latrogenic Creutzfeldt-Jakob disease with florid plaques. Brain Pathol. *13*, 245-249.

Krobitsch,S. and Lindquist,S. (2000). Aggregation of huntingtin in yeast varies with the length of the polyglutamine expansion and the expression of chaperone proteins. Proc. Natl. Acad. Sci. U. S. A *97*, 1589-1594.

Kuppinger, O., Krebs, S., and Förster, M. (2002). Prionenvariabilität im Deutschen Fleckvieh und Braunvieh. Vortragstagung der DGfZ und GfT am 18./19. September in Halle.

Kutzer,T., Pfeiffer,I., and Brenig,B. (2002). Identification of new allelic variants in the ovine prion protein (PrP) gene. J Anim Breed Genet *119*, 201-208.

Lang,C.J., Heckmann,J.G., and Neundorfer,B. (1998). Creutzfeldt-Jakob disease via dural and corneal transplants. J Neurol. Sci. *160*, 128-139.

Lee,H.S., Sambuughin,N., Cervenakova,L., Chapman,J., Pocchiari,M., Litvak,S., Qi,H.Y., Budka,H., del Ser,T., Furukawa,H., Brown,P., Gajdusek,D.C., Long,J.C., Korczyn,A.D., and Goldfarb,L.G. (1999). Ancestral origins and worldwide distribution of the PRNP 200K mutation causing familial Creutzfeldt-Jakob disease. Am. J Hum. Genet *64*, 1063-1070.

Lee,H.S., Brown,P., Cervenakova,L., Garruto,R.M., Alpers,M.P., Gajdusek,D.C., and Goldfarb,L.G. (2001). Increased susceptibility to Kuru of carriers of the PRNP 129 methionine/methionine genotype. J Infect. Dis. *183*, 192-196.

Lee,I.Y., Westaway,D., Smit,A.F., Wang,K., Seto,J., Chen,L., Acharya,C., Ankener,M., Baskin,D., Cooper,C., Yao,H., Prusiner,S.B., and Hood,L.E. (1998). Complete genomic sequence and analysis of the prion protein gene region from three mammalian species. Genome Res. *8*, 1022-1037.

Leggett,M.M., Dukes,J., and Pirie,H.M. (1990). A spongiform encephalopathy in a cat. Vet. Rec. *127*, 586-588.

Legname,G., Baskakov,I.V., Nguyen,H.O., Riesner,D., Cohen,F.E., DeArmond,S.J., and Prusiner,S.B. (2004). Synthetic mammalian prions. Science *305*, 673-676.

Leone,P., Castiglioni,B., Sechi,T., Cassini,P., and Stella,A. (2002). Prion gene octarepeat variability in Italian cattle breeds. Proceedings of the 7th World Congress on Genetics Applied to Livestock Production,Montpellier, 19-23 August 2002, Session 13 13-40.

Leucht,C., Simoneau,S., Rey,C., Vana,K., Rieger,R., Lasmezas,C.I., and Weiss,S. (2003). The 37 kDa/67 kDa laminin receptor is required for PrP(Sc) propagation in scrapie-infected neuronal cells. EMBO Rep. *4*, 290-295.

Lezmi,S., Bencsik,A., Monks,E., Petit,T., and Baron,T. (2003). First case of feline spongiform encephalopathy in a captive cheetah born in France: PrP(sc) analysis in various tissues revealed unexpected targeting of kidney and adrenal gland. Histochem. Cell Biol. *119*, 415-422.

Lezmi,S., Martin,S., Simon,S., Comoy,E., Bencsik,A., Deslys,J.P., Grassi,J., Jeffrey,M., and Baron,T. (2004). Comparative molecular analysis of the abnormal prion protein in field scrapie cases and experimental bovine spongiform encephalopathy in sheep by use of Western blotting and immunohistochemical methods. J Virol. *78*, 3654-3662.

Liberski,P.P. and Gajdusek,D.C. (1997). Kuru: forty years later, a historical note. Brain Pathol. *7*, 555-560.

Ligios,C., Jeffrey,M., Ryder,S.J., Bellworthy,S.J., and Simmons,M.M. (2002). Distinction of scrapie phenotypes in sheep by lesion profiling. J Comp Pathol. *127*, 45-57.

Lipp,O., Ritzmann,M., Kixmoller,M., Heinritzi,K., Hensel,A., and Truyen,U. (2004). Homogeneity of the prion protein gene in various European and Asian pig breeds. J Vet. Med. B Infect. Dis. Vet. Public Health *51*, 97-98.

Llewelyn,C.A., Hewitt,P.E., Knight,R.S., Amar,K., Cousens,S., Mackenzie,J., and Will,R.G. (2004). Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion. Lancet *363*, 417-421.

Lloyd,S.E., Onwuazor,O.N., Beck,J.A., Mallinson,G., Farrall,M., Targonski,P., Collinge,J., and Fisher,E.M. (2001). Identification of multiple quantitative trait loci linked to prion disease incubation period in mice. Proc. Natl. Acad. Sci. U. S. A *98*, 6279-6283.

Lloyd,S.E., Uphill,J.B., Targonski,P.V., Fisher,E.M., and Collinge,J. (2002). Identification of genetic loci affecting mouse-adapted bovine spongiform encephalopathy incubation time in mice. Neurogenetics. *4*, 77-81.

Lloyd,S.E., Thompson,S.R., Beck,J.A., Linehan,J.M., Wadsworth,J.D., Brandner,S., Collinge,J., and Fisher,E.M. (2004). Identification and characterization of a novel mouse prion gene allele. Mamm. Genome *15*, 383-389.

Lloyd,S.E., Linehan,J.M., Desbruslais,M., Joiner,S., Buckell,J., Brandner,S., Wadsworth,J.D., and Collinge,J. (2004). Characterization of two distinct prion strains

derived from bovine spongiform encephalopathy transmissions to inbred mice. J Gen. Virol. *85*, 2471-2478.

Lopez,G.F., Zahn,R., Riek,R., and Wuthrich,K. (2000). NMR structure of the bovine prion protein. Proc. Natl. Acad. Sci. U. S. A *97*, 8334-8339.

Lorenzo,A. and Yankner,B.A. (1994). Beta-amyloid neurotoxicity requires fibril formation and is inhibited by congo red. Proc. Natl. Acad. Sci. U. S. A *91*, 12243-12247.

Luhken,G., Buschmann,A., Groschup,M.H., and Erhardt,G. (2004). Prion protein allele A136 H154Q171 is associated with high susceptibility to scrapie in purebred and crossbred German Merinoland sheep. Arch. Virol. *149*, 1571-1580.

Maignien,T., Lasmezas,C.I., Beringue,V., Dormont,D., and Deslys,J.P. (1999). Pathogenesis of the oral route of infection of mice with scrapie and bovine spongiform encephalopathy agents. J Gen. Virol. *80 (Pt 11)*, 3035-3042.

Mallucci,G., Dickinson,A., Linehan,J., Klohn,P.C., Brandner,S., and Collinge,J. (2003). Depleting neuronal PrP in prion infection prevents disease and reverses spongiosis. Science *302*, 871-874.

Manolakou,K., Beaton,J., McConnell,I., Farquar,C., Manson,J., Hastie,N.D., Bruce,M., and Jackson,I.J. (2001). Genetic and environmental factors modify bovine spongiform encephalopathy incubation period in mice. Proc. Natl. Acad. Sci. U. S. A *98*, 7402-7407.

Marsh,R.F. and Hanson,R.P. (1969). Physical and chemical properties of the transmissible mink encephalopathy agent. J Virol. *3*, 176-180.

Marsh,R.F., Bessen,R.A., Lehmann,S., and Hartsough,G.R. (1991). Epidemiological and experimental studies on a new incident of transmissible mink encephalopathy. J Gen. Virol. *72 (Pt 3)*, 589-594.

Marsh,R.F. and Bessen,R.A. (1993). Epidemiologic and experimental studies on transmissible mink encephalopathy. Dev. Biol. Stand. *80*, 111-118.

Martin,T., Hughes,S., Hughes,K., and Dawson,M. (1995). Direct sequencing of PCR amplified pig PrP genes. Biochim. Biophys. Acta *1270*, 211-214.

Martins,V.R. and Brentani,R.R. (2002). The biology of the cellular prion protein. Neurochem. Int. *41*, 353-355.

Massimino,M.L., Ballarin,C., Bertoli,A., Casonato,S., Genovesi,S., Negro,A., and Sorgato,M.C. (2004). Human Doppel and prion protein share common membrane microdomains and internalization pathways. Int. J Biochem. Cell Biol. *36*, 2016-2031.

Matsubara,E., Frangione,B., and Ghiso,J. (1995). Characterization of apolipoprotein J-Alzheimer's A beta interaction. J Biol. Chem. *270*, 7563-7567.

McHattie,S. and Edington,N. (1999). Clusterin prevents aggregation of neuropeptide 106-126 in vitro. Biochem. Biophys. Res. Commun. *259*, 336-340.

McHattie,S., Wells,G.A., Bee,J., and Edington,N. (1999). Clusterin in bovine spongiform encephalopathy (BSE). J Comp Pathol. *121*, 159-171.

Meyer,R.K., McKinley,M.P., Bowman,K.A., Braunfeld,M.B., Barry,R.A., and Prusiner,S.B. (1986). Separation and properties of cellular and scrapie prion proteins. Proc. Natl. Acad. Sci. U. S. A *83*, 2310-2314.

Miele,G., Manson,J., and Clinton,M. (2001). A novel erythroid-specific marker of transmissible spongiform encephalopathies. Nat. Med. *7*, 361-364.

Miele,G., Jeffrey,M., Turnbull,D., Manson,J., and Clinton,M. (2002). Ablation of cellular prion protein expression affects mitochondrial numbers and morphology. Biochem. Biophys. Res. Commun. *291*, 372-377.

Miller,M.W. and Wild,M.A. (2004). Epidemiology of chronic wasting disease in captive white-tailed and mule deer. J Wildl. Dis. *40*, 320-327.

Minor,D.L., Jr. and Kim,P.S. (1996). Context-dependent secondary structure formation of a designed protein sequence. Nature *380*, 730-734.

Mobley,D.L., Cox,D.L., Singh,R.R., Kulkarni,R.V., and Slepoy,A. (2003). Simulations of oligomeric intermediates in prion diseases. Biophys. J *85*, 2213-2223.

Montagna,P., Gambetti,P., Cortelli,P., and Lugaresi,E. (2003). Familial and sporadic fatal insomnia. Lancet Neurol. *2*, 167-176.

Moore,R.C., Hope,J., McBride,P.A., McConnell,I., Selfridge,J., Melton,D.W., and Manson,J.C. (1998). Mice with gene targetted prion protein alterations show that Prnp, Sinc and Prni are congruent. Nat. Genet *18*, 118-125.

Moore,R.C., Lee,I.Y., Silverman,G.L., Harrison,P.M., Strome,R., Heinrich,C., Karunaratne,A., Pasternak,S.H., Chishti,M.A., Liang,Y., Mastrangelo,P., Wang,K.,

Smit,A.F., Katamine,S., Carlson,G.A., Cohen,F.E., Prusiner,S.B., Melton,D.W., Tremblay,P., Hood,L.E., and Westaway,D. (1999). Ataxia in prion protein (PrP)-deficient mice is associated with upregulation of the novel PrP-like protein doppel. J Mol. Biol. *292*, 797-817.

Morot-Gaudry-Talarmain,Y., Rezaei,H., Guermonprez,L., Treguer,E., and Grosclaude,J. (2003). Selective prion protein binding to synaptic components is modulated by oxidative and nitrosative changes induced by copper(II) and peroxynitrite in cholinergic synaptosomes, unveiling a role for calcineurin B and thioredoxin. J Neurochem. *87*, 1456-1470.

Morrissey,M.P. and Shakhnovich,E.I. (1999). Evidence for the role of PrP(C) helix 1 in the hydrophilic seeding of prion aggregates. Proc. Natl. Acad. Sci. U. S. A *96*, 11293-11298.

Mouillet-Richard,S., Ermonval,M., Chebassier,C., Lehmann,S., Launay,J.M., and Kellermann,O. (2000). Signal transduction through prion protein. Science *289*, 1925-1928.

Mulcahy,E.R. and Bessen,R.A. (2004). Strain-specific kinetics of prion protein formation in vitro and in vivo. J Biol. Chem. *279*, 1643-1649.

National Wildlife Health Center (2003). Chronic Wasting Disease Map http://www.nwhc.usgs.gov/research/chronic_wasting/chronic_wasting_map.html

Neibergs,H.L., Ryan,A.M., Womack,J.E., Spooner,R.L., and Williams,J.L. (1994). Polymorphism analysis of the prion gene in BSE-affected and unaffected cattle. Anim Genet. *25*, 313-317.

Neutra,M.R., Frey,A., and Kraehenbuhl,J.P. (1996). Epithelial M cells: gateways for mucosal infection and immunization. Cell *86*, 345-348.

Nonno,R., Esposito,E., Vaccari,G., Conte,M., Marcon,S., Di Bari,M., Ligios,C., Di Guardo,G., and Agrimi,U. (2003). Molecular analysis of cases of Italian sheep scrapie and comparison with cases of bovine spongiform encephalopathy (BSE) and experimental BSE in sheep. J Clin. Microbiol. *41*, 4127-4133.

O'Rourke,K.I., Besser,T.E., Miller,M.W., Cline,T.F., Spraker,T.R., Jenny,A.L., Wild,M.A., Zebarth,G.L., and Williams,E.S. (1999). PrP genotypes of captive and free-ranging Rocky Mountain elk (Cervus elaphus nelsoni) with chronic wasting disease. J Gen. Virol. *80 (Pt 10)*, 2765-2769.

O'Rourke,K.I., Spraker,T.R., Hamburg,L.K., Besser,T.E., Brayton,K.A., and Knowles,D.P. (2004). Polymorphisms in the prion precursor functional gene but not the pseudogene are associated with susceptibility to chronic wasting disease in white-tailed deer. J Gen. Virol. *85*, 1339-1346.

Oesch,B., Westaway,D., Walchli,M., McKinley,M.P., Kent,S.B., Aebersold,R., Barry,R.A., Tempst,P., Teplow,D.B., Hood,L.E., and . (1985). A cellular gene encodes scrapie PrP 27-30 protein. Cell *40*, 735-746.

Oidtmann,B., Simon,D., Holtkamp,N., Hoffmann,R., and Baier,M. (2003). Identification of cDNAs from Japanese pufferfish (Fugu rubripes) and Atlantic salmon (Salmo salar) coding for homologues to tetrapod prion proteins. FEBS Lett. *538*, 96-100.

Orge,L., Simas,J.P., Fernandes,A.C., Ramos,M., and Galo,A. (2000). Similarity of the lesion profile of BSE in Portuguese cattle to that described in British cattle. Vet. Rec. *147*, 486-488.

Palmer,M.S., Dryden,A.J., Hughes,J.T., and Collinge,J. (1991). Homozygous prion protein genotype predisposes to sporadic Creutzfeldt-Jakob disease. Nature *352*, 340-342.

Pan,K.M., Baldwin,M., Nguyen,J., Gasset,M., Serban,A., Groth,D., Mehlhorn,I., Huang,Z., Fletterick,R.J., Cohen,F.E., and . (1993). Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. Proc. Natl. Acad. Sci. U. S. A *90*, 10962-10966.

Parchi,P., Castellani,R., Cortelli,P., Montagna,P., Chen,S.G., Petersen,R.B., Manetto,V., Vnencak-Jones,C.L., McLean,M.J., Sheller,J.R., and . (1995). Regional distribution of protease-resistant prion protein in fatal familial insomnia. Ann. Neurol. *38*, 21-29.

Parchi,P., Castellani,R., Capellari,S., Ghetti,B., Young,K., Chen,S.G., Farlow,M., Dickson,D.W., Sima,A.A., Trojanowski,J.Q., Petersen,R.B., and Gambetti,P. (1996). Molecular basis of phenotypic variability in sporadic Creutzfeldt-Jakob disease. Ann. Neurol. *39*, 767-778.

Parchi,P., Giese,A., Capellari,S., Brown,P., Schulz-Schaeffer,W., Windl,O., Zerr,I., Budka,H., Kopp,N., Piccardo,P., Poser,S., Rojiani,A., Streichemberger,N., Julien,J., Vital,C., Ghetti,B., Gambetti,P., and Kretzschmar,H. (1999). Classification of sporadic Creutzfeldt-Jakob disease based on molecular and phenotypic analysis of 300 subjects. Ann. Neurol. *46*, 224-233.

Parchi,P., Zou,W., Wang,W., Brown,P., Capellari,S., Ghetti,B., Kopp,N., Schulz-Schaeffer,W.J., Kretzschmar,H.A., Head,M.W., Ironside,J.W., Gambetti,P., and Chen,S.G. (2000). Genetic influence on the structural variations of the abnormal prion protein. Proc. Natl. Acad. Sci. U. S. A *97*, 10168-10172.

Pattison,I.H. (1965). Scrapie in the welsh mountain breed of sheep and its experimental transmission to goats. Vet. Rec. *77*, 1388-1390.

Pearson,G.R., Wyatt,J.M., Gruffydd-Jones,T.J., Hope,J., Chong,A., Higgins,R.J., Scott,A.C., and Wells,G.A. (1992). Feline spongiform encephalopathy: fibril and PrP studies. Vet. Rec. *131*, 307-310.

Peden,A.H., Head,M.W., Ritchie,D.L., Bell,J.E., and Ironside J.W. (8004). Preclinical vCJD after blood transfusion in a *PRNP* codon 129 heterozygous patient. Lancet *264*, 527-529.

Peet,R.L. and Curran,J.M. (1992). Spongiform encephalopathy in an imported cheetah (Acinonyx jubatus). Aust. Vet. J *69*, 171.

Petchanikow,C., Saborio,G.P., Anderes,L., Frossard,M.J., Olmedo,M.I., and Soto,C. (2001). Biochemical and structural studies of the prion protein polymorphism. FEBS Lett. *509*, 451-456.

Premzl,M., Bozic,P., and Gamulin,V. (2000). PRNP octarepeat allele genotype frequencies among the modern and rare cattle breeds in Croatia. Anim Genet *31*, 408-409.

Prusiner,S.B. (1982). Novel proteinaceous infectious particles cause scrapie. Science *216*, 136-144.

Prusiner,S.B., McKinley,M.P., Bowman,K.A., Bolton,D.C., Bendheim,P.E., Groth,D.F., and Glenner,G.G. (1983). Scrapie prions aggregate to form amyloid-like birefringent rods. Cell *35*, 349-358.

Prusiner,S.B. (1991). Molecular biology of prion diseases. Science *252*, 1515-1522.

Puoti,G., Giaccone,G., Rossi,G., Canciani,B., Bugiani,O., and Tagliavini,F. (1999). Sporadic Creutzfeldt-Jakob disease: co-occurrence of different types of PrP(Sc) in the same brain. Neurology *53*, 2173-2176.

Rezaei,H., Marc,D., Choiset,Y., Takahashi,M., Hui Bon,H.G., Haertle,T., Grosclaude,J., and Debey,P. (2000). High yield purification and physico-chemical properties of full-length recombinant allelic variants of sheep prion protein linked to scrapie susceptibility. Eur. J Biochem. *267* , 2833-2839.

Rezaei,H., Choiset,Y., Eghiaian,F., Treguer,E., Mentre,P., Debey,P., Grosclaude,J., and Haertle,T. (2002). Amyloidogenic unfolding intermediates differentiate sheep prion protein variants. J Mol. Biol. *322*, 799-814.

Rieger,R., Edenhofer,F., Lasmezas,C.I., and Weiss,S. (1997). The human 37-kDa laminin receptor precursor interacts with the prion protein in eukaryotic cells. Nat. Med. *3*, 1383-1388.

Rieger,R., Lasmezas,C.I., and Weiss,S. (1999). Role of the 37 kDa laminin receptor precursor in the life cycle of prions. Transfus. Clin. Biol. *6*, 7-16.

Riek,R., Hornemann,S., Wider,G., Billeter,M., Glockshuber,R., and Wuthrich,K. (1996). NMR structure of the mouse prion protein domain PrP(121-321). Nature *382*, 180-182.

Riek,R., Wider,G., Billeter,M., Hornemann,S., Glockshuber,R., and Wuthrich,K. (1998). Prion protein NMR structure and familial human spongiform encephalopathies. Proc. Natl. Acad. Sci. U. S. A *95*, 11667-11672.

Rivera-Milla,E., Stuermer,C.A., and Malaga-Trillo,E. (2003). An evolutionary basis for scrapie disease: identification of a fish prion mRNA. Trends Genet *19*, 72-75.

Roberts,B.T., Moriyama,H., and Wickner,R.B. (2004). [URE3] prion propagation is abolished by a mutation of the primary cytosolic Hsp70 of budding yeast. Yeast *21*, 107-117.

Robinson,M.M., Hadlow,W.J., Huff,T.P., Wells,G.A., Dawson,M., Marsh,R.F., and Gorham,J.R. (1994). Experimental infection of mink with bovine spongiform encephalopathy. J Gen. Virol. *75 (Pt 9)*, 2151-2155.

Rudd,P.M., Endo,T., Colominas,C., Groth,D., Wheeler,S.F., Harvey,D.J., Wormald,M.R., Serban,H., Prusiner,S.B., Kobata,A., and Dwek,R.A. (1999). Glycosylation differences between the normal and pathogenic prion protein isoforms. Proc. Natl. Acad. Sci. U. S. A *96*, 13044-13049.

Rudd,P.M., Wormald,M.R., Wing,D.R., Prusiner,S.B., and Dwek,R.A. (2001). Prion glycoprotein: structure, dynamics, and roles for the sugars. Biochemistry *40*, 3759-3766.

Ryder,S.J., Hawkins,S.A., Dawson,M., and Wells,G.A. (2000). The neuropathology of experimental bovine spongiform encephalopathy in the pig. J Comp Pathol. *122*, 131-143.

Ryder,S.J., Wells,G.A., Bradshaw,J.M., and Pearson,G.R. (2001). Inconsistent detection of PrP in extraneural tissues of cats with feline spongiform encephalopathy. Vet. Rec. *148*, 437-441.

Sabuncu,E., Petit,S., Le Dur,A., Lan,L.T., Vilotte,J.L., Laude,H., and Vilette,D. (2003). PrP polymorphisms tightly control sheep prion replication in cultured cells. J Virol. *77*, 2696-2700.

Safar,J., Wille,H., Itri,V., Groth,D., Serban,H., Torchia,M., Cohen,F.E., and Prusiner,S.B. (1998). Eight prion strains have PrP(Sc) molecules with different conformations. Nat. Med. *4*, 1157-1165.

Sakaguchi,S., Katamine,S., Shigematsu,K., Nakatani,A., Moriuchi,R., Nishida,N., Kurokawa,K., Nakaoke,R., Sato,H., Jishage,K., and . (1995). Accumulation of proteinase K-resistant prion protein (PrP) is restricted by the expression level of normal PrP in mice inoculated with a mouse-adapted strain of the Creutzfeldt-Jakob disease agent. J Virol. *69*, 7586-7592.

Sakaguchi,S., Katamine,S., Nishida,N., Moriuchi,R., Shigematsu,K., Sugimoto,T., Nakatani,A., Kataoka,Y., Houtani,T., Shirabe,S., Okada,H., Hasegawa,S., Miyamoto,T., and Noda,T. (1996). Loss of cerebellar Purkinje cells in aged mice homozygous for a disrupted PrP gene. Nature *380*, 528-531.

Sakudo,A., Lee,D.C., Yoshimura,E., Nagasaka,S., Nitta,K., Saeki,K., Matsumoto,Y., Lehmann,S., Itohara,S., Sakaguchi,S., and Onodera,T. (2004). Prion protein suppresses perturbation of cellular copper homeostasis under oxidative conditions. Biochem. Biophys. Res. Commun. *313*, 850-855.

Salman,M.D. (2003). Chronic wasting disease in deer and elk: scientific facts and findings. J Vet. Med. Sci. *65*, 761-768.

Sander,P., Hamann,H., Pfeiffer,I., Wemheuer,W., Brenig,B., Groschup,M.H., Ziegler,U., Distl,O., and Leeb,T. (2004). Analysis of sequence variability of the bovine prion protein gene (PRNP) in German cattle breeds. Neurogenetics. *5*, 19-25.

Sasaki,K., Doh-ura,K., Ironside,J.W., and Iwaki,T. (2002). Increased clusterin (apolipoprotein J) expression in human and mouse brains infected with transmissible spongiform encephalopathies. Acta Neuropathol. (Berl) *103*, 199-208.

Schlapfer,I., Saitbekova,N., Gaillard,C., and Dolf,G. (1999). A new allelic variant in the bovine prion protein gene (PRNP) coding region. Anim Genet. *30*, 386-387.

Scott,A.C., Wells,G.A., Stack,M.J., White,H., and Dawson,M. (1990). Bovine spongiform encephalopathy: detection and quantitation of fibrils, fibril protein (PrP) and vacuolation in brain. Vet. Microbiol. *23*, 295-304.

Scott,M., Groth,D., Foster,D., Torchia,M., Yang,S.L., DeArmond,S.J., and Prusiner,S.B. (1993). Propagation of prions with artificial properties in transgenic mice expressing chimeric PrP genes. Cell *73*, 979-988.

Scott,M.R., Safar,J., Telling,G., Nguyen,O., Groth,D., Torchia,M., Koehler,R., Tremblay,P., Walther,D., Cohen,F.E., DeArmond,S.J., and Prusiner,S.B. (1997). Identification of a prion protein epitope modulating transmission of bovine spongiform encephalopathy prions to transgenic mice. Proc. Natl. Acad. Sci. U. S. A *94*, 14279-14284.

Scott,M.R., Will,R., Ironside,J., Nguyen,H.O., Tremblay,P., DeArmond,S.J., and Prusiner,S.B. (1999). Compelling transgenetic evidence for transmission of bovine spongiform encephalopathy prions to humans. Proc. Natl. Acad. Sci. U. S. A *96*, 15137-15142.

Seabury,C.M. and Derr,J.N. (2003). Identification of a novel ovine PrP polymorphism and scrapie-resistant genotypes for St. Croix White and a related composite breed. Cytogenet. Genome Res. *102*, 85-88.

Sherman,M.Y. and Goldberg,A.L. (2001). Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases. Neuron *29*, 15-32.

Shmakov,A.N., Bode,J., Kilshaw,P.J., and Ghosh,S. (2000). Diverse patterns of expression of the 67-kD laminin receptor in human small intestinal mucosa: potential binding sites for prion proteins? J. Pathol. *191*, 318-322.

Shyng,S.L., Heuser,J.E., and Harris,D.A. (1994). A glycolipid-anchored prion protein is endocytosed via clathrin-coated pits. J Cell Biol. *125*, 1239-1250.

Shyu,W.C., Harn,H.J., Saeki,K., Kubosaki,A., Matsumoto,Y., Onodera,T., Chen,C.J., Hsu,Y.D., and Chiang,Y.H. (2002). Molecular modulation of expression of prion protein by heat shock. Mol. Neurobiol. *26*, 1-12.

Si,K., Lindquist,S., and Kandel,E.R. (2003). A neuronal isoform of the aplysia CPEB has prion-like properties. Cell *115*, 879-891.

Sigurdson,C.J., Williams,E.S., Miller,M.W., Spraker,T.R., O'Rourke,K.I., and Hoover,E.A. (1999). Oral transmission and early lymphoid tropism of chronic wasting disease PrPres in mule deer fawns (Odocoileus hemionus). J Gen. Virol. *80 (Pt 10)*, 2757-2764.

Simoneau,S., Haik,S., Leucht,C., Dormont,D., Deslys,J.P., Weiss,S., and Lasmezas,C. (2003). Different isoforms of the non-integrin laminin receptor are present in mouse brain and bind PrP. Biol. Chem. *384* , 243-246.

Spencer,M.D., Knight,R.S., and Will,R.G. (2002). First hundred cases of variant Creutzfeldt-Jakob disease: retrospective case note review of early psychiatric and neurological features. BMJ *324*, 1479-1482.

Spielhaupter,C. and Schatzl,H.M. (2001). PrPC directly interacts with proteins involved in signaling pathways. J Biol. Chem. *276*, 44604-44612.

Stefani,M. and Dobson,C.M. (2003). Protein aggregation and aggregate toxicity: new insights into protein folding, misfolding diseases and biological evolution. J Mol. Med. *81*, 678-699.

Stephenson,D.A., Chiotti,K., Ebeling,C., Groth,D., DeArmond,S.J., Prusiner,S.B., and Carlson,G.A. (2000). Quantitative trait loci affecting prion incubation time in mice. Genomics *69*, 47-53.

Stuermer,C.A.O., Lanhorst,M.F., Wiechers,M.F., Legler,D.F., Hannbeck von Hanwehr,S., Guse,A.H., and Plattner,H. (2004). PrPC capping in T cells promotes its association withthe lipid raft proteins reggie-1 and reggie-2 and leads to signal transduction. FASEB J.

Stewart,R.S. and Harris,D.A. (2003). Mutational analysis of topological determinants in prion protein (PrP) and measurement of transmembrane and cytosolic PrP during prion infection. J Biol. Chem. *278*, 45960-45968.

Takasuga,A., Abe,T., Ito,T., Watanabe,T., Kamatani,N., and Sugimoto,Y. (2003). Novel prion protein polymorphisms in cattle. Anim Genet *34*, 396-397.

Taraboulos,A., Jendroska,K., Serban,D., Yang,S.L., DeArmond,S.J., and Prusiner,S.B. (1992). Regional mapping of prion proteins in brain. Proc. Natl. Acad. Sci. U. S. A *89*, 7620-7624.

Tateishi,J., Koga,M., and Mori,R. (1981). Experimental transmission of Creutzfeldt-Jakob disease. Acta Pathol. Jpn. *31*, 943-951.

Telling,G.C., Scott,M., Hsiao,K.K., Foster,D., Yang,S.L., Torchia,M., Sidle,K.C., Collinge,J., DeArmond,S.J., and Prusiner,S.B. (1994). Transmission of Creutzfeldt-Jakob disease from humans to transgenic mice expressing chimeric human-mouse prion protein. Proc. Natl. Acad. Sci. U. S. A *91*, 9936-9940.

Telling,G.C., Scott,M., Mastrianni,J., Gabizon,R., Torchia,M., Cohen,F.E., DeArmond,S.J., and Prusiner,S.B. (1995). Prion propagation in mice expressing human and chimeric PrP transgenes implicates the interaction of cellular PrP with another protein. Cell *83*, 79-90.

Thadani,V., Penar,P.L., Partington,J., Kalb,R., Janssen,R., Schonberger,L.B., Rabkin,C.S., and Prichard,J.W. (1988). Creutzfeldt-Jakob disease probably acquired from a cadaveric dura mater graft. Case report. J Neurosurg. *69*, 766-769.

Thomas,T., Thomas,G., McLendon,C., Sutton,T., and Mullan,M. (1996). beta-Amyloid-mediated vasoactivity and vascular endothelial damage. Nature *380*, 168-171.

Thuring,C.M., Erkens,J.H., Jacobs,J.G., Bossers,A., van Keulen,L.J., Garssen,G.J., van Zijderveld,F.G., Ryder,S.J., Groschup,M.H., Sweeney,T., and Langeveld,J.P. (2004). Discrimination between scrapie and bovine spongiform encephalopathy in sheep by molecular size, immunoreactivity, and glycoprofile of prion protein. J Clin. Microbiol. *42*, 972-980.

Tobler,I., Gaus,S.E., Deboer,T., Achermann,P., Fischer,M., Rulicke,T., Moser,M., Oesch,B., McBride,P.A., and Manson,J.C. (1996). Altered circadian activity rhythms and sleep in mice devoid of prion protein. Nature *380*, 639-642.

Tranulis,M.A. (2002). Influence of the prion protein gene, Prnp, on scrapie susceptibility in sheep. APMIS *110*, 33-43.

Trevitt,C.R. and Singh,P.N. (2003). Variant Creutzfeldt-Jakob disease: pathology, epidemiology, and public health implications. Am. J Clin. Nutr. *78*, 651S-656S.

Valleron,A.J., Boelle,P.Y., Will,R., and Cesbron,J.Y. (2001). Estimation of epidemic size and incubation time based on age characteristics of vCJD in the United Kingdom. Science *294*, 1726-1728.

van Keulen,L.J., Schreuder,B.E., Vromans,M.E., Langeveld,J.P., and Smits,M.A. (2000). Pathogenesis of natural scrapie in sheep. Arch. Virol. Suppl 57-71.

van Keulen,L.J., Vromans,M.E., and van Zijderveld,F.G. (2002). Early and late pathogenesis of natural scrapie infection in sheep. APMIS *110*, 23-32.

Vanik,D.L. and Surewicz,W.K. (2002). Disease-associated F198S mutation increases the propensity of the recombinant prion protein for conformational conversion to scrapie-like form. J Biol. Chem. *277*, 49065-49070.

Vorberg,I., Chan,K., and Priola,S.A. (2001). Deletion of beta-strand and alpha-helix secondary structure in normal prion protein inhibits formation of its protease-resistant isoform. J Virol. *75*, 10024-10032.

Wadsworth,J.D., Joiner,S., Hill,A.F., Campbell,T.A., Desbruslais,M., Luthert,P.J., and Collinge,J. (2001). Tissue distribution of protease resistant prion protein in variant Creutzfeldt-Jakob disease using a highly sensitive immunoblotting assay. Lancet *358*, 171-180.

Wegrzyn,R.D., Bapat,K., Newnam,G.P., Zink,A.D., and Chernoff,Y.O. (2001). Mechanism of prion loss after Hsp104 inactivation in yeast. Mol. Cell Biol. *21*, 4656-4669.

Weissmann,C. (1991). A 'unified theory' of prion propagation. Nature *352*, 679-683.

Weissmann,C. (1999). Molecular genetics of transmissible spongiform encephalopathies. J Biol. Chem. *274*, 3-6.

Wells,G.A., Hawkins,S.A., Green,R.B., Austin,A.R., Dexter,I., Spencer,Y.I., Chaplin,M.J., Stack,M.J., and Dawson,M. (1998). Preliminary observations on the pathogenesis of experimental bovine spongiform encephalopathy (BSE): an update. Vet. Rec. *142*, 103-106.

Wells,G.A., Hawkins,S.A., Austin,A.R., Ryder,S.J., Done,S.H., Green,R.B., Dexter,I., Dawson,M., and Kimberlin,R.H. (2003). Studies of the transmissibility of the agent of bovine spongiform encephalopathy to pigs. J Gen. Virol. *84*, 1021-1031.

Wells,G.A., Hawkins,S.A., Austin,A.R., Ryder,S.J., Done,S.H., Green,R.B., Dexter,I., Dawson,M., and Kimberlin,R.H. (2003). Studies of the transmissibility of the agent of bovine spongiform encephalopathy to pigs. J Gen. Virol. *84*, 1021-1031.

Westaway,D., Goodman,P.A., Mirenda,C.A., McKinley,M.P., Carlson,G.A., and Prusiner,S.B. (1987). Distinct prion proteins in short and long scrapie incubation period mice. Cell *51*, 651-662.

Westaway,D., Cooper,C., Turner,S., Da Costa,M., Carlson,G.A., and Prusiner,S.B. (1994). Structure and polymorphism of the mouse prion protein gene. Proc. Natl. Acad. Sci. U. S. A *91*, 6418-6422.

Whyte,S.M., Sylvester,I.D., Martin,S.R., Gill,A.C., Wopfner,F., Schatzl,H.M., Dodson,G.G., and Bayley,P.M. (2003). Stability and conformational properties of doppel, a prion-like protein, and its single-disulphide mutant. Biochem. J *373*, 485-494.

Wilesmith,J.W., Wells,G.A., Ryan,J.B., Gavier-Widen,D., and Simmons,M.M. (1997). A cohort study to examine maternally-associated risk factors for bovine spongiform encephalopathy. Vet. Rec. *141*, 239-243.

Will,R.G., Ironside,J.W., Zeidler,M., Cousens,S.N., Estibeiro,K., Alperovitch,A., Poser,S., Pocchiari,M., Hofman,A., and Smith,P.G. (1996). A new variant of Creutzfeldt-Jakob disease in the UK. Lancet *347* , 921-925.

Will,R.G., Zeidler,M., Stewart,G.E., Macleod,M.A., Ironside,J.W., Cousens,S.N., Mackenzie,J., Estibeiro,K., Green,A.J., and Knight,R.S. (2000). Diagnosis of new variant Creutzfeldt-Jakob disease. Ann. Neurol. *47*, 575-582.

Will,R.G. (2003). Acquired prion disease: iatrogenic CJD, variant CJD, kuru. Br. Med. Bull. *66*, 255-265.

Wille,H., Michelitsch,M.D., Guenebaut,V., Supattapone,S., Serban,A., Cohen,F.E., Agard,D.A., and Prusiner,S.B. (2002). Structural studies of the scrapie prion protein by electron crystallography. Proc. Natl. Acad. Sci. U. S. A *99*, 3563-3568.

Williams,E.S. and Young,S. (1992). Spongiform encephalopathies in Cervidae. Rev. Sci. Tech. *11*, 551-567.

Willoughby,K., Kelly,D.F., Lyon,D.G., and Wells,G.A. (1992). Spongiform encephalopathy in a captive puma (Felis concolor). Vet. Rec. *131*, 431-434.

Windl,O., Dempster,M., Estibeiro,J.P., Lathe,R., de Silva,R., Esmonde,T., Will,R., Springbett,A., Campbell,T.A., Sidle,K.C., Palmer,M.S., and Collinge,J. (1996). Genetic basis of Creutzfeldt-Jakob disease in the United Kingdom: a systematic

analysis of predisposing mutations and allelic variation in the PRNP gene. Hum. Genet *98*, 259-264.

Wong,B.S., Brown,D.R., Pan,T., Whiteman,M., Liu,T., Bu,X., Li,R., Gambetti,P., Olesik,J., Rubenstein,R., and Sy,M.S. (2001). Oxidative impairment in scrapie-infected mice is associated with brain metals perturbations and altered antioxidant activities. J Neurochem. *79*, 689-698.

Wong,E., Thackray,A.M., and Bujdoso,R. (2004). Copper induces increased beta-sheet content in the scrapie-susceptible ovine prion protein PrPVRQ compared with the resistant allelic variant PrPARR. Biochem. J *380*, 273-282.

Wood,J.L., McGill,I.S., Done,S.H., and Bradley,R. (1997). Neuropathology of scrapie: a study of the distribution patterns of brain lesions in 222 cases of natural scrapie in sheep, 1982-1991. Vet. Rec. *140*, 167-174.

Wrathall,A.E., Brown,K.F., Sayers,A.R., Wells,G.A., Simmons,M.M., Farrelly,S.S., Bellerby,P., Squirrell,J., Spencer,Y.I., Wells,M., Stack,M.J., Bastiman,B., Pullar,D., Scatcherd,J., Heasman,L., Parker,J., Hannam,D.A., Helliwell,D.W., Chree,A., and Fraser,H. (2002). Studies of embryo transfer from cattle clinically affected by bovine spongiform encephalopathy (BSE). Vet. Rec. *150*, 365-378.

Wyatt,J.M., Pearson,G.R., Smerdon,T.N., Gruffydd-Jones,T.J., Wells,G.A., and Wilesmith,J.W. (1991). Naturally occurring scrapie-like spongiform encephalopathy in five domestic cats. Vet. Rec. *129*, 233-236.

Yamaguchi,N., Sakaguchi,S., Shigematsu,K., Okimura,N., and Katamine,S. (2004). Doppel-induced Purkinje cell death is stoichiometrically abrogated by prion protein. Biochem. Biophys. Res. Commun. *319*, 1247-1252.

Yin,S.M., Sy,M.S., Yang,H.Y., and Tien,P. (2004). Interaction of Doppel with the full-length laminin receptor precursor protein. Arch. Biochem. Biophys. *428*, 165-169.

Zahn,R., Liu,A., Luhrs,T., Riek,R., von Schroetter,C., Lopez,G.F., Billeter,M., Calzolai,L., Wider,G., and Wuthrich,K. (2000). NMR solution structure of the human prion protein. Proc. Natl. Acad. Sci. U. S. A *97*, 145-150.

Zahn,R. (2003). The octapeptide repeats in mammalian prion protein constitute a pH-dependent folding and aggregation site. J Mol. Biol. *334*, 477-488.

Zhu,Y.J., Lin,H., and Lal,R. (2000). Fresh and nonfibrillar amyloid beta protein(1-40) induces rapid cellular degeneration in aged human fibroblasts: evidence for AbetaP-channel-mediated cellular toxicity. FASEB J *14*, 1244-1254.

Ziegler,J., Sticht,H., Marx,U.C., Muller,W., Rosch,P., and Schwarzinger,S. (2003). CD and NMR studies of prion protein (PrP) helix 1. Novel implications for its role in the PrPC-->PrPSc conversion process. J Biol. Chem. *278*, 50175-50181.

Zou,W.Q., Capellari,S., Parchi,P., Sy,M.S., Gambetti,P., and Chen,S.G. (2003). Identification of novel proteinase K-resistant C-terminal fragments of PrP in Creutzfeldt-Jakob disease. J Biol. Chem. *278*, 40429-40436.