Dissertation

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The 37 kDa/67 kDa laminin receptor as a therapeutic target in prion diseases: potency of antisense LRP RNA, siRNAs specific for LRP mRNA and a LRP decoy mutant

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Erklärung

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Man kann niemanden überholen,
wenn man in seine Fußstapfen tritt.

Francois Truffaut (1932 - 1984)
frz. Regisseur, Schauspieler und Produzent

Die vorliegende Arbeit wurde in der Zeit von September 2002 bis Oktober 2005 im Labor von PD Dr. Stefan Weiß am Genzentrum der Ludwig-Maximilians-Universität angefertigt.

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Prion diseases are a group of rare, fatal neurodegenerative diseases, also known as transmissible spongiform encephalopathies (TSEs), that affect both animals and humans and include bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep, chronic wasting disease in deer and elk and Creutzfeldt-Jakob disease (CJD) in humans. TSEs are usually rapidly progressive and clinical symptoms comprise dementia and loss of movement coordination. A common hallmark of TSEs is the accumulation of an abnormal isoform (PrP^{Sc}) of the host-encoded prion protein (PrP^{c}) in the brains of affected animals and humans. PrP^{c} is a highly conserved cell surface sialoglycoprotein that is expressed in several cell types, mainly neuronal cells, but its normal physiological function is still not known. However, PrP^{c} is elementary for the acquisition and the replication of prion diseases. Several inhibitors of the PrP^{Sc} formation have been reported, but none of them showed great potency in an *in vivo* application. Thus, the identification of the 37kDa/67kDa laminin receptor (LRP/LR) as the cell surface receptor for prions opened a new direction for the development of a TSE therapy.

In the 1980s, the appearance of a new form of a neurological disease in the UK attracted attention of the scientific community. Since then, more than 180.000 BSE cases have been reported in the UK. The route of transmission of BSE is still not proven, but transmission of BSE to humans has been shown to cause the new variant of Creutzfeldt-Jakob disease (vCJD). Currently, no treatment to slow down or stop the disease process in humans with any form of CJD is established. In June 2004, the PRION-1 clinical trial (3 years) was started to assess the activity and safety of Quinacrine in human prion disease since there are no other drugs available which are considered suitable for human evaluation. However, several strategies have been investigated to find an anti-prion treatment including development of a vaccination therapy and screening for potent chemical compounds.

The discovery that the 37kDa/67kDa laminin receptor (LRP/LR) acts as the cell surface receptor for the cellular prion protein (PrP^{c}) opened a new perspective for the development of an anti-prion therapy. In scrapie-infected neuronal cells, which represent a widely used and well characterized *in vitro* model for transmissible spongiform encephalopathies, the accumulation of PrP^{Sc} has been prevented by transfection of (i) antisense LRP RNA, (ii) small interfering RNAs targeting the LRP mRNA and (iii) incubation with the polyclonal anti-LRP antibody W3. Furthermore, the knock down of surface LRP/LR resulted in a reduction of the cellular PrP levels, suggesting an interference with the PrP internalization process. Thus, LRP/LR is required for the PrP^{Sc} propagation *in vitro* and involved in the PrP^{c} metabolism.

Due to the existence of several LR genes, a major step to investigate the role of the 37kDa/67kDa laminin receptor in scrapie pathogenesis *in vivo* is the generation of transgenic mice exhibiting a lower level of LRP/LR. Hemizygous transgenic mice that express LRP/LR antisense RNA under the control

of the neuron-specific enolase (NSE) promoter were generated and showed a reduced LRP/LR protein level in the cerebellum and the hippocampus. Intracerebral inoculation of these transgenic mice with the scrapie agent will show, whether the accumulation of pathogenic PrP^{Sc} in the brain is delayed or prevented due to a reduced LRP/LR level.

A further therapeutic anti-prion approach is given by LRP/LR deletion mutants that can be secreted to the cell culture medium and might act as decoys. Previously, it has been demonstrated that a transmembrane deletion mutant is able to prevent PrP^{c} binding and internalization. *In vitro* studies using an N-terminally truncated LRP mutant, representing the extracellular domain of LRP/LR (LRP102-295::FLAG), revealed a reduced binding of (i) recombinant cellular PrP to mouse neuroblastoma cells, (ii) infectious moPrP 27-30 to BHK21 cells and (iii) interfered with the PrP^{Sc} propagation in chronically scrapie-infected mouse neuroblastoma cells. Furthermore, a cell free binding assay demonstrated the direct binding of the LRP102-295::FLAG mutant to both PrP^{c} and PrP^{Sc}. These results together with the finding that that endogenous LRP levels remain unaffected by the expression of the mutant indicate that the secreted LRP102-295::FLAG mutant may act in a trans-dominant negative manner as a decoy by trapping PrP molecules. Thus, the LRP mutant might represent a potential therapeutic tool for the treatment of TSEs.

To investigate the therapeutic potential of the LRP102-295::FLAG decoy mutant *in vivo* transgenic mice were generated ectopically expressing LRP102-295::FLAG in the brain. Animals showed no phenotype and transgene expression was detected in cortical and cerebellar brain regions. An intracerebral prion inoculation of these mice will prove whether the expression of the LRP102-295::FLAG mutant can impair the PrP^{Sc} accumulation in the brain and can thus, act as a alternative therapeutic tool in prion diseases.

Since there is no efficient TSE-treatment available, great expenses have been driven to discover an effective prion disease treatment. The recent finding that experimental introduction of RNA can be used to interfere with the function of an endogenous gene (RNA interference) provided a new tool for the development of gene-specific therapeutics. In order to evaluate a gene transfer therapeutic TSE strategy, human immunodeficiency virus (HIV)-derived vectors that express short hairpin RNA (shRNA) directed against the LRP mRNA were used. Following integration of LRP-shRNA-expressing lentiviral vectors into the genome of neuronal cells efficient LRP/LR downregulation was observed. In scrapie infected neuronal cells, downregulation of the LRP gene expression resulted in a diminishment of PrP^{Sc} propagation, providing a further therapeutic strategy in the development of a TSE treatment.

CHAPTER I

Introduction

1 Prion diseases

Prion diseases or transmissible spongiform encephalopathies (TSEs) are incurable neurodegenerative disorders, which occur both in humans and animals. Human TSEs include Kuru (Gajdusek and Zigas, 1957), Gerstmann-Sträussler-Scheinker syndrome (GSS) (Gerstmann *et al*., 1936), fatal familial insomnia (FFI) (Lugaresi *et al*., 1986) and Creutzfeldt-Jakob disease (CJD) (Creutzfeldt, 1920), which is the most prominent prion disease in humans. In animals, scrapie in sheep and goats (McGowan 1922), feline spongiform encephalopathy (FSE) in cats (Wyatt, 1990), transmissible mink encephalopathy (TME) (Burger and Hartsough, 1965), chronic wasting disease in wild ruminants (CWD) (Williams and Young, 1980), bovine spongiform encephalopathy (BSE) in cattle (Wells *et al*., 1987) and encephalopathies of a number of zoo animals (exotic ungulate encephalopathy, EUE) (Jeffrey and Wells, 1988; Kirkwood *et al*., 1990) have been described. A hallmark of all prion diseases is the accumulation of an abnormal, partially proteinase-resistant isoform of the cellular prion protein (PrP^{c}), which is a cell-surface glycosylphosphatidylinositol (GPI) anchored protein (Stahl and Prusiner, 1991). PrP^{c} is highly conserved among mammals (Schätzl *et al*., 1995) and is expressed in many tissues, notably high levels in the brain of animals and humans (Kretzschmar *et al*., 1986; Moudjou *et al*., 2001). The conversion of the host-encoded PrP^{c} into the abnormal disease-causing isoform (PrP^{Sc}) involves a conformational change and is important in the pathogenesis of these diseases (Prusiner, 1994). Several hypotheses about the nature of the infectious agent have been proposed. Initially, the agent was thought to be a slow virus (Sigurdsson, 1954; Thormar, 1971) because of several criteria: a long incubation period of month to decades until the onset of disease, limitation of the infection to a single host and pathoanatomical changes in a single organ or tissue. Further research revealed that the agent differed substantially from viruses and was extremely resistant to ultraviolett and ionizing radiation and treatment with nucleases. Such findings led to the suggestion that the transmissible agent may be devoid of nucleic acid (Alper *et al*., 1967). In 1967, J.S.Griffith postulated the hypothesis that the causative agent might be a protein (Griffith, 1967). The theory of a self-propagating proteinaceous agent (Prusiner, 1982) was proposed after the isolation of a protease-resistant sialoglycoprotein specifically associated with infectivity, designated the prion protein (PrP) (Bolton *et al*., 1982). The term prion, which was devised by Stanley Prusiner, is the abbreviation for "proteinaceous infectious particle" and was defined as "small proteinaceous infectious particle that resists inactivation by procedures which modify nucleic acids" (Prusiner, 1982).

1.1 History of prion diseases

During the18^{th} century scrapie was first recognized in sheep and earned its name due to the itching and rubbing of affected sheep. In the 1920s, Hans Gerhard Creutzfeldt and Alfons Maria Jakob described the first case of a progressive neurological disturbance in humans (Creutzfeldt, 1920; Jakob, 1921)

(Table 1) and in 1922 the eponym “Creutzfeldt-Jakob disease” was first used to characterize a number of neurodegenerative diseases. In 1936, scrapie was demonstrated to be transmissible by inoculation between sheep following prolonged incubation periods (Cuillé and Chelle, 1936) (Table 1). In the 1950s, a similar disease known as Kuru was identified among the Fore people, a tribe of remote highland natives of Papua New Guinea (Gajdusek and Zigas, 1957) (Table 1). Two years later, in 1959, the neuropathological similarities between both Kuru and scrapie (Hadlow, 1959) and Kuru and CJD (Klatzo *et al*., 1959) were observed (Table 1). In the 1960s, the term transmissible spongiform encephalopathy (TSE) was applied after the discovery of the transmissible ability of both Kuru (Gajdusek *et al*., 1966) and Creutzfeldt-Jakob disease (CJD) (Gibbs *et al*., 1968) to chimpanzees (Table 1). In 1976 Daniel Carleton Gajdusek was awarded the Nobel Prize for medicine for his work on “slow virus” infection. Reports in the early 1980s of a spongiform encephalopathy in captive mule and elk from the USA did not attract much attention (Williams and Young, 1980). In April 1985, english farmers reported for the first time that cattle showing random fear and aggression and uncoordinated movements. Postmortem analysis of their brains revealed multiple lesions that had turned the gray matter into a spongelike mass (Wells *et al*., 1987) (Table 1). The appearance of bovine spongiform encephalopathy (BSE) in cattle in the UK in 1987 rapidly evolved into a major epidemic (Wilesmith, 1988). Renewed interest and research on this disease gave rise to considerable concern about the possibility of transmission the infectious agent from animal species to human. This fear was emphasized by the appearance of spongiform encephalopathies in captive zoo animals and in domestic housecats in the UK. About a decade after the first BSE case was recognized, a new form of the degenerative brain disorder known as Creutzfeldt-Jakob disease (CJD) appeared in UK, named new variant CJD (vCJD) (Will *et al*., 1996) (Table 1). By February 2006 a total number of 159 vCJD patients have been registered in the UK (www.vetolavie.chez.tiscali.fr), and it has been shown that these are linked to BSE (Hill *et al*., 1997) (Table 1).

Table 1. Chronology of essential prion research

Year	Research discovery
Mid 18th century	First description of scrapie
1920	First description of CJD (Creutzfeldt, 1920)
1936	Report of experimental transmission of scrapie (Cuillé and Chelle, 1936)
1955-1957	Discovery of Kuru in New Guinea (Gajdusek and Zigas, 1957)
1959	Similarities between Kuru and scrapie noted (Hadlow, 1959)
	Similarities between Kuru and CJD noted (Klatzo *et al.*, 1959)
1961	Experimental transmission of scrapie to mice (Chandler, 1961)
1966	Experimental transmission of Kuru to chimpanzees (Gajdusek *et al.*, 1966)
1967	First notification of the protein-only hypothesis (Griffith, 1967)
1968	Experimental transmission of CJD to chimpanzees (Gibbs *et al.*, 1968)
1974	First documentation of iatrogenic prion transmission (Duffy *et al.*, 1974)
1982	Manifestation of the prion concept (Prusiner, 1982)
1985	Cloning of the PrP^{c} encoding gene (Chesebro *et al.*, 1985; Oesch *et al.*, 1985)
1986	PrP^{c} and PrP^{Sc} isoforms shown to be encoded by the same host gene (Basler *et al.*, 1986)
1987	First report of BSE in cattle (Wells *et al.*, 1987)
1993	$Prnp^{0/0}$ mice are resistant to scrapie inoculation (Bueler *et al.*, 1993)
1996	Identification of new variant CJD (Will *et al.*, 1996)
1997	First evidence that vCJD is caused by the BSE agent (Hill *et al.*, 1997)
1999	Discovery of the PrP^{c} homolog Dpl (Moore *et al.*, 1999)
2000	Resolution of the structure of the human prion protein by NMR (Zahn *et al.*, 2000)
2001	Identification of the crystal structure of human prion protein (Knaus *et al.*, 2001)
	Identification of pharmacotherapeutics for prion diseases (Korth *et al.*, 2001)
2002	Interaction of cell-surface prion protein with glycosaminoglycans (Pan *et al.*, 2002)
2003	Cell-free conversion of bacterial recombinant PrP (Kirby *et al.*, 2003)
2004	Proof that PrP^{c} is sufficient for spontaneous formation of prions (Legname *et al.*, 2004)
2005	Effective treatment of scrapie-infected mice using a mucosal anti-PrP vaccination (Goni *et al.*, 2005)

1.2 Human prion diseases

Human transmissible spongiform encephalopathies (Table 2) are a group of rapidly progressive disorders characterized by a defined spectrum of clinical abnormalities.

Table 2. Human prion diseases

TSE	Year of first report
Creutzfeldt-Jakob disease (CJD)	1920
Sporadic Creutzfeldt-Jakob disease (sCJD)	1921
Familial Creutzfeldt-Jakob disease (fCJD)	1924
Iatrogenic Creutzfeldt-Jakob disease (iCJD)	1974
New variant Creutzfeldt-Jakob disease (vCJD)	1996
Gerstmann-Sträussler-Scheinker syndrome (GSS)	1928
Kuru	1957
Fatal familial insomnia (FFI)	1986
Sporadic fatal insomnia (sFI)	1999

They all share a spongiform degeneration of the brain and a variable amyloid plaque formation. Four categories of human prion diseases have been described and can present as sporadic, inherited or iatrogenic disorders: Kuru, Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker syndrome and fatal insomnia (Table 3).

Kuru (means “trembling with fear” in the native language), the prototype of human spongiform encephalopathy, is restricted to the ‘Fore’ people in the Eastern Highlands of New Guinea, where prions were transmitted by ritualistic cannibalism (Gajdusek, 1977). Among the natives the disease was originally termed “negi nagi” (meaning a silly person) which led to the designation as the ‘laughing death’ (Klatzo *et al*., 1959). The disease occurred mostly in children and women, because they consumed the brain of deceased family members. In 1959, the government banned the cannibalistic practice.

Creutzfeldt-Jakob disease (CJD) presents as a sporadic (classical), hereditary (familial) or iatrogenic (acquired) illness. Approximately 85% of all CJD cases occur sporadically, geographically ubiquitously with an incidence rate of 0.5-2 cases per one million people per year. The median age of the onset of disease represents the seventh decade (64 years) and men and women are equally affected (Kretzschmar, 1993; Ravilochan *et al*., 1983). Typically, memory loss and progressive dementia with cerebellar degeneration are diagnosed in classical CJD patients. Homozygosity for amino acid Methionin or Valin at position 129 has been identified as a predisposing factor in the majority of sporadic and iatrogenic CJD cases (Collinge *et al*., 1991; Palmer *et al*., 1991).

Iatrogenic CJD (iCJD) is a very rare disease and has resulted from neurosurgery, corneal grafting, human dura mater implants or exposure, and the use of human growth hormone (hGH) and pituitary-derived gonadotropin (hGNH). Iatrogenic CJD was first recognized in 1974 in a US patient who received a corneal transplant from a donor later proven to have died from CJD (Duffy *et al*., 1974). Today, the most common source of iCJD has been contaminated human growth hormone and a total of 131 cases of iCJD following treatment with hGH have been reported (February 2006, www.eurocjd.ed.ac.uk) in the UK, France and the Netherlands.

Familial CJD (fCJD), representing 5-15% of all CJD cases, is classified into many haplotypes based on the *Prn-p* mutation and codon 129 on the mutant allele (Lee *et al*., 1999). The majority of familial CJD cases (>70%) have been associated with a codon 200 mutation in the *Prn-p* gene (E200K) (Bertoni *et al*., 1992; Goldfarb *et al*., 1990; Goldgaber *et al*., 1989) or with a codon 178 mutation (D178N) (Goldfarb *et al*., 1991; Haltia *et al*., 1991; Nieto *et al*., 1991) (Figure 1). The symptoms of the familial form of CJD vary, depending on the type of PrP mutation involved (Mastrianni *et al*., 2001).

In contrast to CJD, **Gerstmann-Sträussler-Scheinker syndrome** (GSS) is almost always described in a familial context. Only a few sporadic cases resembling GSS have been described (Masters *et al*., 1981). GSS is a rare form of prion disease and occurs at a rate of one per 100 million people per year worldwide (Ghetti *et al*., 1995). The syndrome was first described in 1928 by the Austrian neurologist Josef Gerstmann (1887-1969), followed by a more detailed report in collaboration with his colleagues Erwin Sträussler and Isaak Scheinker (Gerstmann *et al*., 1936). Most of the patients show first symptoms in the fourth or fifth decade of life. Investigations have shown that missense mutations are present in the *Prn-p* gene of GSS patients. To date, six mutations have been identified at codons 102 (P102L) (Hsiao *et al*., 1989), 105 (P105L) (Yamada *et al*., 1993), 117 (A117V) (Doh-ura *et al*., 1989), 131 (G131V) (Panegyres *et al*., 2001), 198 (F198S) (Dlouhy *et al*., 1992) and 217 (Q217R) (Hsiao *et al*., 1992), but the majority of affected families with GSS have a point mutation at codon 102 (Figure 1).

Fatal familial insomnia (FFI) was first described in 1986 in a 53 years old man (Lugaresi *et al*., 1986). Since then, it has been reported in several European countries (Almer *et al*., 1999; Carota *et al*., 1996; Padovani *et al*., 1998), Australia (McLean *et al*., 1997) and Japan (Nagayama *et al*., 1996). The occurrence of fatal familial insomnia is associated with the same codon 178 mutation (D178N) also observed in a subtype of familial CJD (Medori *et al*., 1992). The phenotype caused by the D178N mutation depends on a polymorphism at codon 129. The Met 129-Asn 178 allele segregates with FFI, while the Val 129-Asn 178 allele segregates with fCJD (Goldfarb *et al*., 1992).

Recently, the first cases of a sporadic form of fatal insomnia (sFI) have been reported in a 44-year-old man and a 58-year old woman (Mastrianni *et al.*, 1999; Scaravilli *et al.*, 2000). Both patients were homozygous for methionine at codon 129, but had no mutations in the prion protein gene.

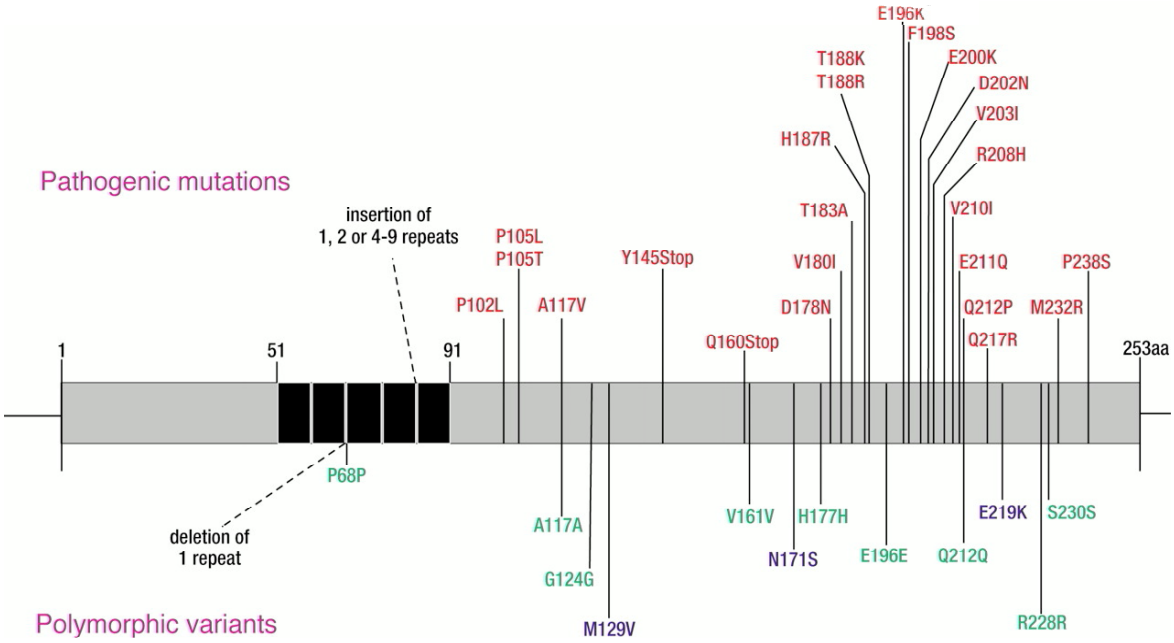


Figure 1. Pathogenic mutations and polymorphic variants of the human prion protein

Prn-p mutations can be divided into two groups: (i) point mutations resulting in amino acid substitutions or production of a stop-codon (one case) in the prion protein and (ii) insertions encoding additional integral copies of an octapeptide repeat or deletion of an octapeptide repeat. (adopted from Collinge, 2001).

In 1995 and early 1996, a small number of remarkably young CJD patients were diagnosed in the United Kingdom (UK). Due to its similarity to sCJD, this human disease was termed "**new variant of CJD**" (vCJD) (Will *et al.*, 1996). In contrast to sCJD, the median age of onset of the disease in vCJD patients is 28 years (sCJD 68 years) and the clinical course is prolonged (median 14 month, sCJD 6 months). The appearance of vCJD in the United Kingdom and the experimental evidence that vCJD is caused by the same prion strain that causes BSE raised the possibility that an epidemic of vCJD might occur (Cousens *et al.*, 1997; Ghani *et al.*, 1998). Laboratory transmission studies in transgenic mice demonstrated that the characteristics of vCJD, including incubation period and neuropathological changes, are very similar in BSE and vCJD (Scott *et al.*, 1999). The favored hypothesis for transmission of BSE to humans is a dietary exposure to prion-contaminated bovine tissues (likely CNS) in the 1980s (Will, 1999). Variant CJD is difficult to distinguish from other neurological disorders, so that a definitive diagnosis has relied on neuropathology. It has been demonstrated that vCJD can be diagnosed by PrP^{Sc}-immunostaining on a tonsil biopsy (Collinge, 1996; Hill *et al.*, 1999).

To date, vCJD disease has only been recognized in individuals homozygous for methionine at codon 129 in the *Prn-p* gene (Collinge *et al*., 1996).

The human genotype at codon 129 of the *Prn-p* gene is known be a key determinant in human transmissible spongiform encephalopathies. This polymorphism modulates phenotype and disease susceptibility to acquired or sporadic prion infection (Cervenakova *et al*., 1998). The large majority of prion-disease-affected individuals are homozygous at codon 129 for either methionine or valine (Collinge *et al*., 1991; Palmer *et al*., 1991; Windl *et al*., 1996). The prevalence of Met/Met is only 39% in the normal Caucasian population while the frequency for Met/Val is about 50% and for Val/Val 11% (Collinge *et al*., 1991). The protective effect of *Prn-p* codon 129 heterozygosity is seen in some of the inherited prion diseases (Baker *et al*., 1991; Hsiao *et al*., 1992).

Table 3. Pathogenesis of human prion diseases

Disease	Mechanism of pathogenesis
Kuru	infection through ritualistic cannibalism
Sporadic Creutzfeldt-Jakob disease (sCJD)	somatic mutation
Familial Creutzfeldt-Jakob disease (fCJD)	germline mutation in the *Prn-p* gene
Iatrogenic Creutzfeldt-Jakob disease (iCJD)	infection from prion-contaminated medication, grafts and instruments
New variant Creutzfeldt-Jakob disease (vCJD)	infection from bovine prions
Gerstmann-Sträussler-Scheinker syndrome (GSS)	germline mutation in the *Prn-p* gene
Fatal familial insomnia (FFI)	germline mutation in the *Prn-p* gene
Sporadic fatal insomnia (sFI)	not known

1.3 Animal prion diseases

Transmissible spongiform encephalopathies (TSEs) have also been described in different animal species (Table 4).

Scrapie was first recognized as a disease of sheep in Great Britain over 200 years ago and is known as the prototype of prion diseases in animals. It is a slowly progressive disease and causes degeneration of the central nervous system (McGowan, 1922). The name “scrapie” is derived from the tendency of affected sheep to scrape off their wool. Although the worldwide distribution is unknown, Australia and New Zealand are the only countries being scrapie-free.

The first case of **bovine spongiform encephalopathy** (BSE) was diagnosed in 1987 (Table 4) (Wells *et al*., 1987). BSE has a long incubation period of four to five years and is characterized by a spongiform degeneration of the brain with severe and fatal neurological symptoms. Between

November 1996 and February 2006 180857 cases of BSE were confirmed in the UK (www.vetolavie.chez.tiscali.fr). Epidemiological studies suggest that the source of BSE was cattle food that was contaminated by the scrapie agent (from meat and bone meal from sheep) due to an improper sterilization procedure. In July 1988, the UK banned the use of ruminant sources in the preparation of animal feed, but sale from UK to other countries was allowed. Six years later, the European Union (EU) banned mammalian meat and bone meal to ruminants (cattle, sheep). Starting in 1996, bans prevented the sale of food and food products containing beef from the UK to other countries. However, in 1999, the EU repealed the ban for meat fulfilling specific requirements. Since then, cattle were continuously monitored for BSE.

Transmissible mink encephalopathy (TME) has sporadically appeared in farmed mink and was first reported in 1947 in Wisconsin and Minnesota (USA) (Table 4). Epidemiological studies revealed that the disease is causally linked to the ingestion of prion-contaminated meat, potentially scrapie (Marsh and Bessen, 1993). To investigate potential food-borne sources of TME, mink were intracerebrally exposed to sheep scrapie brain homogenate and showed high susceptibility to the sheep scrapie (Hanson *et al.*, 1971). Nevertheless, mink did not develop disease from ingesting scrapie brain (Marsh and Hanson, 1979). However, further experiments demonstrated that TME was transmitted to cattle and that brain from these cattle transmitted the TME agent efficiently to mink (Marsh *et al.*, 1991).

Feline spongiform encephalopathy (FSE) has been described in captive cheetah, puma, ocelot, tiger and lion (Table 4) and was likely due to the ingestion of BSE-infected material (Kirkwood and Cunningham, 1994). In addition, domestic cats have also been diagnosed with FSE (Ryder *et al.*, 2001). By February 2006, there were a total of 89 confirmed cases peaking at 1994 (www.defra.gov.uk). Cases have mainly been confined to the UK, but there have been sporadic cases reported in Northern Ireland, Norway, Lichtenstein and Switzerland (www.defra.gov.uk). The most widely accepted hypothesis for transmission of the infectious agent is that the affected domestic cats were exposed to BSE material present in contaminated commercial cat food.

Chronic wasting disease (CWD) is the only prion disease known affecting free ranging wildlife including elk, white-tailed deer and mule deer. The disease was first recognized in captive mule deer (*Odocoileus hemionus*) 39 years ago in Colorado (Table 4), but not diagnosed as a TSE until 1978 (Williams and Young, 1980). Typically, affected animals are 3-5 years old. The origin of CWD in captive or free-ranging deer remains enigmatic (Miller *et al.*, 2000; Williams and Miller, 2002), because these animals had never been fed with meat- or bone-meal (Williams and Young, 1992). The mode of transmission of CWD is unknown.

In parallel to the first BSE cases, cases of spongiform encephalopathy in hoofed zoo species (**exotic ungulate encephalopathy**, EUE) were diagnosed in British zoos (Table 4). The exotic zoo ruminants

that died of TSE include greater kudu, eland, nyala, gemsbok, Arabian oryx, a scimitar-horned oryx and a bison (Kirkwood and Cunningham, 1994). The affected exotic ruminants appear to be linked to the BSE epidemic via the consumption of contaminated feed (Kirkwood and Cunningham, 1994).

The first case of a spontaneously developed spongiform encephalopathy in a rhesus monkey (*Macaca mulatta*) (**primate spongiform encephalopathy**, PSE) was reported in 1996 in France (Table 4). Immunohistochemistry revealed severe vacuolation of neurons related to those seen in the brains of CJD patients (Bons *et al*., 1996). In the 1990s, further TSE cases of primates (lemurs) were reported. Indeed, lemurs experimentally infected with BSE showed similar neuropathology and distribution of PrP^{Sc} as spontaneously infected zoo lemurs (Bons *et al*., 1999)

Table 4. Animal prion diseases

Species	TSE	Year of first report
sheep, goats	Scrapie	1732
european minks	Transmissible mink encephalopathy (TME)	1947
captive mule and elk	Chronic wasting diseases (CWD)	1980
captive cats	Feline spongiform encephalopathy (FSE)	1986
domestic cats	Feline spongiform encephalopathy (FSE)	1990
cattle	Bovine spongiform encephalopathy (BSE)	1987
zoo antelopes	Exotic ungulate encephalopathy (EUE)	1986
non-human primates	Primate spongiform encephalopathy (PSE)	1996

2 The prion protein (PrP)

The prion protein (PrP^{c}) is a normal cellular glycolipid anchored protein and is highly conserved among mammalian species (Schätzl *et al*., 1995; Wopfner *et al*., 1999). It has been identified in various animals including birds (Harris *et al*., 1993), pisces (Gibbs and Bolis, 1997) and marsupials (Windl *et al*., 1995), and may be present in all vertebrates. PrP mRNA is constitutively expressed in the brains of adult animals with a high expression in neurons (Kretzschmar *et al*., 1986). Substantial amounts have also been found in heart (Brown *et al*., 1990), skeletal muscle (Bosque *et al*., 2002; Brown *et al*., 1998), lymphoid tissue and leukocytes (Liu *et al*., 2001; Paltrinieri *et al*., 2004), intestinal tissues (Morel *et al*., 2004) and uterus and testis (Tanji *et al*., 1995). In 1986, the human PrP gene (*Prn-p*) has been mapped to 20p12-pter (short arm of chromosome 20) (Liao *et al*., 1986; Robakis *et al*., 1986; Sparkes *et al*., 1986). The conformation of the cellular isoform of murine PrP was first determined by nuclear magnetic resonance (NMR) studies (Riek *et al*., 1996). Since then, NMR measurements on the prion protein from various species, e.g. Syrian hamster (Donne *et al*.,

1997), human (Zahn *et al.*, 2000), cattle (Lopez Garcia *et al.*, 2000), sheep, cat, dog and pig (Lysek *et al.*, 2005) as well as chicken, turtle, frog (Calzolai *et al.*, 2005) and elk (Gossert *et al.*, 2005), were performed and revealed that they all have global architecture similarities. The prion protein has a flexible, unstructured N-terminal region and a well-ordered C-terminal globular domain, which includes three α-helices and two antiparallel β-sheet structures (Riek *et al.*, 1996). The N-terminal region contains a segment of several octapeptide-repeat regions that preferentially bind copper (Hornshaw *et al.*, 1995) (Figure 2).

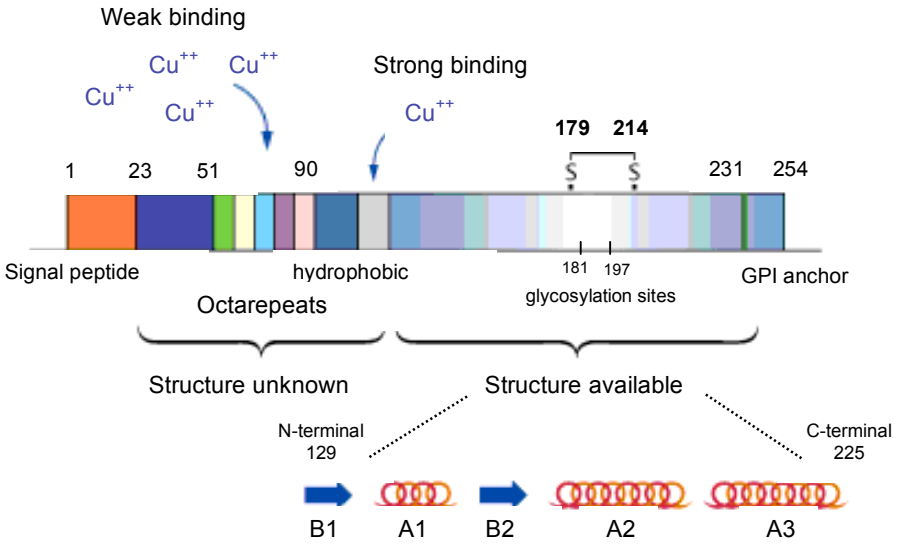


Figure 2. Genomic organization of the Syrian hamster cellular prion protein

Cellular PrP (PrP^c) is a GPI-anchored protein of 254 amino acid residues. During PrP^c processing a 22 amino acid N-terminal signal peptide is removed and 23 C-terminal residues are cleaved upon the addition of the glycosylphosphatidylinositol anchor to Ser-231. The C-terminus is highly structured and possesses two glycosylation sites (Asn-181 and Asn-197) and cysteine residues 179 and 214 act as sites to form a disulfide bound. The N-terminal region contains a series of 5 octapeptide repeats that have been implicated in the binding of metal ions. (www.chemsoc.org, modified)

Infrared spectroscopy and circular dichroism demonstrated that the secondary structure of PrP^c is mainly composed of α-helices (42%), whereas PrP^{Sc} consists mainly of β-sheets (Cohen *et al.*, 1994). In Syrian hamster and mice, PrP^c is synthesized as a precursor of 254 amino acids (aa) while the human *Prn-p* encodes a prion protein 253 aa in length. During trafficking within the cell the N-terminal signal peptide (22 amino acids) is cleaved off in the endoplasmatic reticulum (Hope *et al.*, 1986) and 23 C-terminal residues demerge upon addition of the GPI anchor at serine (Ser) 231 (Stahl *et al.*, 1987). The hamster prion protein has two glycosylations sites at Asparagines (Asn) 181 and Asn 197, and a disulphide bond is formed between Cysteine (Cys) 179 and Cys 214 (Turk *et al.*, 1988). Cell culture studies revealed that PrP^c constitutively cycles between the cell surface and an endocytic

compartment with a transit time of approximately 60 minutes. More than 95% of the internalized protein is recycled back to the cell surface, where it is anchored via the GPI moiety (Shyng *et al.*, 1993).

2.1 Role of PrP^c

The exact physiological role of the cellular prion protein PrP^c remains still obscure, although some possible biological functions have been described. The proposed functions include a neuroprotective function due to antiapoptotic activity (Bounhar *et al.*, 2001; Diarra-Mehrpour *et al.*, 2004), a functional role in copper metabolism due to its copper-binding capacity (Brown *et al.*, 1997), involvement in signal transduction (Koch *et al.*, 1991; Mouillet-Richard *et al.*, 2000), memory formation (Coitinho *et al.*, 2003) and neuritogenesis (Graner *et al.*, 2000). Mice lacking PrP ($Prnp^{0/0}$) showed no obvious phenotype (Bueler *et al.*, 1992), although they have abnormalities in synaptic physiology (Collinge *et al.*, 1994) and in circadian rhythm and sleep (Tobler *et al.*, 1996). $Prnp^{0/0}$ mice were shown to be completely resistant to prion disease (Bueler *et al.*, 1993). Several lines of PrP knockout mice have been generated to unveil the function of PrP^c (Weissmann and Flechsig, 2003).

The molecular mechanism of PrP **protection against oxidative stress** is still unclear, but PrP may reduce copper-mediated oxidative stress due to its copper-binding activity (Vassallo and Herms, 2003). PrP^c binds copper via conserved histidine residues within the N-terminal region of the protein that contains an octapeptide repeat region (Brown *et al.*, 1997). It has been proposed that PrP^c acts as a sensor for copper and triggers intracellular calcium signals that finally modulate synaptic transmission (Vassallo and Herms, 2003). Since PrP knockout mice exhibit approx. 50% lower copper concentration in synaptosomal fractions than wild-type mice, it was suggested that PrP^c might regulate the copper concentration in the synaptic region and may play a role in the re-uptake of copper into the presynapse (Kretzschmar *et al.*, 2000).

Furthermore, it has been shown that PrP^c harbors a copper/zinc-dependent superoxide-dismutase (SOD) that provides PrP^c with **antioxidant activity**. By deletion of the octapeptide repeat region involved in copper binding, the SOD activity was abolished (Brown *et al.*, 1999). *In vivo* experiments revealed that protein and lipid oxidation is increased in skeletal muscle, heart and liver in $Prnp^{0/0}$ mice suggesting a PrP^c function related to cellular antioxidant defenses (Klamt *et al.*, 2001).

PrP^c is known to be attached to the plasma membrane through a glycosylphosphatidylinositol (GPI) anchor and may act as a cell-surface receptor mediating **cell-surface signaling** or cell adhesion. Recently, a coupling of PrP^c to the non-receptor tyrosine kinase Fyn was observed (Mouillet-Richard *et al.*, 2000). Fyn tyrosine kinase is a member of the Src family of tyrosine kinases and is required for morphological differentiation of oligodendrocytes (Osterhout *et al.*, 1999).

Furthermore, several experimental findings suggest a major role for PrP^c in cell survival or cell death. In a yeast two-hybrid system, PrP^c was demonstrated to interact selectively with the Bcl-2 protein (Kurschner and Morgan, 1995), that is a suppressor of the programmed cell death. Recently, the **antiapoptotic activity** of PrP has been shown in a human breast carcinoma cell line (Diarra-Mehrpour *et al*., 2004).

Despite all knowledge, there is some controversy around the protective function of PrP^c. It has been demonstrated that in some cell lines overexpression of PrP^c increase the susceptibility of these cells to staurosporine-induced apoptosis (Paitel *et al*., 2002; Paitel *et al*., 2003). In addition, it was proposed that endogenous cellular prion protein sensitizes neurons to apoptotic stimuli through a p53-dependent caspase 3 mediated activation (Paitel *et al*., 2003; Paitel *et al*., 2004). But there are several possibilities to explain this discrepancy. First, in some cases overexpression of PrP may interfere with the cellular protein synthesis machinery leading to saturation following toxicity. Second, overexpression of PrP may lead to the formation of topological forms of toxic PrP and third, overexpressed PrP may be converted into a lethal protein or into an aberrant isoform.

2.2 Trafficking of PrP^c

PrP is predominantly found on the cell surface where it is attached by a GPI anchor (Stahl *et al*., 1987). The biosynthetic pathway of PrP is similar to that of other membrane proteins. PrP^c is synthesized in the rough endoplasmatic reticulum (rER) and is further trafficking along the secretory pathway to the cell surface. Biosynthesis of PrP^c includes cleavage of the N-terminal signal peptide, addition of N-linked oligosaccharide chains, formation of a disulfide bound, attachment of the GPI-anchor and cleavage of the C-terminal hydrophobic peptide (Haraguchi *et al*., 1989; Stahl *et al*., 1987; Turk *et al*., 1988) followed by traveling to the plasma membrane. PrP^c is associated with lipid rafts (Naslavsky *et al*., 1997), which are special membrane domains that are rich in cholesterol and sphingolipids (Simons and Ikonen, 1997) and thought to represent sites for signal transduction events. Previously published studies suggest, that misfolded or improperly matured PrP is retro-translocated from the ER into the cytosol and degraded by the proteasome (Bonifacino and Weissman, 1998; Ma and Lindquist, 2001; Ma and Lindquist, 2002; Ma *et al*., 2002; Tsai *et al*., 2002; Yedidia *et al*., 2001).

Most PrP^c molecules are attached to the cell surface, but at the endoplasmatic reticulum membrane PrP can be synthesized in several topological forms. These topological forms are ordinary generated during the PrP biosynthesis and span the membrane once, with either the N-terminus or the C-terminus on the cytoplasmic side of the lipid bilayer, designated ^{Ntm}PrP and ^{Ctm}PrP (Harris, 2003) (Figure 3). The possibility that alternative topological variants of PrP may play a role in prion diseases arose a great interest. In fact, it has been demonstrated that ^{Ctm}PrP is a key neurotoxic intermediate in certain

prion diseases and might cause neurodegeneration (Hegde *et al.*, 1998; Hegde *et al.*, 1999; Stewart *et al.*, 2001).

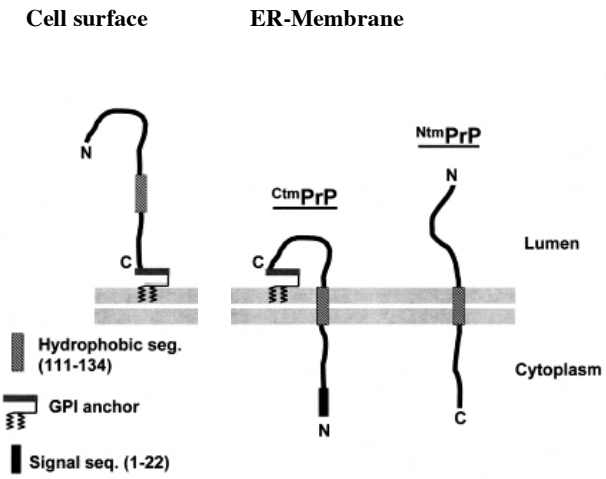


Figure 3. Topological forms of PrP

The majority of newly synthesized PrP^c is attached to the cell surface through a C-terminal glycosyl-phosphatidylinositol (GPI). During PrP biosynthesis in the endoplasmatic reticulum, the prion protein can also be synthesized in different topological form including two transmembrane forms designated ^{Ntm}PrP and ^{Ctm}PrP. The transmembrane species span the lipid bilayer once via a highly conserved hydrophobic region (amino acid 111-134) with either the N- or C-terminus on the extracytoplasmic side of the membrane. (Harris, 2003, modified)

PrP^c is constitutively internalized into an endocytic compartment and the majority is recycled to the cell surface (Shyng *et al.*, 1993). It has been shown that copper rapidly and reversibly stimulates endocytosis of PrP^c from the cell surface (Pauly and Harris, 1998). Furthermore, it has been demonstrated that PrP^c, which is internalized in response to copper, is delivered to early endosomes (Brown and Harris, 2003). The mechanism of PrP^c internalization is currently under investigation, and caveolae, rafts and clathrin-coated pits have been shown to be involved (Figure 4). Localization studies of PrP^c indicated that PrP^c clusters in caveolae or caveolae-like domains (CLDs) (Vey *et al.*, 1996). It has also been shown that PrP^c is present in detergent insoluble glycosphingolipid-rich membrane domains (DIGs) in mouse brain (Parkin *et al.*, 1999). Furthermore, in Chinese ovary hamster (CHO) cells caveolin-related endocytosis has been implicated (Peters *et al.*, 2003). Interestingly, some GPI-anchored proteins may use endosomal organelles that contain caveolin-1 (termed caveosomes), which is distinct from classical endosomes, to traffic to the Golgi (Nichols, 2002).

A more commonly studied pathway for the endocytic uptake of PrP^c involves clathrin-coated pits (Figure 4). Initial experiments using a chicken homologue of PrP^c indicated that internalization of PrP^c is dependent on clathrin-mediated endocytosis (Shyng *et al.*, 1994). Later on it has been demonstrated

that the N-terminal region of the prion protein is essential for its internalization via clathrin-coated pits (Shyng *et al.*, 1995). More recently, it was shown that the N-terminal region is not only important for endocytosis but also for delivery of PrP^c to the cell surface (Nunziante *et al.*, 2003). In neurons, it has been shown that PrP^c is located in the Golgi and in endosomal intracytoplasmic organelles (Laine *et al.*, 2001). Recent immunoelectron microscopy experiments in neuronal cells have demonstrated, that PrP^c is rapidly endocytosed and predominantly found in clathrin-coated pits (Sunyach *et al.*, 2003). Investigation of the vesicular transport revealed, that different Rab proteins are associated with endosomes (Sonnichsen *et al.*, 2000) that are implicated in the endocytic transport of PrP^c.

Finally, non-clathrin and non-caveolin but raft-dependent endocytosis has been proposed to participate in the internalization of PrP^c (Figure 4). The association of GPI-anchored proteins with cholesterol-rich rafts is generally a dynamic phenomenon and might facilitate maturation and correct folding of PrP^c (Sarnataro *et al.*, 2004). Overall, PrP^c may use distinct intracellular routes for internalization but there is growing evidence for a role of classical endosomal organelles in PrP^c intracellular trafficking.

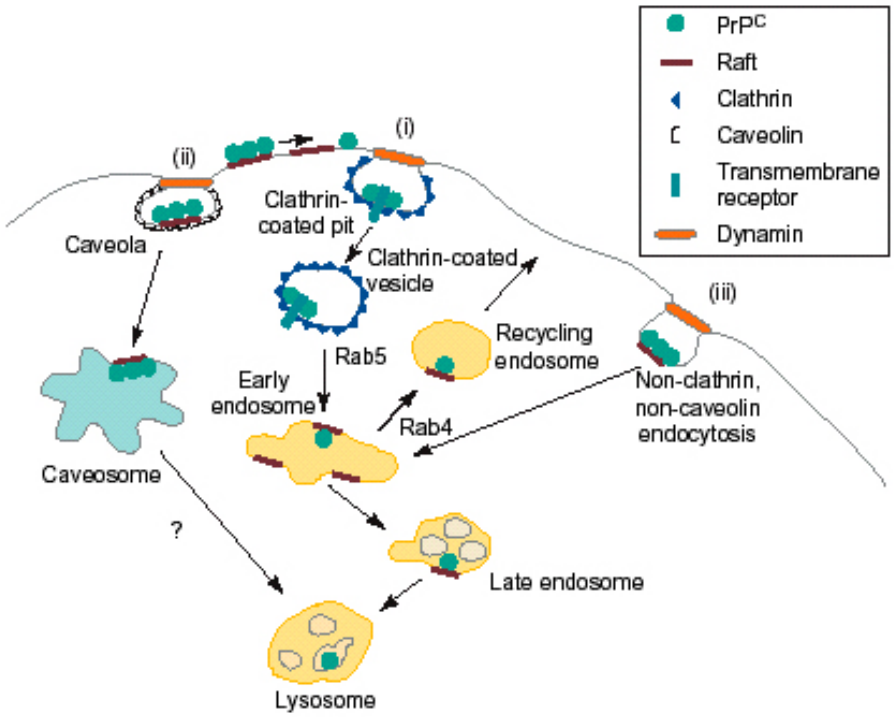


Figure 4. Cellular trafficking of PrP^c

At the cell surface, PrP^c can be constitutively internalized into an endocytic compartment from which most molecules are recycled to the plasma membrane. (i) In neuronal cells, PrP^c internalization seems to depend on clathrin-mediated endocytosis. Rab5 positive endosomes have been shown to participate in the trafficking of PrP^c. (ii) Caveolin-related endocytosis and trafficking have also been suggested for PrP^c, particularly in CHO cells. (iii) Like other GPI-anchored proteins PrP^c is associated with rafts. Therefore, a non-clathrin and non-caveolin, but raft-dependent endocytosis has been implicated to participate in the internalization of PrP^c. (adopted from Campana *et al.*, 2005)

2.3 Characteristics of PrP^{Sc}

In TSEs, the cellular prion protein PrP^{c} can be converted into a pathogenic isoform referred to as PrP^{Sc} that shows great resistance to radiation and nucleases (Alper *et al*., 1967). The high proportion of β-sheets in PrP^{Sc} (Figure 5) renders it insoluble and markedly resistant to proteases (Cohen and Prusiner, 1998).

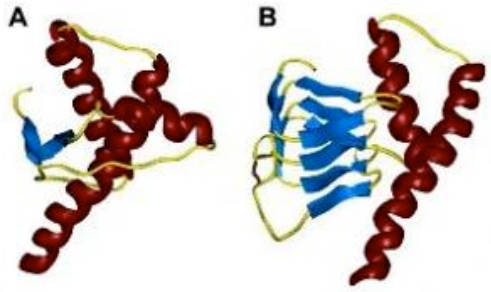


Figure 5. Models of the three-dimensional structures of PrP isoforms

The structure of the normal prion protein, known as cellular PrP^{c} (A), contains mostly α-helical structures (brown ribbons) and a small portion of flattened β-sheet (blue arrows). The increase in β-sheet upon conversion into the prion form is illustrated in (B) by the theoretical structural model of PrP^{Sc}. The 90-160 region of amino acid sequence has been modeled onto α-helical architecture (blue arrows) while the C-terminal helices are preserved as in PrP^{c} (brown ribbons). (adopted from F. Cohen, University of California, San Francisco)

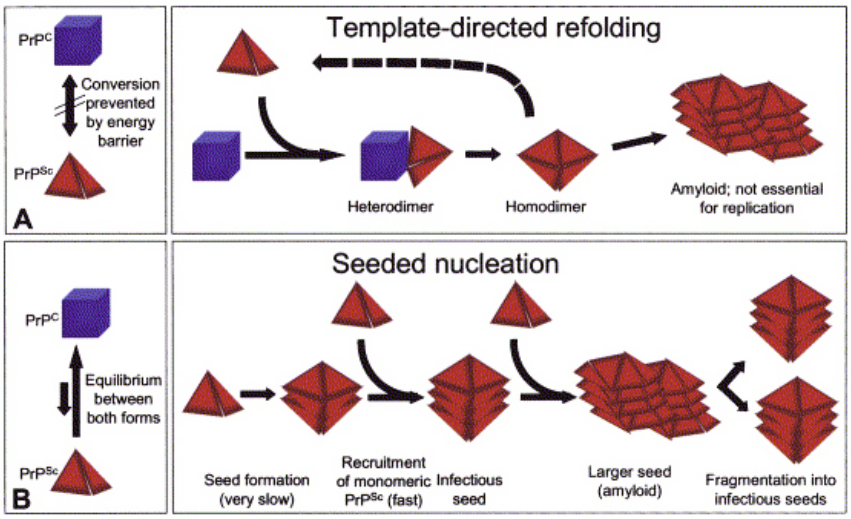
Digestion with proteases result in a 27-30kDa fragment, often termed PrPres (Bolton *et al*., 1982). PrP27-30 is unusual stable at high temperatures and can only be inactivated by protein denaturants that modify the structure of PrP27-30 (Prusiner *et al*., 1993). During prion diseases PrP27-30 accumulates into rod-shaped polymers that are insoluble in aqueous and organic solvents as well as nonionic detergents. In contrast, PrP^{Sc} (the full-length infectious conformer of PrP^{c}) has a tendency to form aggregates but not amyloid fibrils (McKinley *et al*., 1991) (Table 5).

Table 5. Properties of different PrP isoforms

PrP isoform	PrP^{c}	PrP^{Sc}	PrP27-30
infectivity	non infectious	infectious	infectious
protease status	sensitive	partially resistant	resistant
solubility	soluble	insoluble	insoluble
aggregation status	monomer	aggregates	amyloid fibrils
secondary structure	α-helices (42%), β-sheets (3%)	α-helices (30%), β-sheets (45%)	α-helices (21%), β-sheets (54%)

2.4 Conversion of PrP^{c} to PrP^{Sc}

Given the same primary structure of PrP^{c} and PrP^{Sc} (Basler *et al.*, 1986), the different properties of PrP^{c} and PrP^{Sc} seemed likely to involve post-translational modifications. Extensive biochemical investigations have failed to reveal any covalent difference between PrP^{c} and PrP^{Sc} (Stahl *et al.*, 1993). By contrast, spectroscopic studies demonstrated a conformational difference between PrP^{c} and PrP^{Sc}. It was shown, that PrP^{c} has a high α-helical content of approx. 40%, with little or no β-sheets (approx. 3%), whereas PrP^{Sc} contains approx. 30% α-helices and approx. 45% β-sheets (Pan *et al.*, 1993) (Table 5). PrP^{Sc} formation is supposed to occur via the interaction between PrP^{c} and PrP^{Sc}, which is able to convert the host protein into a likeness of itself (Griffith, 1967; Prusiner, 1982). The mechanism by which PrP^{Sc} triggers further PrP^{Sc} production is unknown, although two major models have been proposed (Figure 6). The catalytic model (Prusiner, 1991) proposes that the presence of PrP^{Sc} catalyzes the conversion of PrP^{c} to PrP^{Sc}. Alternatively, it has been proposed that the formation of PrP^{Sc} is a nucleation-dependent process (Lansbury and Caughey, 1995). The conversion between PrP^{c} and PrP^{Sc} is reversible, but the PrP^{Sc} monomer is less stable than PrP^{c} and several monomeric PrP^{Sc} molecules are stabilized by binding to PrP^{c} or other PrP^{Sc} aggregates. The cell-free *in vitro* conversion process was shown to be consistent with the nucleated polymerization mechanism of PrP^{Sc} formation and inconsistent with the heterodimer mechanism (Caughey *et al.*, 1995).



Prusiner, 1991

Lansbury and Caughey, 1995

Figure 6. Conversion models of PrP^{c} to PrP^{Sc}

(A) The "refolding (or catalytic) model" postulates that PrP^{Sc} is inherently more stable than PrP^{c}, but kinetically inaccessible. But PrP^{Sc} could promote conversion of a PrP^{c} molecule to the more stable PrP^{Sc} conformation. Infectivity then relies on the ability of the PrP^{Sc} molecule to bind to and catalyze the conversion of existing intermediate molecules. (B) The nucleation-polymerization or "seeding" model proposes, that PrP^{c} and PrP^{Sc} are in a reversible thermodynamic equilibrium, but the PrP^{Sc} monomer is less stable than PrP^{c}. Only if several monomeric PrP^{Sc} molecules are mounted into a higly ordered seed, further monomeric PrP^{Sc} can be recruited and form amyloid aggregates. Fragmentation of PrP^{Sc} aggregates increases the number of seeds, which can recruit further PrP^{Sc}, and thus results in replication of the agent. (adopted from Aguzzi and Polymenidou, 2004)

However, the precise subcellular localization of PrP^{Sc} propagation remains controversial. But there is evidence, that either late-endosome-like organelles or lysosomes are involved (Arnold *et al.*, 1995; Mayer *et al.*, 1992). Recent studies report the involvement of rafts in different diseases (Fantini *et al.*, 2002) and a role in pathogen invasion (van der Goot and Harder, 2001). A role for lipid rafts in the formation of PrP^{Sc} is deduced from the finding that both PrP^{c} and PrP^{Sc} are present in rafts isolated from infected cells (Baron and Caughey, 2003; Botto *et al.*, 2004). Although the exact mechanisms of how rafts control the formation of PrP^{Sc} are unidentified, there exist three different models. Firstly, rafts might be involved in targeting PrP^{c} to the specific compartments in which PrP^{Sc} transconformation occurs. Secondly, rafts might contain factors comprising the transconformation machinery. And thirdly, rafts might provide the membrane environment for the transconformation process by concentrating PrP^{c} and PrP^{Sc} within defined parts of the plasma membrane (Campana *et al.*, 2005).

2.5 The prion-like protein Doppel (Dpl)

In 1999, a novel gene 16kb downstream from the mouse prion protein gene (*Prn-p*), located on chromosome 2, was identified and termed *Prn-d* (prion protein dublet). The human *Prn-d* gene is localized similarly, 27kb downstream of *Prn-p* on 20pter-p12 (Moore *et al.*, 1999). The *Prn-d* gene generates two major transcripts of 1.7kb and 2.7kb and encodes a 179 amino acid residue PrP-like protein designated "Doppel" (Dpl). Like PrP, Dpl is expressed during embryogenesis, in heart and testis, but in contrast to PrP, it is minimally expressed in the adult central nervous system (CNS). The Dpl and PrP proteins show approx. 25% sequence identity.

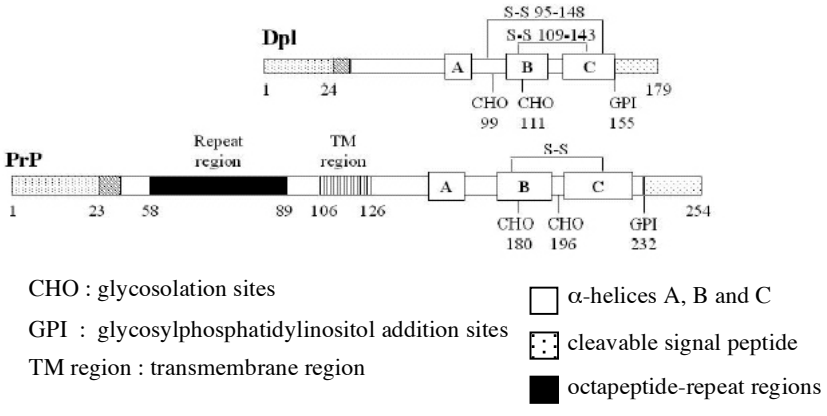


Figure 7. Organization of murine Dpl and PrP

PrP and Dpl are both GPI-anchored proteins and show structural similarities. Like PrP^{c}, Dpl is a protein with two N-glycosylation sites and a globular C-terminus containing three α-helices and two short β-strands. Dpl is distinguished from PrP^{c} by a shorter N-terminal region, a kink in helix B and a different location and stoichiometry of the Cu^{2+}-binding site. (adopted from Whyte *et al.*, 2003)

However, Doppel lacks the flexible N-terminal octapetide repeat region of PrP^c that binds copper ions and the highly conserved region between amino acid 106 and 126 that is thought to be essential for prion replication (Weissmann and Aguzzi, 1999) (Figure 7). Investigation of the physiological functions of Dpl revealed, that Dpl deficiency resulted in male sterility in mice (Behrens *et al*., 2002). Furthermore, ectopic expression of Dpl in the brain of PrP knockout mice has been proposed to cause neurotoxicity (Moore *et al*., 1999; Rossi *et al*., 2001).

Very little is known about potential binding partners of Doppel. Very recently, it was shown that Dpl binds the full-length 37kDa laminin receptor precursor protein (LRP) (Yin *et al*., 2004). In addition, Yin *et al*. claimed that the middle segment of LRP (residues 100-220) is sufficient for the binding of LRP to Dpl (Yin *et al*., 2004). However, a LRP mutant lacking the first 43 amino acids (LRP44-295) did not interact with Dpl (Hundt and Weiss, 2004). These contradictory results might be explained by the fact that the binding site in the middle segment of LRP (residues 100-220) is not properly exposed in a N-terminal deleted LRP mutant. In a yeast two-hybrid system, Dpl also failed to interact with itself and the cellular prion protein (Hundt and Weiss, 2004), suggesting that Dpl might play no essential role in prion diseases.

Together with the findings that (i) expression of Dpl in the CNS of mice did not modulate TSEs (Tuzi *et al*., 2002), (ii) Dpl expression was not modified in scrapie-infected cells and in the brain of CJD patients (Peoc'h *et al*., 2003) and (iii) Dpl is dispensable for prion disease progression and PrP^{Sc} (Behrens *et al*., 2001) generation an essential function of Dpl in prion diseases is unlikely.

3 Prion-like elements in yeast, fungi and aplysia

Prion-like proteins are found naturally in many plants and animals. Hence, scientists reasoned that such proteins provide some evolutionary advantage to their host. In yeast *Saccaromyces cerevisiae* (Weissmann, 1994) and in the filamentosus fungus *Podospora anserina* (Coustou *et al*., 1997) genetic elements with an unusual pattern of inheritance were described.

In yeast, the prion-like genetic elements were named [PSI^+] and [URE3] and are associated with the proteins Sup35p* and Ure2p*, respectively (Wickner, 1994; Masison and Wickner, 1995; Wickner *et al*., 1995), equivalent to mammalian prions such as PrP^{Sc}. The corresponding non-prion-associated genetic elements are termed [*psi*$^-$] and [*ure3*], the protein determinants Sup35p and Ure2p, respectively. Sup35p is an essential subunit of the translation release factor that recognizes termination codons and releases the complete peptide from the last tRNA (Hawthorne and Mortimer, 1968), while Ure2p acts as a negative regulator of nitrogen metabolism (Coschigano and Magasanik, 1991; Courchesne and Magasanik, 1988). [PSI^+] results in expression of the prion form Sup35p* causing a reduction of translational fidelity leading to read-trough of stop-codons (Cox *et al*., 1988). [URE3] leads to expression of Ure2p*, promoting the uptake of ureidosuccinate (Lacroute, 1971).

Genomic searches led to the identification of further prion proteins, Rnq1p (Sondheimer and Lindquist, 2000) and New1p (Michelitsch and Weissman, 2000). Like other prions, Rnq1 and New1p exist in two heritable states, a soluble form and an aggregated form, [RNQ^+] (also known as the epigenetic element [PIN^+]) and [NU^+], respectively.

Recently, it was demonstrated that in the sea slug *Aplysia californica* CPEB (cytoplasmic polyadenylation element binding protein) has prion-like properties, forming small aggregates or multimers in its active state (Si *et al*., 2003). CPEB was initially identified in *Xenopus* oocytes as a translational regulator that activates dormant mRNAs by elongating their poly (A) tails (Hake and Richter, 1994). Si *et al*. speculated that in *Aplysia* CPEB has at least two conformational states: one inactive and one active. The prion-like state of neuronal CPEB might be more active, might have altered substrate specificity, or might be devoid of the inhibitory function of the basal state. The prion-like state would thereby promote expression of several proteins establishing new synaptic connections and thus, maintain long-term synaptic changes associated with memory storage (Si *et al*., 2003).

4 Prion strains

Initially, prion strains were isolated based on different clinical symptoms in goats with scrapie (Pattison and Millson, 1961). Newly, prion strains are distinguishable by their biological properties such as behavior of the affected animals, incubation period, neuropathological profiles, plaque morphology and proteinase K susceptibility of PrP^{Sc}. To date, different scrapie strains have been isolated from natural sheep scrapie by passage into mice (Table 6). Well studied are two strains of transmissible mink encephalopathie (TME) termed drowsy (DY) and hyper (HY) prions. These prion strains were isolated from mink by passage in Syrian hamsters. Studies of the DY and HY prion strains showed, that two strains produced PrP27-30 molecules of different molecular sizes (Bessen and Marsh, 1994). Furthermore, new strains have been produced upon passage from one species to another (Kimberlin *et al*., 1987). Interestingly, several human PrP^{Sc} types have been isolated that are associated with different phenotypes of CJD (Collinge *et al*., 1996; Parchi *et al*., 1996). Three PrP^{Sc} types among cases of sporadic and itrogenic CJD and a type 4 in all cases of vCJD were described by Collinge and colleagues (Collinge *et al*., 1996). Knowing that vCJD is associated with a PrP^{Sc} type that is distinct from those seen in classical CJD, molecular strain typing can be applied to molecular diagnosis of Creutzfeldt-Jakob disease. This technique may also be applicable to determine whether BSE has been transmitted to other species, and thereby possesses implications for epidemiological studies. (Hill *et al*., 1997).

Table 6. Established TSE strains in mice and hamsters

Species	TSE strain
mouse-adapted scrapie	139A, RML, Chandler, ME7, 22L, 87V, C506-M3, 79A, 111A
mouse-adapted BSE	301C, 301V, 6PB1
mouse-adapted CJD	Fukuoka-1
hamster-adapted scrapie	263K, 22A-H, ME7-H, Sc237
hamster-adapted BSE	BSE-H
hamster-adapted TME	DY, HY

5 Prion transmission barriers

Early studies proposed that transmission of prion diseases between different mammalian species is restricted by a "species barrier" (Pattison, 1965). By the use of experimental mouse models it has been illustrated that the "species barrier" limits or even prevents the interspecies transmission of prion diseases. For example, mice are normally resistant to an infection with the 263K strain of hamster prion, but become susceptible to that strain of PrP^{Sc} by transgenic introduction of the hamster PrP gene (Scott *et al.*, 1989). To study TSE transmission different mouse models were created, including transgenic mice expressing human PrP (Telling *et al.*, 1994), bovine PrP (Buschmann *et al.*, 2000) and ovine PrP (Crozet *et al.*, 2001; Vilotte *et al.*, 2001). These transgenic mice showed a strongly reduced species barrier following inoculation of human, bovine or sheep prions, respectively, allowing transmission of natural prion disease to wild-type mice. A second approach to facilitate the transmission of TSEs in mice was the overexpression of murine PrP in mice. Transmission studies in tga20 mice, that express 10-fold higher levels of PrP^{c} than wild-type mice, revealed a significant reduction of the incubation periods following inoculation with natural scrapie isolates (Vilotte *et al.*, 2001). Furthermore, prion inoculation of tga20 mice has been shown to shorten the incubation period by the half (Fischer *et al.*, 1996; Thackray *et al.*, 2002). Recent studies of TSE transmissions in mice revealed further complexity of the genetic control of prion diseases. Genetic loci other than *Prn-p* influencing the incubation periods of mouse-adapted scrapie strains were identified on chromosomes 2, 11, 12 and 9 (Lloyd *et al.*, 2001; Stephenson *et al.*, 2000).

6 In search of binding proteins and receptors for prions

More than 10 years ago the existence of a cellular receptor for prions was proposed. It was reasoned that the cellular prion protein PrP^{c} would require a transmembrane protein to connect with the

intracellular space (Shyng *et al*., 1994). Employing complementary hydropathy, a 66kDa membrane protein that binds PrP^c both *in vitro* and *in vivo* was found (Martins *et al*., 1997) and it was reasoned that this protein might act as a cellular prion protein receptor. By the same group, years later the 66kDa protein was identified as stress-inducible-protein1 (STI1), phasing a role in neurite outgrowth and neuroprotection (Zanata *et al*., 2002). Using the yeast two-hybrid technology in *Saccharomyces cerevisiae*, the 37kDa laminin receptor precursor (LRP) was identified interacting with the cellular prion protein PrP^c (Rieger *et al*., 1997). Moreover, it has been shown that several glycosaminoglycans (GAG) bind to PrP^c (Pan *et al*., 2002; Priola and Caughey, 1994) and stimulate the PrPres formation in a cell-free system (Wong *et al*., 2001). Heparan sulfates are a component of amyloid plaques in prion diseases (Snow *et al*., 1990). Different proteins (Figure 8, Table 7) have been shown to interact with the cellular prion protein including laminin (Graner *et al*., 2000), which is an extracellular matrix protein, N-CAM (Schmitt-Ulms *et al*., 2001), a cell surface component with an important role in neuronal aggregation, and the laminin receptor (Rieger *et al*., 1997).

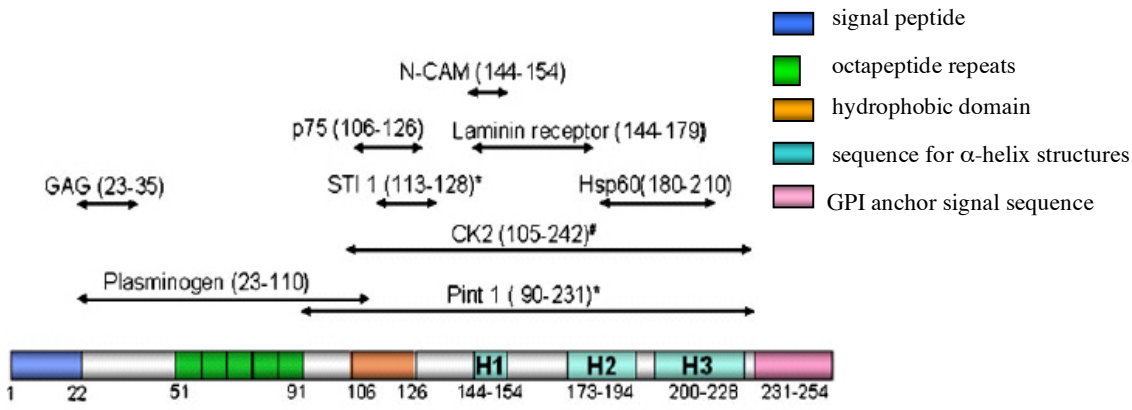


Figure 8. Representation of the binding domains of PrP^c ligands on the human PrP^c molecule

Numbers below the boxes correspond to the amino acids in each domain. Double arrows indicate the binding site for each PrP^c-binding molecule, which is also represented by amino acid numbers in parentheses. (adopted from Lee *et al*., 2003)

Table 7. Binding partners for the cellular prion protein and subcellular binding sites

PrP^c binding molecules	proposed subcellular binding sites
Synapsin 1b	intracellular vesicles (Spielhaupter and Schätzl, 2001)
Grb2	intracellular vesicles (Spielhaupter and Schätzl, 2001)
Pint 1	unknown (Spielhaupter and Schätzl, 2001)
Caveolin-1	caveolae raft (Mouillet-Richard *et al.*, 2000)
CK2	caveolae raft (Meggio *et al.*, 2000)
STI 1	cell surface (Zanata *et al.*, 2002)
Bcl-2	unknown (Kurschner and Morgan, 1995)
p75	caveolae raft (Della-Bianca *et al.*, 2001)
Laminin	cell surface (Graner *et al.*, 2000)
GAG	cell surface (Pan *et al.*, 2002)
N-CAM	caveolae-like domain (Schmitt-Ulms *et al.*, 2001)
Hsp60	unknown (Edenhofer *et al.*, 1996)
Nrf2	unknown (Yehiely *et al.*, 1997)
Aplp1	cell surface (Yehiely *et al.*, 1997)
Laminin receptor	cell surface (Gauczynski *et al.*, 2001b)
NRAGE	cytosol (Bragason and Palsdottir, 2005)

6.1 Molecular chaperones

Misfolded proteins can lead to intracellular aggregate formation, disruption of multiple cellular processes and cell death. In prion diseases, it is proposed that the conversion of PrP^c to PrP^{Sc} is mediated by molecular chaperones. In a yeast two-hybrid screen the chaperonin Hsp60 was identified as an interaction partner for the Syrian hamster prion protein (Edenhofer *et al.*, 1996). *In vitro* binding studies with recombinant PrP confirmed the specificity of the PrP-Hsp60 interaction. Interestingly, heat shock protein Hsp104 has been linked to the function of prion-like elements in yeast (Chernoff *et al.*, 1995). Furthermore, Hsp104 and GroEL, the bacterial homologue of Hsp60, were found to promote conversion of PrP^c into the proteinase K resistant state. Other heat shock proteins such as GroES, Hsp40, Hsp70 and Hsp90 don't seem to influence the conversion process (DebBurman *et al.*, 1997).

6.2 Protein X

Protein X represents a prominent protein proposed to participate in the conversion mechanism of PrP^{c} to PrP^{Sc}, although its identity is still unknown. Protein X might act as a molecular chaperone in the formation of PrP^{Sc} (Telling *et al.*, 1995). Evidence for the participation of protein X in prion propagation arose from transmission studies in transgenic mice. Transgenic mice expressing high levels of human prion protein (Tg(HuPrP)$Prnp^{+/+}$) failed to develop CNS dysfunctions following inoculation with human CJD prions (PrP^{CJD}), whereas mice expressing a chimeric human/mouse PrP transgene (Tg(MHu2M)$Prnp^{+/+}$) were highly susceptible to human prions (Telling *et al.*, 1994). One explanation for the difference in susceptibility of (Tg(MHu2M)$Prnp^{+/+}$) and (Tg(HuPrP)$Prnp^{+/+}$) mice to human prions might be that mouse chaperones catalyzing the refolding of PrP^{c} into PrP^{Sc} might readily interact with the MHu2MPrP^{c}/HuPrP^{CJD} complex but not with HuPrP^{c}/HuPrP^{CJD}. Another possibility might be that sequences at the N- or C-terminus of HuPrP inhibit the formation of PrP^{Sc} in murine cells (Telling *et al.*, 1994). Overall, it was concluded that different susceptibilities of Tg(HuPrP) and Tg(MHu2M) mice to human prions indicate that additional species-specific factors might be involved in prion replication (Telling *et al.*, 1994).

The fact that N-terminal truncations of PrP^{c} still permits the formation of PrP^{Sc} suggested that the binding site for the protein X is located in the C-terminal segment of PrP (residues 169-230) (Fischer *et al.*, 1996; Rogers *et al.*, 1993). The finding that substitution of a basic residue at positions 167, 171 or 218 of human PrP, respectively, prevented PrP^{Sc} formation, suggests that these mutant PrPs might act as "dominant negatives" by binding protein X and rendering it unavailable for prion propagation (Kaneko *et al.*, 1997).

6.3 The 37kDa/67kDa laminin receptor (LRP/LR)

In a yeast two-hybrid screen, the 37kDa laminin receptor precursor (LRP) was identified as an interaction partner for the prion protein (Rieger *et al.*, 1997). Further *in vitro* studies on neuronal and non-neuronal cells validated that the laminin receptor LRP/LR act as the receptor for the cellular prion protein (Gauczynski *et al.*, 2001b). Yeast two-hybrid studies and cell biological mapping analysis identified domains on PrP and LRP involved in the PrP-LRP interaction (Figure 9). Two binding domains for LRP on PrP were identified: a direct binding domain (PrPLRPbd1, aa144-179) and an indirect binding domain (PrPLRPbd2, aa53-93), which depends on the presence of heparan sulfate proteoglycans (HSPGs), functioning as co-factors or co-receptors for the binding of PrP^{c} to LRP/LR (Hundt *et al.*, 2001) (Figure 9). Furthermore, the yeast two-hybrid system localized the direct PrP-binding domain on LRP between amino acid residues 161 and 179 (Hundt *et al.*, 2001) and a second HSPG-dependent binding site, that might be located between amino acid 180 and 285 (Figure 9).

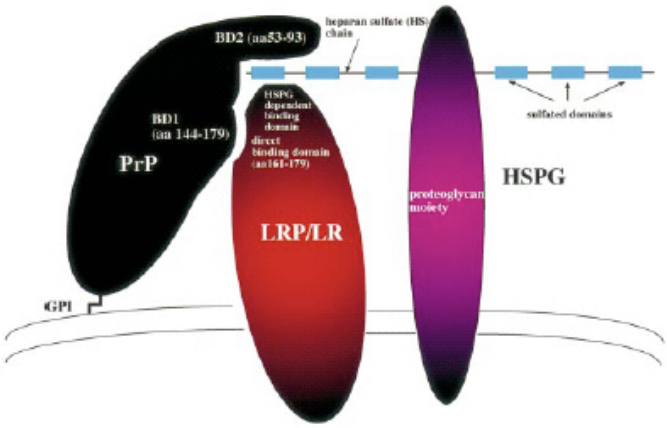


Figure 9. Model for the function of the 37kDa/67kDa LRP/LR as a receptor for PrP
The PrP molecule binds directly to LRP/LR via PrPLRPbd1 (aa144-179) and indirectly via PrPLRPbd2 (aa53-93). Direct binding of LRP/LR to PrP occurs via the direct binding site on LRP/LR (aa161-179), whereas the indirect HSPG-dependent binding domain may reside between aa 180 and 285. (adopted from Hundt *et al.*, 2001)

HSPGs are multifunctional macromolecules characterized by a core polypeptide to which glycosaminoglycans (GAGs) are covalently attached and have been also shown to be associated with Aβ deposits in Alzheimer's disease (AD) (Snow *et al.*, 1994). In addition, cell surface proteoglycans are known to act as cellular receptors for some bacteria and several animal viruses (Rostand and Esko, 1997), including foot-and-mouth disease type O virus (Jackson *et al.*, 1996), HSV types 1 and 2 (Shieh *et al.*, 1992) and dengue virus (Chen *et al.*, 1997). Furthermore, HSPGs were identified as primary receptor for AAV (Summerford and Samulski, 1998) and shown to mediate HIV-cell attachment and virus entry (Patel *et al.*, 1993). HSPGs play also a crucial role in regulating key developmental signaling pathways, such as the Wnt, TGF-β (transforming growth factor-β) and FGF (fibroblast growth factor) pathways (Lin, 2004).

The 37kDa LRP is thought to be the precursor of the 67kDa high-affinity laminin receptor (LR), which was first isolated from melanoma cells due to its high binding capacity to laminin (Rao *et al.*, 1983). Although LRP consists of a transmembrane domain (amino acid residue 86-101, Castronovo *et al.*, 1991), it is abundantly localized in the cytoplasm (Romanov *et al.*, 1994). In mammalian cells, it has been demonstrated that both the 37kDa LRP and the 67kDa LR are present in plasma membrane fractions (Gauczynski *et al.*, 2001b). The exact mechanism by which the 37kDa precursor forms the mature 67kDa isoform is up to now still unclear. Data from the yeast two-hybrid analysis showed that LRP failed to interact with itself (Hundt *et al.*, 2001), which is an argument against the hypothesis of a direct homodimerization. Analysis of the membrane-bound 67kDa LR indicated, that acylation of LRP is involved in the processing of the receptor (Landowski *et al.*, 1995). Additional studies suggested that the 67kDa LR is a heterodimer stabilized by fatty acid-mediated interactions (Buto *et al.*, 1998).

Mammalian genomes contain multiple copies of the LRP gene, particularly 6 copies in the mouse and 26 copies in the human genome (Jackers *et al.*, 1996b). Sequencing revealed that over 50% of the 37kDa LRP gene copies were pseudogenes most probably generated by retropositional events. The finding of multiple pseudogenes for the 37kDa LRP might suggest that the accumulation of several copies of this gene might have given a survival advantage to the cell in the course of evolution (Jackers *et al.*, 1996a).

Interestingly, the 37kDa LRP appears to be a multifunctional protein involved in the translational machinery (Auth and Brawerman, 1992) and has also been identified as p40 ribosome-associated protein (Makrides *et al.*, 1988). LRP has also been found in the nucleus, where it is tightly associated with nuclear structures (Sato *et al.*, 1996). The 37kDa/67kDa LRP/LR has been described to act as a receptor for laminin, elastin and carbohydrates (Ardini *et al.*, 1998) as well as a receptor for Venezuelan equine encephalitis virus (VEE) (Ludwig *et al.*, 1996), Sindbis virus (Wang *et al.*, 1992) and Dengue virus (Tio *et al.*, 2005). Due to the co-localization of LRP/LR and PrP on the surface of mammalian cells, a possible role of LRP/LR for PrP binding and internalization was assumed. Using a cell-binding assay with recombinant PrP a LRP/LR dependent binding of PrP has been shown (Gauczynski *et al.*, 2001b). The strict LRP/LR specificity for the PrP binding could be confirmed in competition assays with different anti-LRP antibodies. Furthermore, it has been demonstrated, that the PrP internalization process represents an active receptor-mediated event (Gauczynski *et al.*, 2001b). Due to the identification of various LRP/LR isoforms, additional studies have been performed to detect the isoforms that are present in the central nervous system and bind PrP. Therefore, mouse brain fractions enriched in the laminin receptor were purified and overlay assays with recombinant PrP were performed (Simoneau *et al.*, 2003). Several LRP/LR isoforms corresponding to different maturation states of the receptor were identified, including a 44kDa, 60kDa, 67kDa and a 220kDa form. Furthermore, it has been demonstrated, that all of these isoforms were able to bind PrP, supporting a physiological role for the laminin receptor/PrP interaction in the brain (Simoneau *et al.*, 2003). A closer insight into the fine cellular distribution of LRP/LR in the central nervous system was obtained by using immunohistochemistry in adult rat brain (Baloui *et al.*, 2004). It has been shown that the 67kDa LR is the major receptor form, which is expressed within the cytoplasm and at the plasma membrane in most neurons and in a subset of glia cells. In contrast, the 37kDa LRP is much less abundant in adult than in postnatal central nervous system and its expression is restricted to a subclass of cortical interneurons known to be particularly sensitive to abnormal prion accumulation and rapidly degenerate during early stages of CJD (Belichenko *et al.*, 1999). In addition, recent studies showed that LRP/LR is not only involved in the PrP^{c} metabolism, but has also a crucial role in prion propagation. Using antisense LRP RNA and small interfering (si) RNAs specific for LRP mRNA, PrP^{Sc} levels in scrapie-infected neuronal cells were reduced indicating a necessity for the laminin receptor LRP/LR for PrP^{Sc} propagation in cultured cells (Leucht *et al.*, 2003).

Due to the facts that a (natural) infection with prions mostly occur via an oral route and that LRP/LR acts as receptor for prion proteins (Gauczynski *et al*., 2001b), potential binding sites for PrP in the intestinal mucosa were examined. Studies of the tissue expression of LR in human duodenal and jejunal biopsy samples led to the discovery that this receptor is expressed in the apical brush border of small intestinal epithelial cells (Shmakov *et al*., 2000). Employing immunohistochemistry LR expression has also been observed in the paranuclear/Golgi apparatus region and in the Paneth cell secretory granules (Shmakov *et al*., 2000). These findings suggest an involvement of LR in both secretory and endocytotic functions of human small intestinal epithelium. Moreover, it was speculated that the major implication of intestinal expression of the 67kDa LR might be an increased susceptibility to an oral infection with prions (Shmakov *et al*., 2000). It has been demonstrated that the oral transmission of infectious prion particles led to a rapid accumulation of PrP^{Sc} in Peyer's patches (Maignien *et al*., 1999). PrP^{Sc} has also been detected in enterocytes of the villous epithelium of the small intestine in primates after oral exposure to prions (Bons *et al*., 1999). Enterocytes represent the major cell population of the intestinal epithelium (Booth and Potten, 2000) and are known to actively participate in endocytosis. Since expression of PrP^{c} was demonstrated to be necessary for prion replication, expression of the cellular prion protein in the gastrointestinal tract has been analyzed and indeed it has been shown that PrP^{c} is present in human enterocytes (Morel *et al*., 2004). These results led to the hypothesis that enterocytes might play an important role for the uptake of infectious prion particles. Very recently, it has been demonstrated that bovine PrP^{Sc} is internalized by human enterocytes via an LRP/LR-mediated endocytosis (Morel *et al*., 2005). In this study, the Caco-2/TC7 cell model system, which is morphologically and functionally similar to normal human enterocytes, was used to investigate the PrP^{Sc} uptake in human enterocytes. Analysis of the presence of PrP^{Sc} after supply of prion-contaminated brain homogenate from different sources in Caco-2/TC7 enterocytes revealed that BSE prions were specifically internalized and accumulate in human enterocytes, whereas murine-adapted scrapie-prions were not endocytosed. Due to the demonstration, that the laminin receptor LRP/LR is required for the endocytosis of PrP^{c} and the PrP^{Sc} propagation in neuronal cells, the involvement of this receptor in the endocytosis of prions in human enterocytes was investigated. Immunofluorescence analysis of PrP^{Sc}-containing vesicles revealed a co-localization with LRP/LR in the sub-apical compartment of Caco-2/TC7 cells after 5 minutes incubation with BSE brain homogenate. In addition, blocking of the LRP/LR by an anti-LRP antibody showed a significant decrease of PrP^{Sc} endocytosis, approving that prion endocytosis in human enterocytes is mediated by the 37kDa/67kDa laminin receptor LRP/LR. Interestingly, prions from a mouse-adapted scrapie-strain were not internalized, suggesting a specific uptake of bovine BSE prions by human enterocytes (Morel *et al*., 2005). For a role in the uptake of pathogens from the gut, the 37kDa/67kDa LRP/LR must be internalized after ligand binding. In terms of internalization, it has been shown that the 67kDa LR functioned as the major receptor for virus entry into mammalian cells (Wang *et al*., 1992). Association

of the laminin receptor with glycolipid-enriched microdomains in the cell membrane might lead to a clustering with other proteins in this region to provide a mechanism for internalization (Hooper, 1999). In summary, understanding the LRP/LR-PrP interaction in human intestine may enlighten the pathogenesis of prion diseases since an important role of the 37kDa/67kDa LRP/LR in mediating binding and internalization of the prion protein and its involvement in pathological mechanisms was demonstrated.

7 Therapeutic approaches for the treatment of prion diseases

Since the appearance of variant Creutzfeldt-Jakob disease, numerous strategies and targets have been proposed for a therapy of prion diseases, including:

(i) stabilization of the structure of PrP^{c} to prevent the transconformation from PrP^{c} to PrP^{Sc}

(ii) interference of the binding of PrP^{Sc} to PrP^{c}

(iii) inhibition of the formation of the abnormal form of PrP

(iv) destruction of PrP^{Sc} aggregates

(v) inhibition of the prion protein receptor(s)

The inhibition of the PrP^{Sc} accumulation, however, is the most studied target. There are a number of compounds which have been shown to efficiently interfere with the PrP^{Sc} accumulation, such as Congo red (Ingrosso *et al.*, 1995) and analogs (Demaimay *et al.*, 2000), certain cyclic tetrapyrrols such as porphyrins and phtalocyanines (Priola *et al.*, 2000) and sulfated polyanions such as dextran sulfate 500 (Farquhar and Dickinson, 1986), pentosan polysulfate (Caughey and Raymond, 1993) and suramin (Gilch *et al.*, 2001). Many other compounds have been identified to have an effect on the formation of pathological PrP^{Sc} *in vitro* and *in vivo*, but only flupirtine, an analgetic, is possibly beneficial in humans (Otto *et al.*, 2004).

To identify novel substances regarding a therapeutic potency, assays for the screening of large compound libraries have to be developed. Recently, a high-throughput assay based on the scanning for intensely fluorescent targets (SIFT) for the identification of drugs, which interfere with the PrP^{c}/PrP^{Sc} interaction, was described (Bertsch *et al.*, 2005). A library of 10,000 compounds was screened and a new lead structure with favorable pharmacological features has been identified (*N*'-benzylidene-benzohydrazides) that represent a novel anti-prion drug.

7.1 Chemical compounds

Although several substances have been identified which inhibit PrP^{Sc} formation, unfortunately most of them show only significant effects when administered long before the clinical onset. At present, there

is no therapy for clinically affected TSE patients, so that TSEs usually culminate in death. Up to now various prion inhibitors have been identified (Table 8), but the development of potent compounds for an *in vivo* therapy in human TSEs is still required. The development of immune intervention strategies on one hand and the use of neuronal stem cells on the other may provide novel approaches for TSEs (Dormont, 2003).

Polyanions are highly charged molecules that interact with cell membranes and are known to inhibit the entry of several viruses by a non-specific mechanism (Moelling *et al*., 1989). Certain polyanion compounds such as heteropolyanion 23 (HPA23) (Kimberlin and Walker, 1983), Dextran Sulfate 500 (DS500) (Farquhar and Dickinson, 1986), pentosan polysulfate (PS) (Diringer and Ehlers, 1991; Farquhar *et al*., 1999) and suramin (Gilch *et al*., 2001) have been shown to be efficient in the treatment of TSEs and prolonged the life span of animals inoculated with the scrapie agent. For instance, intraperitoneal administration of pentosan polysulfate into scrapie-infected mice has been shown to significantly prolong the survival time of these animals, whereas an oral challenge with PS was ineffective in delaying disease (Farquhar *et al*., 1999). Depending on the mouse strain, PS treatment increased the mean incubation period of the scrapie strain or even protected mice completely from disease (Farquhar *et al*., 1999). Due to the prion-curing properties of sulfated polyanions in an early stage of infection (Caughey and Raymond, 1993) various heparan sulfate mimetics (HMs) have been developed and tested for their activity as anti-prion drugs. HM2602 was found to hamper PrPres accumulation *in vitro* and *in vivo* and prolonged significantly the survival of scrapie infected hamsters (Adjou *et al*., 2003).

The azoic dye **Congo red**, which specifically binds to amyloid structures, potently inhibits the accumulation of PrPres *in vitro* without affecting the metabolism of the cellular prion protein (Caughey and Race, 1992). In scrapie-infected hamsters, treatment with Congo red resulted in a prolonged incubation time, depending on the amount of drug administration (Ingrosso *et al*., 1995). The maximal effect was observed, when the scrapie agent and Congo red were co-injected, suggesting that the timing of drug administration is a key-determinant.

Polyene antibiotics such as Amphotericin B and its synthetic derivative MS-8209 are antifungal agents and have been evaluated as anti-TSE agents in infected hamsters, delaying scrapie disease and PrP^{Sc} accumulation (Adjou *et al*., 1995; Pocchiari *et al*., 1987). These substances are thought to interact with cholesterol present in mammalian cell membranes, disturbing the PrP^{Sc} endocytosis (Bolard, 1986; Taraboulos *et al*., 1995) or directly affect the conversion of PrP^{c} to PrP^{Sc} (Demaimay *et al*., 1997). Another polyene antibiotic, filipin, has also been shown to inhibit PrPres formation *in vitro* (Marella *et al*., 2002).

In contrast to polyene antibiotics, **cationic amphiphilic drugs** act on the clathrin-dependent pathway and inhibit receptor-mediated endocytosis by reducing the number of coated pit-associated receptors at the cell surface (Sofer and Futerman, 1995; Wang *et al.*, 1993). It has been demonstrated that derivatives of phenothiazines such as promazine, chlorpromazine and azepromazine, are sufficient for PrP^{Sc} inhibition in cultured cells chronically infected with prions (Korth *et al.*, 2001). Chlorpromazine has been used in humans as an antipsychotric drug (treatment of schizophrenia) since the 1950s (Delay *et al.*, 1952) and is known to cross the blood-brain-barrier (Korth *et al.*, 2001). Nevertheless, combined chlorpromazine and quinacrine therapy in FFI patients did not improve their clinical conditions (Benito-Leon, 2004).

Another class of inhibitors of the PrPres accumulation comprises derivatives of **acridines/ bis-acridines**, in which quinacrine is the most prominent representative. Quinacrine, an antimalarial drug, has been shown to be 10-fold more potent than chlorpromazine in inhibiting PrP^{Sc} formation *in vitro* (Korth *et al.*, 2001). In a murine model, oral treatment with quinacrine after intracerebral prion inoculation did not lead to any increased survival time compared to control animals (Barret *et al.*, 2003), which was consistent with the results obtained in CJD patients (Haik *et al.*, 2004).

Tetrapyrrole compounds, which include porphyrins and phtalocyanines, are known to bind selectively to proteins and induce conformational changes. Certain molecules have been identified to inhibit the formation of PrPres *in vitro* (Caughey *et al.*, 1998) and increased the survival time of TSE-infected animals (Priola *et al.*, 2000). Among different tested compounds, phtalocyanine tetrasulfonate (PcTS), *meso*-tetra(4-*N*-methylpyridyl)porphine iron (III) (TMPP-Fe^{3+}) and deuteroporphyrin IX 2,4-bis-(ethylene glycol) iron (III) (DPG_2-Fe^{3+}) were the most potent inhibitors. Tetrapyrrols have also been shown to inhibit PrPres formation in a cell-free conversion reaction, suggesting a direct interaction of the tetrapyrrol with PrP molecules (Caughey *et al.*, 1998).

The finding, that transfection of scrapie-infected mouse neuroblastoma (ScN2a) cells using the SuperFect reagent, which is a mixture of branched **polyamines**, reduced the level of pre-existing PrP^{Sc} led to the testing of several other polyamines for their ability to interfere with the PrP^{Sc} propagation in chronically infected ScN2a cells (Supattapone *et al.*, 2001). The branched polymers investigated include polyethyleneimine (PEI), polypropyleneimine (PPI) and polyamidoamide (PAMAM) dentrimers. All these compounds have been demonstrated to eliminate PrP^{Sc} in an *in vitro* assay using prion-infected cells. Furthermore, the cationic lipopolyamine DOSPA was shown to interfere with the accumulation of PrP^{Sc} in scrapie-infected neuroblastoma cells (Winklhofer and Tatzelt, 2000).

Anthracyclins are a class of chemotherapeutic agents normally used to treat a wide range of cancers. Administration of a derivative of doxorubicin, 4´-iodo-4´-deoxy-doxorubicin (IDX), in a murine model of reactive amyloidosis reduced amyloid deposits (Merlini *et al.*, 1995). In prion diseases, IDX

was shown to bind to PrP amyloid and prolonged the survival time of scrapie-infected hamsters, when administered intracerebrally at the same time as the scrapie agent (Tagliavini *et al.*, 1997). It has been proposed that IDX bind to the abnormal form of PrP, decreasing the availability of template molecules for the conversion process of the normal prion protein (Tagliavini *et al.*, 1997).

Table 8. Chemical compounds exhibiting therapeutic antiprion effects

Class of compounds	Example
polysulfonated, polyanionic substances	dextran sulfate, suramin, pentosan polysulfate, heparan sulfate mimetics
amyloidotropic intercalators	congo red
polyene antibiotics	amphotericin B, filipin
cyclic tetrapyrrols	porphyrines, phtalocyanines
polyamines	DOSPA, SuperFect, polyethyleneimine
anthracyclines	IDX
phenothiazines	chlorpromazine
acridines/ bis-acridines	quinacrine, chloroquine
designer peptides	β-sheet breaker
aptamers	RNA aptamer DP7

7.2 Immunotherapy

The absence of detectable immune response during transmissible spongiform encephalopathies is likely due to the fact that the prion protein is a self-antigen expressed on the surface of many cells of the host. Experimentally, the problem of auto-tolerance was bypassed using $PrP^{0/0}$ mice to generate monoclonal antibodies that recognize PrP^{c} (Krasemann *et al.*, 1996). Exposure of scrapie-infected neuroblastoma cells to the monoclonal anti-PrP antibody 6H4 was found to inhibit PrP^{Sc} replication and even prevent infection of susceptible N2a cells by pre-treatment with the antibody (Enari *et al.*, 2001). Furthermore, application of Fabs (antibody antigen-binding fragment) to scrapie-infected N2a cells dramatically reduced PrP^{Sc} formation in prion-infected cells (Peretz *et al.*, 2001). The most effective antibody, Fab D18, recognized an epitope between amino acid residues 132 and 156 of murine PrP. Surprisingly, this region is well known as an important site for molecular interactions modulating PrP^{c}-PrP^{Sc} conversion (Korth *et al.*, 1997). To overcome the tolerance against PrP^{c} *in vivo*, genes encoding the heavy chain variable region of 6H4 were expressed in $PrP^{0/0}$ mice (Heppner *et al.*,

2001). Reintroduction of a single copy of the *Prn-p* gene resulted in 6H4PrP$^{+/-}$ mice that were intraperitoneally challenged with murine scrapie agent. *In vivo* expression of anti-PrP antibodies were found to prevent PrP^{Sc} replication in the spleen and the brain, providing immunological protection against prion diseases (Heppner *et al*., 2001).

Recently, it was demonstrated that passive intraperitoneal immunization with anti-PrP monoclonal antibodies dramatically reduced PrP^{Sc} levels and delayed the onset of prion disease in scrapie-infected mice (White *et al*., 2003). In contrast, intracerebral injection of PrP^{c}-specific monoclonal antibodies was found to trigger neuronal cell death by cross-linking cellular prion protein (Solforosi *et al*., 2004). To avoid that problem, recombinant single-chain antibody (scFv) fragments derived from monoclonal anti-PrP antibody 6H4 have been generated. ScFv fragments are small in size (approx. 30kDa) and exhibit only a monovalent antigen binding site. Secretion of anti-PrP scFv by mammalian cells has been shown to cure chronically prion-infected neuroblastoma cells (Donofrio *et al*., 2005), suggesting scFv as candidates for gene transfer-based immunotherapy of prion diseases. Active immunization with synthetic peptides, resembling different portions of the (hamster) prion protein, has been shown to prolong the survival time of hamsters infected intraperitoneally with 263K scrapie agent (Magri *et al*., 2005).

To induce a native PrP^{c}-specific antibody response, a novel strategy has been developed. Retroviral PrP-expressing particles were generated and upon intravenous injection in PrP$^{0/0}$ and wild-type mice, PrP retroparticles induced a humoral immune response against the native form of cellular PrP^{c} (Nikles *et al*., 2005). Another effective strategy to overcome the auto-tolerance to PrP *in vivo* is the use of an attenuated *Salmonella* vaccine strain that express mouse PrP (Goni *et al*., 2005). It has been demonstrated, that oral immunization using *Salmonella typhimurium* as a delivery and adjuvant system led to a humoral immune response against PrP and significantly prolonged the incubation period or prevent infection in mice following oral exposure to the prion agent (Goni *et al*., 2005).

Delivery of antibodies by gene therapy may circumvent the limitations of either passive or active immunization by providing a steady antibody supply. Viruses are naturally evolved vehicles that efficiently transfer their genes into host cells. Particular viruses such as retroviruses, adenovirus, adeno-associated virus (AAV), herpesvirus and poxvirus have been selected as gene delivery vehicles because of their capacities to carry foreign genes and their ability to efficiently deliver these genes associated with efficient gene expression (Walther and Stein, 2000). Among these vector systems, retroviral vectors represent the most prominent delivery system, since these vectors have high gene transfer efficiency in both dividing and non-dividing cells and mediate high expression of therapeutic genes (Walther and Stein, 2000).

In respect of the treatment of neurological disorders such as TSEs, recombinant adenovirus and adeno-associated virus (AAV) are also highly effective vehicles for gene transfer into CNS cells. For the treatment of neurodegenerative diseases such as Alzheimer's disease, AAV-mediated gene delivery

has already been investigated (Zhang *et al.*, 2003; Hara *et al.*, 2004; Feng *et al.*, 2004; Sanftner *et al.*, 2005). The first therapeutic gene delivery attempt for prion diseases was initiated using AAV particles expressing an anti-LRP scFv (Rey *et al.*, submitted). Intracerebrally injection of AAV encoding anti-LRP scFv resulted in expression of the anti-LRP single chain antibodies in mouse brain (Rey *et al.*, submitted). Current prion infection experiments will prove if the level of anti-LRP scFv produced in the brain is sufficient to delay prion disease and thus, representing a novel therapeutic strategy in the treatment of TSEs.

7.3 RNA interference (RNAi) and antisense RNA

RNA silencing occurs naturally in several organisms such as plants, fungi and protozoa and is believed to evolve as an innate defense mechanism to antagonize viral infection and integration of transposable elements. RNA interference (RNAi) is triggered by the presence of double-stranded (ds) RNA in the cell and results in the rapid destruction of mRNA containing the identical sequence (Tuschl, 2001) (Figure 10).

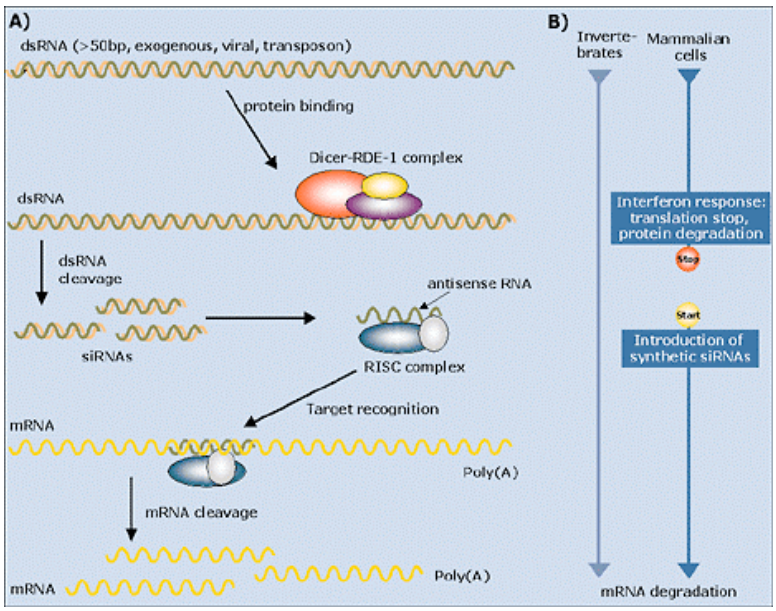


Figure 10. Mechanism of the RNAi mediated gene silencing pathway

RNAi triggers sequence-specific repression of gene expression. (A) The dsRNA is recognized by the cellular machinery and bound by the Dicer-RDE-1 (RNAi deficient-1) protein complex, that cleaves long dsRNA in a RNaseIII-like fashion into 19-22bp siRNA oligonucleotides. The antisense strand of the siRNA is used by an RNA-induced silencing complex (RISC) to guide mRNA cleavage, therefore promoting mRNA degradation. (B) In invertebrates the dsRNA-induced gene silencing process can be started via gene-specific, full-length dsRNA. In mammalian cells, long dsRNA (>30bp) induces an anti-viral response mechanism leading to non-specific RNA degradation. A gene-specific silencing process is started if short siRNAs are introduced into mammalian cells. (adopted from www.amaxa.com)

The discovery that the introduction of dsRNA longer than 30bp into somatic mammalian cells induced the anti-viral interferon-response (Figure 10) led to the application of small interfering RNA (siRNA) duplexes, which are specific gene silencing molecules of 19-22 nucleotides that bypass the anti-viral response.

Delivery of siRNAs into mammalian cells was usually achieved via transfection of double-stranded (ds) oligonucleotides or plasmids encoding small hairpin RNA (shRNA) (Elbashir *et al*., 2001), until recently, retroviral vectors have been used for the delivery of siRNAs *in vitro* to reach long-term and stable expression of siRNAs. Both adeno-associated viral (AAV) vectors (Tomar *et al*., 2003) and lentiviral vectors (Matta *et al*., 2003) have been described to be efficient vectors for delivery of siRNAs into mammalian cells.

Recently, a method for *in vivo* delivery of siRNAs to the liver, spleen, lung, kidney and pancreas of mice has been described (Lewis *et al*., 2002), using rapid injection of synthetically prepared siRNA duplexes into the tail vein. As a therapeutic strategy to deliver nucleic acids, cationic-lipid-based carriers have emerged as the most popular nonviral method (Sioud and Sorensen, 2003; Sorensen *et al*., 2003). Unfortunately, liposomal carriers localize the encapsulated siRNA complexes mainly to the blood compartment, but efficient brain delivery systems need to be developed since neurons are the main cells targeted in prion diseases. To address the targeted delivery of RNAi-based therapeutics, encapsulation of the shRNA expression plasmids in pegylated immunoliposomes (PILs) bearing surface targeting ligands was performed (Zhang *et al*., 2003a). It has been demonstrated that the PIL gene delivery system was effective in primates as well as in rodents (Zhang *et al*., 2003a; Zhang *et al*., 2003b).

Since current treatments do not prevent the appearance of clinical symptoms and death in experimental scrapie-infected animals, the development of the siRNA technology offers a promising approach for a therapy of prion diseases. Suggesting that suppression of PrP expression might be a potential therapeutic strategy against prion diseases (Bueler *et al*., 1993), the effect of specific *Prn-p* gene silencing using RNAi has been studied (Daude *et al*., 2003; Tilly *et al*., 2003). It has been demonstrated that transfection of siRNA duplexes corresponding to the murine *Prn-p* triggered specific *Prn-p* gene silencing in scrapie-infected neuroblastoma cells and caused a rapid loss of their PrPres content (Daude *et al*., 2003). Due to the transient nature of transfection, a long-term effect of *Prn-p*-specific siRNA duplexes on the PrP^{Sc} accumulation has not been achieved (Daude *et al*., 2003). A further strategy to interfere with the PrP^{Sc} propagation is the knockdown of LRP/LR, which was identified as the cell-surface receptor for PrP^{c} (Gauczynski *et al*., 2001b). In scrapie-infected neuronal cells, it has been shown that transfection of LRP-specific siRNAs resulted in a strong reduction of the PrP^{Sc} level correlated with LRP downregulation (Leucht *et al*., 2003), demonstrating a requirement of LRP/LR for PrP^{Sc} propagation in cultured cells. The discovery that (i) knockdown of LRP/LR prevents

PrP^{Sc} binding and internalization *in vitro* (Leucht *et al*., 2003) and (ii) LRP/LR is the receptor for the infectious bovine prion protein (Morel *et al*., 2005) provide a perspective for the treatment of TSEs.
As demonstrated in an ALS (amyotrophic lateral sclerosis) animal model, lentivirus-mediated RNAi represent a promising therapeutic tool to specifically knockdown disease-relevant genes (Ralph *et al*., 2005; Raoul *et al*., 2005). Therefore, a lentivirus-based RNAi gene therapy might be promising for the treatment of prion diseases. *In vitro* studies using HIV-derived vectors expressing LRP-specif siRNAs revealed an efficient reduction of the LRP/LR gene expression in neuronal cells (Vana *et al*., submitted). Furthermore, application of vector-encoded LRP-specific siRNAs resulted in a decrease of PrP^{Sc} levels in scrapie-infected neuronal cells (Vana *et al*., submitted), confirming an essential role of LRP/LR for prion propagation. Injection of lentivirus particles expressing LRP-specific siRNAs into mice following prion inoculation will demonstrate if the knockdown of LRP/LR might prolong the onset of prion disease *in vivo*.
Another strategy interfering with the PrP^{Sc} accumulation is the application of antisense RNA targeting either PrP or LRP/LR, resulting in gene silencing. Hitherto, no PrP antisense RNA approach has been performed, which might be due to the discovery of PrP-specific small interfering RNAs as an effective therapeutic tool against prion diseases (Daude *et al*., 2003; Tilly *et al*., 2003). However, transfection of LRP antisense RNA has been shown to ablate LRP/LR expression and prevent PrP^{Sc} propagation in scrapie-infected neuronal cells (Leucht *et al*., 2003), suggesting that the 37kDa/67kDa LRP/LR might act as potent target for the development of new therapies against prion diseases.

7.4 Trans-dominant negative mutants

Several reports have discussed strategies for inhibiting PrP^{Sc} accumulation. One major problem with drugs shown to exert anti-scrapie effects is their intrinsic property to induce a wide variety of side effects (Priola and Caughey, 1994; Tagliavini *et al*., 1997). Alternatively, deleted PrP molecules inhibiting the accumulation of PrP^{Sc} in a trans-dominant fashion were designed (Hölscher *et al*., 1998). Employing scrapie-infected mouse neuroblastoma cells as a model system, it has been shown that a deletion of eight amino acids in mouse PrP^{c} ($PrP^{c}\Delta$114-121) abrogates the conversion of the mutant protein into PrP^{Sc}. In addition, $PrP^{c}\Delta$114-121 overexpression resulted in inhibition of PrP^{Sc} accumulation (Hölscher *et al*., 1998). Assuming that any side effects of $PrP^{c}\Delta$114-121 should be minimal compared to those of the chemical compounds, an alternative therapeutic approach can be envisaged using trans-dominant PrP mutants.
In vitro, several dominant-negative PrP mutants have been investigated for their therapeutic potential in prion diseases and have been shown to prevent PrP^{Sc} formation (Kaneko *et al*., 1997). Further studies using transgenic mice expressing dominant-negative PrP mutants (Q167R and Q218K)

demonstrated that expression of dominant-negative PrP strongly reduced PrP^{Sc} accumulation *in vivo* (Perrier *et al.*, 2002).

Delivery of PrP containing dominant negative mutations has been achieved using lentiviral gene transfer (Crozet *et al.*, 2004). By taking advantage of "prion resistant" polymorphisms that naturally exist in sheep and humans (Q171R and E219K), corresponding residues were mutated in the murine *Prn-p* gene (Q167R and Q218K) and subcloned into lentiviral vectors. Transduction of prion-infected neuroblastoma cells with lentiviral vectors carrying the dominant negative PrP mutants showed a strong expression of the transgene and a potent inhibition of PrP^{Sc} accumulation (Crozet *et al.*, 2004). Moreover, for the treatment of Alzheimer's disease a gene therapy strategy has been recently evaluated (Marr *et al.*, 2003), suggesting lentiviral vectors as an useful tool in the development of a therapeutic approach in prion diseases.

Because PrP^{c} is a major cellular requirement for the propagation of infectivity (Bueler *et al.*, 1993), it represents an attractive therapeutic target. However, identification of the 37kDa/67kDa laminin receptor LRP/LR as the receptor for prions (Gauczynski *et al.*, 2001b; Morel *et al.*, 2005) led to an alternative target for the development of TSE therapeutics. Recently, it has been shown that a LRP mutant encompassing only the extracellular domain of LRP/LR (LRP102-295::FLAG) might act in a trans-dominant negative manner as a decoy by trapping PrP molecules (Vana and Weiss, 2006). *In vitro* studies revealed that the LRP mutant is able to reduce the PrP^{Sc} accumulation in scrapie-infected neuronal cells (Vana and Weiss, 2006) and thus, might have potential for the development of a TSE therapy. *In vivo* prion inoculation studies in transgenic mice expressing LRP102-295::FLAG brainspecifically will verify if the trans-dominant negative LRP mutant can interfere with the PrP^{Sc} formation and prolong the onset of prion disease (Vana and Weiss, submitted).

Creutzfeldt-Jakob disease and other transmissible spongiform encephalopathies are fatal and to date, there is no effective treatment to cure prion diseases. However, there are a number of potential treatments in development or under consideration. Nevertheless, no treatment has been shown to slow down or halt the disease process in CJD patients. Laboratory findings are only simply indications of the possible therapeutic value. Therefore, further work is essential to establish treatments that efficiently medicate prion diseases.

CHAPTER II

BSE-die gebannte Gefahr?
Epidemiologie, Therapie, Suszeptibilität und Übertragbarkeit bei Prionerkrankungen

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Prionkrankheiten sind neurodegenerative, stets tödlich verlaufende Erkrankungen, die zur Gruppe der spongiformen, übertragbaren Enzephalopathien zählen. Dazu gehören BSE bei Rindern, die Traberkrankheit (Scrapie) bei Schafen und Ziegen sowie die Creutzfeldt-Jakob Erkrankung (CJD) beim Menschen. BSE trat erstmals in Großbritannien Mitte der achtziger Jahre auf und gegenwärtig geht man davon aus, dass durch den Konsum von BSE-kontaminierten Produkten die neue Variante der Creutzfeldt-Jakob Krankheit (vCJD) beim Menschen ausgelöst wurde. Diskutiert werden neue Therapiemöglichkeiten zur Behandlung menschlicher Prionerkrankungen und die Risiken an BSE zu erkranken.

Durch einen EU-weiten Beschluß ist seit Januar 2001 für alle kranken und verhaltensauffälligen Rinder über 30 Monate ein BSE-Test verpflichtend (in Deutschland werden BSE-Tests ab einem Alter von 24 Monaten durchgeführt). Aufgrund eines solchen Tests wurde am 24. November 2000 erstmals bei einer in Deutschland geborenen Kuh BSE diagnostiziert. Seitdem sind in Deutschland 382 Rinder (Stand 29.09.2005) erkrankt (Abb. 1). Die Gesamtzahl der seit 1986 registrierten BSE-Fälle in Großbritannien ist mit 180837 beachtlich (Stand 29.09.2005) (Abb. 2). BSE zeigt Inkubationszeiten von 4-6 Jahren und verursacht Störungen des Verhaltens, der Bewegung und der Sensibilität. Trotz des Auftretens von klinischen Symptomen ist bis dato eine eindeutige Diagnose nur post mortem durch eine Hirnbiopsie möglich, in der ein charakteristisches, mit der Krankheit assoziiertes Protein, das Scrapie-Prion-Protein (PrP^{Sc}), nachgewiesen wird.

Das Prion-Protein

PrP kommt in zwei Konformationen vor: einer zellulären (PrP^{c}) und einer abnormalen Isoform (PrP^{Sc}). PrP^{c} wird in jedem Säugetier exprimiert, wobei seine physiologische Funktion bisher noch unklar ist. PrP^{c} bindet Kupfer und spielt bei synaptischen Übertragungen und der Regulation von zirkadianer Aktivität eine Rolle. Durch die Umwandlung der zellulären Form PrP^{c} in die pathologische Form PrP^{Sc} kommt es zur Aggregat-Bildung im Gehirn und zur Entstehung der Prionerkrankungen. PrP^{Sc} ist extrem hitze- und druckbeständig und resistent gegenüber Proteasen. Im Laufe der Erkrankung kommt es zu Ablagerungen von Amyloiden im Zentralnervensystem, zum Anschwellen von Hirnzellen (Astrogliose) und zur vermehrten Bildung von Vakuolen durch abgestorbene Zellen (Spongiose).

Therapieansätze zur Behandlung von Prionerkrankungen

Trotz intensiver Forschung gibt es bislang keine effektiven Behandlungsmöglichkeiten für Prionerkrankungen, welche deswegen stets letal sind. Hinweise auf einen Anti-Prion Effekt einiger Substanzen *in vivo* erhielten Forscher, nachdem sie Hamster mit DMSO (Dimethylsulfoxid) behandelten und eine Infektion mit PrP^{Sc} erschwert wurde (Shaked *et al.*, 2003). Auch in Tierversuchen mit Heparansulfatanaloga konnte gezeigt werden, dass die Bildung des pathologischen

Prion-Proteins reduziert war (Adjou *et al.*, 2003). Ein Nachteil der Behandlung mit diesen Substanzen ist jedoch, dass die Manifestation der Erkrankung nur verzögert, aber nicht verhindert wird. Große Hoffnung wurde deswegen in die Entwicklung von Impfstoffen gesetzt, die anfangs allerdings enttäuscht wurde, da das zelluläre Prion-Protein ein körpereigenes Protein und deswegen nur schwach immunogen ist. Dennoch ist es gelungen, Antikörper gegen PrP^{c} zu generieren. Dazu wurden verschiedene Strategien entwickelt, um eine Immunität der Versuchstiere gegenüber einer Prioninfektion zu erreichen. In transgenen Mäusen, welche Anti-PrP-Antikörper produzierten, konnte gezeigt werden, dass eine humorale Immunantwort gegen PrP einen protektiven Effekt gegenüber einer Prioninfektion hat (Heppner *et al.*, 2001). Neuere Therapieansätze fokussieren auf Vakzine aus rekombinanten retroviralen Partikeln, um die Toleranz des Körpers gegenüber dem zellulären Prion-Protein zu überwinden. Erste Untersuchungen in Mäusen haben gezeigt, dass es möglich ist, autoreaktive spezifische PrP-Antikörper zu generieren (Nikles *et al.*, 2005). Weiterführende Studien sollen prüfen, ob die induzierte Antikörpermenge in einem Organismus ausreichend ist, um eine Prioninfektion zu verhindern.

Moderne Therapiemöglichkeiten

Aussichtsreich scheint bei der Entwicklung neuer Therapien gegen Prionerkrankungen der Befund, dass Prionen einen Rezeptor benötigen, um internalisiert zu werden. Wir konnten den 37kDa/67kDa Laminin-Rezeptor (LRP/LR) als Rezeptor für das Prion-Protein identifizieren und zeigen, dass der Rezeptor auch für die Vermehrung von infektiösen Prionen in Zellkultur essentiell ist. Sowohl antisense LRP RNA als auch small interfering RNAs (siRNAs), welche gegen die LRP mRNA, und Antikörper, die gegen den 37kDa/67kDa LRP/LR gerichtet sind, inhibierten eine Akkumulation von PrP^{Sc} in Zellkultur (Leucht *et al.*, 2003). Anschließende *in vivo* Studien sollen nun zeigen, ob transgene Tiere, die antisense-LRP RNA bzw. anti-LRP-siRNAs exprimieren, vor einer Prioninfektion geschützt sind. Alternative Therapiemöglichkeiten bestehen auch im Einsatz von dominant-negativ wirkenden LRP/LR Mutanten, die nach Sekretion durch ein Abfangen des pathogenen Prion-Proteins ein Fortschreiten der Erkrankung verhindern können („Lockvogel"- oder „Decoy"- Effekt) (Vana and Weiss, 2006). Eine Weiterentwicklung der klassischen Antikörper-Therapie stellt die Generierung von sogenannten Anti-LRP-Einzelketten-Antikörpern dar. Einzelketten-Antikörper (scFv) bestehen nur aus den variablen Regionen der leichten (V_L) und schweren Kette (V_H) eines Immunglobulins, welche durch einen Peptidlinker kovalent miteinander verknüpft sind. Kürzlich gelang es in Mäusen durch eine orale Immunisierung mit PrP-exprimierenden Salmonella-Bakterien den Ausbruch einer Prionose zu verhindern. Immunisierte Mäuse zeigten im Vergleich zur Kontrollgruppe 500 Tage nach der Prioninfektion keine klinischen Symptome einer Prionerkrankung. PrP^{Sc} konnte weder im Gehirn noch in der Milz nachgewiesen werden (Goni *et al.*, 2005). Eine Immunisierung gegen PrP scheint damit ein sehr vielversprechender therapeutischer Ansatz zur Bekämpfung von Prionosen zu sein (Abb. 3)

und könnte die Behandlung von menschlichen TSEs, wie der durch die Übertragung von BSE auf den Menschen induzierte neue Variante der Creutzfeldt-Jakob-Erkrankung (vCJD) ermöglichen.

Die neue Variante der Creutzfeldt-Jakob Erkrankung

Die neue Variante der CJD unterscheidet sich von der klassischen (sporadischen) Erkrankung insbesondere hinsichtlich des Alters der Betroffenen (mittleres Alter: 27) und der sehr langsamen Progressivität der Erkrankung. Sie beginnt meist mit psychiatrischen Symptomen wie Ängstlichkeit und Depressionen. Später treten dann neurologische Störungen mit dem Verlust intellektueller Fähigkeiten auf. Die Beobachtung, dass alle vCJD-Patienten eine genetische Disposition an der Position 129 des vom Prion-Gen (*Prn*-p) kodierten Prion-Protein besitzen, führte zur Entwicklung eines kommerziellen genetischen Tests, mit dessen Hilfe überprüft werden kann, welche Aminosäure an der Position 129 des Prion-Proteins eingebaut wurde (www.medigenomix.de). Alle vCJD Patienten kodierten auf beiden Allelen des Prion-Gens P*rn*-p für die Aminosäure Methionin an Position 129 des Prion-Proteins, waren also homozygot für Methionin (Tabelle 1). Diese Variante ist in der kaukasischen Bevölkerung mit einem Anteil von 39% vertreten. 11% der Bevölkerung hingegen sind homozygot für die Aminosäure Valin und 50% der kaukasischen Bevölkerung zeigen eine Methionin-Valin Heterozygotie (Tabelle 1).

Tab.1: Der Codon 129 Polymorphismus des Prion-Protein-Gens (*Prn*-p)

Aminosäuren, welche vom Codon 129 exprimiert werden	Verteilung in der Bevölkerung	Vorkommen bei sCJD	Vorkommen bei vCJD
Met/Met	39%	80%	100%
Met/Val	50%	8%	0%
Val/Val	11%	12%	0%

Das Ergebnis der Sequenzanalyse liefert jedoch keinen Beweis für eine Immunität gegenüber einer Prioninfektion, da Personen, welche Met/Val heterozygot oder Val/Val homozygot sind, die Krankheit möglicherweise später entwickeln könnten als Menschen mit Met/Met Homozygotie. In einer aktuellen Studie untersuchten Züricher Forscher den Einfluß dieser verschiedenen Allelvarianten des Prion-Protein-Gens auf die Gedächtnisleistung. Dabei wurde entdeckt, dass sich sowohl Träger der homozygoten als auch der heterozygoten Methionin-Variante durchschnittlich 17 Prozent mehr Informationen merken konnten als die Personen, welche die homozygote Valin-Variante trugen (Papassotiropoulos *et al*., 2005). Die Forscher nehmen deswegen an, dass das Prion-Protein eine wichtige Rolle in der Ausbildung des Langzeitgedächtnisses spielen könnte und nicht nur für die neurodegenerativen Erkrankungen bei Mensch und Tier verantwortlich ist. In Deutschland ist bisher noch kein Fall der neuen Variante der Creutzfeldt-Jakob-Erkrankung aufgetreten, dennoch kommt der

Entwicklung empfindlicher Tests und wirksamer Therapien gegen Prionerkrankungen eine wichtige Aufgabe zu.

Suszeptibilität und Übertragbarkeit von Prionen - Aktuelle Forschung

Auch wenn die BSE-Fallzahlen in Europa sinken wird die Problematik der Prionerkrankungen in den nächsten Jahren weiterhin öffentliche Beachtung finden. Französische Forscher nehmen an, dass Kinder und Jugendliche besonders anfällig für die neue Variante der Creutzfeldt-Jakob-Erkrankung sein könnten. Ursächlich könnte ein übermäßiger Konsum von Rindfleisch sein, aber auch eine besonders hohe Durchlässigkeit des Darms für Prionen im Jugendalter schließen die Wissenschaftler nicht aus (Boelle *et al.*, 2004). In einer soeben veröffentlichten Studie konnten wir zusammen mit Französischen Kollegen zeigen, dass menschliche Enterozyten im Darm den BSE-Erreger innerhalb weniger Minuten aufnehmen können, wobei der Lamininrezeptor eine essentielle Rolle spielt (Morel *et al.*, 2005). Interessanterweise konnten Scrapie-Prionen des Schafes nicht von den menschlichen Dünndarmzellen aufgenommen werden, was erklären könnte warum BSE, nicht aber Schaf-Scrapie auf den Menschenübertragbar ist (Morel *et al.*, 2005). Obwohl menschlichen Prionerkrankungen gering verbreitet sind, so handelt es sich dennoch um stets tödlich verlaufende Erkrankungen, welche im Fall der vCJD besonders junge Menschen betrifft. Um das Leben der Betroffenen zu retten, suchen Forscher intensiv nach Behandlungsmöglichkeiten, um Prionerkrankungen effizient zu bekämpfen. Eine Heilung von CJD beim Menschen ist nach heutigem Wissenstand zwar noch nicht möglich, scheint aber im Lichte der aktuellen Therapieforschung in naher Zukunft realisierbar.

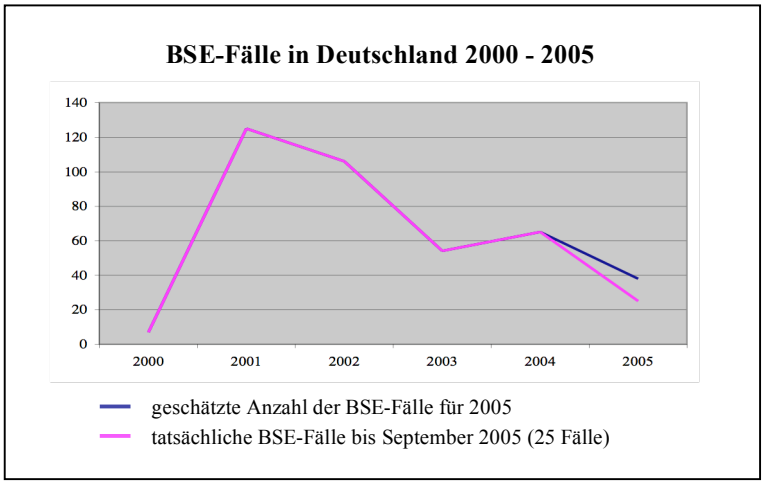

Abb. 1: BSE-Fälle in Deutschland seit Einführung der Meldepflicht für BSE-Fälle durch die EU 1990

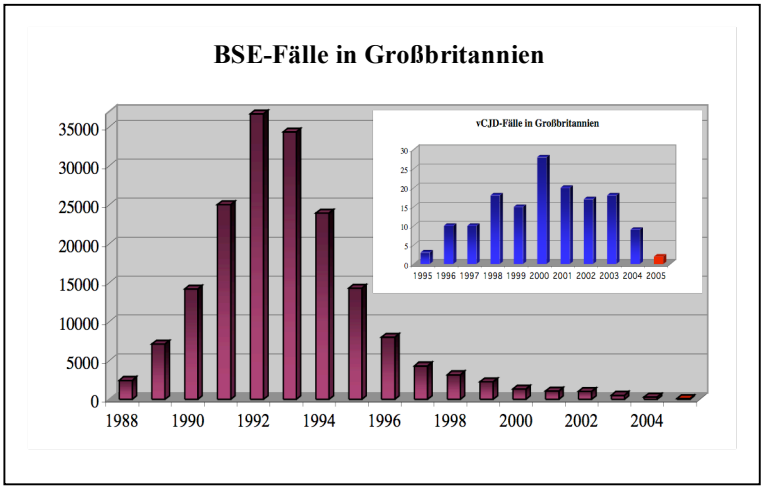

Abb. 2: BSE-und vCJD-Fälle in Großbritannien im Vergleich

Die BSE- und vCJD-Statistik (Inset) zeigt alle bis Dezember 2004 bestätigten Fälle in Großbritannien (gesamt 180837, Stand 29.09.05). Die Zahlen für 2005 entsprechen den bis September 2005 erfassten Fällen: 126 BSE-Fälle und 2 vCJD Erkrankungen.

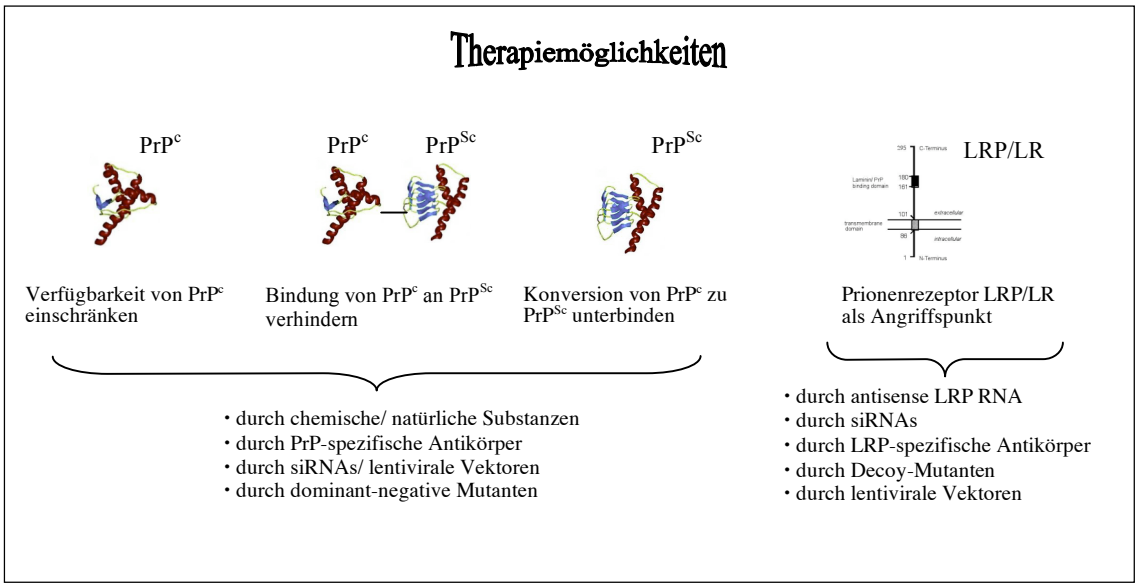

Abb. 3: Mögliche Therapieansätze gegen Prionerkrankungen

CHAPTER III

The 37kDa/67kDa laminin receptor is required for PrP^{Sc} propagation in scrapie-infected neuronal cells

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

The accumulation of PrP^{Sc} in scrapie-infected neuronal cells has been prevented by three approaches: (i) transfection of ScMNB cells with an antisense laminin receptor precursor (LRP) RNA-expression plasmid, (ii) transfection of ScN2a cells and ScGT1 cells with small interfering RNAs (siRNAs) specific for the LRP mRNA, and (iii) incubation of ScN2a cells with an anti-LRP/LR antibody. LRP antisense RNA and LRP siRNAs reduced LRP/LR expression and inhibited the accumulation of PrP^{Sc} in these cells. The treatment also reduced PrP^{c} levels. The anti-LRP/LR antibody, W3, abolished PrP^{Sc} accumulation and reduced PrP^{c} levels after seven days of incubation. Cells remained free of PrP^{Sc} after being cultured for 14 additional days without the antibody, whereas the PrP^{c} level was restored. Our results demonstrate the necessity of the laminin receptor (LRP/LR) for PrP^{Sc} propagation in cultured cells and suggest that LRP/LR-specific antibodies could be used as powerful therapeutic tools in the treatment of transmissible spongiform encephalopathies.

Introduction

Transmissible spongiform encephalopathies (TSEs) are a group of neurodegenerative disorders which include Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle, and scrapie in sheep (Aguzzi and Weissmann, 1998; Lasmezas and Weiss, 2000; Prusiner *et al.*, 1998; Weissmann, 1999). The main pathogenic event in the development of TSEs is the conversion of PrP^{c} to PrPres. A main feature of PrP^{Sc} is its partial resistance to proteases, which makes it biochemically distinguishable from PrP^{c} (Caughey and Raymond, 1991). Recently, we identified the 37kDa/67kDa laminin receptor (LRP/LR) as the cell surface receptor for the cellular prion protein (PrP^{c}) (Gauczynski *et al.*, 2001b). Heparan sulfate proteoglycans (HSPGs) have been shown to function as cofactors or co-receptors for the binding of PrP^{c} to LRP/LR (Hundt *et al.*, 2001b). The LRP/LR laminin receptor has been shown to interact directly with the prion protein in the yeast two-hybrid system (Rieger *et al.*, 1997). This interaction was confirmed by pull down assays in cotransfected COS-7 cells and co-infected insect cells (Rieger *et al.*, 1997). Furthermore, increased levels of LRP were found in the brain, spleen and pancreas of scrapie infected mice and hamsters as well as in scrapie infected neuroblastoma cells, a well characterized *in vitro* model for scrapie infection (Rieger *et al.*, 1997). These data suggest a link between the 37kDa/67kDa laminin receptor and prion propagation. The non-integrin LRP/LR laminin receptor represents a multifunctional protein required for cell differentiation, movement and growth (for review see (Gauczynski *et al.*, 2001a)). Its cDNA encodes a 37 kDa protein, also known as p40, and has been cloned from different species by several groups. This protein has been reported to be ribosome associated, to bind to histones H2A, H2B and H4 and to be the precursor of the metastasis-associated 67kDa mature high-affinity laminin receptor (for a review see (Gauczynski *et al.*, 2001a), Leucht & Weiss 2002). The 67kDa LR is consistently upregulated in aggressive carcinoma suggesting a role in cell homeostasis and cohesion. The amino acid sequence of the receptor is extremely well conserved through evolution with at least

98.3% homology between mouse, human and bovine sequences and 99% homology between rat and human sequences (for review, see (Gauczynski et al., 2001a), Leucht & Weiss, 2002). Published data suggest the existence of at least six LR genes in the mouse genome, one of these is localized on chromosome nine and at least two copies are thought to be functional (Douville and Carbonetto, 1992). Using TRIBE-MCL, an algorithm for detection of protein families (Enright *et al.*, 2002), five LRP/LR genes were identified when the program was used to search the latest mouse draft genome sequence (Mouse Genome Sequencing Consortium, 2003, available at http://www.ensembl.org). The LRP gene on chromosome 9 has seven exons and six introns, in contrast with earlier results (Douville and Carbonetto, 1992), no LRP/LR gene on chromosome six has been identified. Interestingly, genes that affect susceptibility to prions have been identified on mouse chromosome nine (Stephenson *et al.*, 2000). PrP specific antibodies have successfully been used in preventing prion propagation *in vitro* and *in vivo* as follows: first, the accumulation of PrP^{Sc} in scrapie infected neuroblastoma cells was inhibited by PrP-specific antibodies (Peretz *et al.*, 2001), second, scrapie infection was abolished by transgenic expression of PrP-specific antibodies in mice (Heppner *et al.*, 2001). The epitope recognized by the antibody that has the most potent effect on PrP^{Sc}, D18, consists of amino acid residues 132-156 of PrP, which includes helix A (residues 145-155). Because PrP residues 144-179 has been shown to constitute a binding site for the LRP/LR (Hundt *et al.*, 2001a), we investigated whether an antibody directed against LRP/LR, the cellular receptor for PrP^{c} (Gauczynski *et al.*, 2001b), can also be used to interfere with the metabolism of PrP^{Sc}. To ablate LRP/LR expression from all putative LRP/LR-encoding genes, we used an antisense RNA and a small interfering RNA (siRNA) approach. We investigated whether these strategies had an effect on prion propagation in several scrapie-infected cell systems.

Results and Discussion

Antisense LRP RNA prevents PrP^{Sc} propagation

To produce LRP antisense messenger RNA, we cloned a region of LRP complementary DNA from nucleotide positions 65 to 901 into the expression plasmid pCI-neo in the antisense orientation to produce the pCI-neo-asLRP plasmid. After transient transfection of pCI-neo-asLRP into ScMNB cells we confirmed antisense LRP RNA expression in these cells (Fig. 1A). The level of LRP mRNA was greatly reduced 48 h after transfection (Fig. 1B). Using phosphorimaging, the reduction was quantified and LRP mRNA levels were found to be 80-85% of normal LRP/LR mRNA expression levels. A similar reduction in target mRNA levels has been shown in other studies that have used the antisense RNA method to downregulate the expression of the myelic basic protein (Katsuki *et al.*, 1988), and *Wnt-1* (Erickson *et al.*, 1993). At the level of protein expression, no LRP protein was detected by western blotting 48 h after transfection (Fig. 1C). Levels of PrP^{Sc} were unaffected in cells transfected

with pCI-neo as compared with untransfected cells (Fig. 1D). In ScMNB cells we were able to detect only the diglycosylated form of PrP using the SAF70 antibody, whereas in ScN2a and ScGT1 cells we observed the classic three-band pattern. We observed a reduction in PrP^{c} level after antisense LRP RNA transfection (Fig. 1D), which might be caused by an altered PrP^{c} metabolism. Previous studies have indicated that PrP^{c} internalization is highly dependent on the presence of the LRP/LR at the cell surface (Gauczynski *et al*., 2001b), where the LRP/LR binds PrP^{c} through two distinct domains: the octapeptide region and the region encompassing amino acids 144-179 of PrP^{c} (Hundt *et al*., 2001a). This is consistent with a recent study, in which it was found that the octapeptide region is essential for internalization of PrP^{c} (Nunziante *et al.*, 2003). Hence, the altered PrP^{c} levels seen in this study are likely to be due to perturbed metabolism of the protein.

LRP-specific siRNAs prevent PrP^{Sc} propagation

SiRNAs were used to verify the results obtained using the LRP antisense RNA construct. This method has been used successfully in other studies to knock-down target-gene expression levels (Elbashir *et al*., 2001). We tested four different LRP-specific siRNAs for their ability to repress LRP expression in ScN2a cells. All of them repressed LRP synthesis (Fig. 2A). Figure 2B shows data from a time-course experiment carried out to analyze the effect of siRNA-LRP3 on PrP^{Sc} propagation in ScN2a cells. Seventy-hours after transfection, PrP^{Sc} propagation was completely abolished by siRNA-LRP3, whereas siRNA-LRP1, siRNA-LRP4 and a control siRNA (lamin A/C, described in Elbashir *et al*., 2001) had a smaller effect (siRNA-LRP1 + siRNA-LRP4) or no effect (control) on PrP^{Sc} levels. PrP^{c} levels were reduced in the presence of siRNA-LRP3. The same effects were observed with LRP antisense RNA 72 h after transfection. In contrast to PrP^{Sc}, PrP^{c} levels increased 96 h after transfection, probably due to a decrease in siRNA effectiveness with time. We also tested the efficiency of the reduction of LRP expression using siRNAs in ScGT1 cells, which show a robust PrP^{Sc} phenotype (that is, these cells propagate PrP^{Sc} over a long period of time). The results were consistent with those obtained in ScN2a cells, with a strong reduction of PrP^{Sc} correlated with LRP downregulation (Fig. 2C).

Anti-LRP/LR antibody W3 prevents PrP^{Sc} accumulation

LRP/LR specific antibodies have been used successfully to compete with recombinant prion protein for binding to the LRP/LR in different mammalian cell types (Gauczynski *et al*., 2001b), showing that the LRP/LR has a crucial role in the metabolism of PrP^{c}. Using the LRP/LR specific antibody W3 (Rieger *et al.*, 1997) in ScN2a cells we observed a reduction of PrP^{Sc} to a undetectable level (Fig. 3A, B). The antibody was used at concentrations of 6- 64 μg ml^{-1}. At a concentration of 12 μg ml^{-1} a reduction in PrP^{Sc} level was observed. At a higher concentrations (64 μg ml^{-1}), PrP^{Sc} accumulation was completely abolished after incubation for three days, indicating a dose dependent effect (Fig. 3A). In a

time-course experiment, we found a complete clearance of PrP^{Sc} after incubation for one week, using an antibody concentration of 32 µg ml^{-1} (Fig. 3B). These results are consistent with a previous study, in which different anti-PrP antibodies were used to reduce PrP^{Sc} in cultured cells (Peretz *et al.*, 2001),Table I). In that study, PrP antibody concentrations of 1.2 µg/ml to 10 µg ml^{-1} were sufficient to clear PrP^{Sc} from ScN2a cells after one week of incubation (Table I). We also incubated ScN2a cells in which PrP^{Sc} had been previously cleared by W3 for a further two weeks without any antibody, and showed that no PrP^{Sc} reappeared (Fig. 3B). PrP^{c} levels in W3-treated cells were reduced after seven days of incubation with W3, but were completely restored after a further two-week incubation in the absence of the antibody (Fig. 3B).

Table I. Efficacy of anti-PrP and anti-LRP antibodies in clearance of PrP^{Sc} from ScN2a cells

Antibody	**anti-LRP/LR**	**anti-PrP**[1]			
Incubation time	1 week	1 week			
Antibody	W3	D18	D13	R1	R2
Effective Concentration [µg ml^{-1}]	32	1.2	2.5	10	10

[1] Data taken from Peretz *et al.*, 2001

Role of the LRP/LR in PrP^{Sc} propagation in cultured cells

The knock down of the LRP/LR on the cell surface by LRP antisense RNAs or by siRNAs, and the blockage of LRP/LR binding sites by the W3 anti LRP/LR antibody are most likely to interfere with PrP levels by blocking the PrP internalization process. However, some PrP^{c} can still be synthesized and transported through the secretory pathway to the cell surface (Figs 1D, 2B and 3). Conversion of PrP^{c} to PrP^{Sc} is thought to take place either at the cell membrane or in the endocytic pathway. Thus, it is possible that due to the lack of PrP^{c} within the endocytic pathway no PrP^{Sc} can be formed, resulting in a time-dependent reduction of PrP^{Sc} (Figs 2B and 3B). It is also possible that the LRP/LR has a function in the conversion of PrP^{c} to PrP^{Sc}, and that the absence of the LRP/LR from the cell surface affects PrP^{Sc} formation. PrP^{Sc} propagation cannot be restored after cessation of the incubation with anti LRP/LR antibody (Fig. 3B) due to the absence of any PrP^{Sc} to re-initiate the conversion process. In contrast, PrP^{c} levels were completely restored after cessation of incubation with the anti LRP/LR antibody (Fig. 3B). Furthermore, depletion or blockage of the LRP/LR on the cell surface might directly prevent PrP^{Sc} binding and internalization. In summary, our results show that the LRP/LR is not only involved in PrP^{c} metabolism, as demonstrated in previous reports (Gauczynski *et al.*, 2001b; Hundt *et al.*, 2001), but also has a crucial role in prion propagation. The fact that LRP/LR-specific

antibodies are able to clear PrP^{Sc} from neuroblastoma cells provides possibilities for the development of new experimental therapies for TSEs.

Methods

Construction of pCI-neo-asLRP. Bases 65-901 of the LRP cDNA were amplified by PCR with reverse transcription (RT-PCR) from total RNA isolated from N2a cells, introducing the restriction sites *NheI* and *SmaI*. The LRP cassette was cloned in an antisense orientation into the plasmid pCI-neo using the *NheI* and *SmaI* sites to produce pCI-neo-asLRP. Cloning was confirmed by sequencing.

Cell culture. ScMNB and ScN2a cells (both lines are neuroblastoma cells chronically infected with scrapie) were grown in DMEM, 10% fetal bovine serum, 2 mM Glutamax (Invitrogen), 100 units ml^{-1} penicillin and 10 µg ml^{-1} streptomycin sulfate, at 37°C with 5% CO_2. ScN2a cells were produced as described previously (Bosque & Prusiner, 2000). ScGT1-7 cells (GT1 hypothalamic neuronal cells, chronically infected with the Chandler scrapie isolate) were provided by S.Lehmann, and were cultured as described previously (Mange *et al.*, 2000), with the exception that DMEM was replaced with Opti-MEM (Gibco LifeSciences).

Inhibition studies using the W3 antibody. ScN2a cells ($1x10^6$) were incubated in normal growth medium (DMEM, 10% fetal bovine serum, 2 mM Glutamax) supplemented with the purified polyclonal anti LRP/LR antibody, W3, at varying concentrations. After incubation with the antibody, the cells were harvested, lyzed and analyzed by western blotting.

Inhibition studies using small interfering RNAs. Four pairs of complementary 21-nucleotide RNAs corresponding to regions of the LRP cDNA were made (Ambion). As a control, the lamin A/C RNA duplex was used (Elbashir *et al.*, 2001). The single-stranded complementary RNAs were annealed in annealing buffer (provided by the manufacturer) for 1 min at 90°C, followed by incubation for 1 h at 37°C. The RNA duplexes were transfected into ScN2a cells (cultured in Opti-MEM medium, Invitrogen) using Oligofectamine (Invitrogen) in accordance with the manufacturer's instructions. ScGT1-7 cells were seeded in 60-mm petri dishes ($5x10^5$ cells per dish) and transfected the following day with 10 µg of each of the 21-nucleotide RNA pairs using Exgen 500 (Fermentas) in accordance with the manufacturer's instructions.

Ribonuclease protection assays. Total RNA was purified from transfected ScMNB cells and used in a Ribonuclease Protection Assay using the RPA III kit (Ambion). An antisense riboprobe was made by *in vitro* transcription from pCI-neo-asLRP, following linearization of the plasmid with *Eco*RI, in the presence of [α-^{32}P]-UTP. The antisense riboprobe was combined with the total RNA and the mixture was then precipitated. The precipitates were dissolved in hybridization buffer, denatured and hybridized with the total RNA. This was followed by incubation with RNase for 30 min at 37°C, followed by inactivation of the RNase and ethanol precipitation of the RNA. Protected RNA

fragments were separated on a 5% acrylamide/ urea gel and visualized using a Storm 860 phosphorimager equipped with ImageQuant software.

Reverse-transcriptase-PCR. Total RNA was purified from transfected ScMNB cells and cDNA synthesis was carried out using oligo(dT) primer in a RT reaction. The resulting cDNA was then amplified by PCR using a 5'-oligodeoxyribonucleotide corresponding to the 3'-end of the cytomegalovirus promoter and a 3'-oligodeoxyribonucleotide corresponding to a sequence in the 5'-region of the simian virus 40 polyadenylation signal. PCR products were separated on 1% agarose gels and stained with ethidium bromide.

Western blotting. Cytoplasmic lysats were made using a buffer containing 10 mM Tris/HCl pH 7.5, 100 mM NaCl, 10 mM EDTA, 0.5 % Triton X-100, 0.5 % sodium desoxycholate. After centrifugation, the total protein content of the lysats was measured (BCA-Protein Assay, Pierce) and equal amounts of protein from each lysate were analyzed. For PrP^{Sc} detection, cell lysates digested with proteinase K (20 µg ml^{-1}) for 1 h at 37°C . The reaction was stopped by the addition of Pefabloc (1mM) and the proteins were denatured using 6 M guanidine hydrochloride. Samples were boiled in SDS sample buffer and analyzed on an SDS-polyacryamide gel containing 12.5% acrylamide. For PrP^{c} and PrP^{Sc} detection (from ScN2a cells), 10% Bis-Tris gels with MES running buffer (NuPAGE, Invitrogen) were used. Proteins were blotted on a polyvinylidene difluoride membrane, blocked and incubated overnight with the monoclonal antibodies SAF70, SAF32 or SAF84 (diluted 1:5000 in blocking solution) or A7 (diluted 1:2,500 in blocking solution) for PrP detection. The polyclonal anti LRP/LR antibody W3 (Rieger *et al.*, 1997) (1:2,000), or the monoclonal antibody 43512 (1 µg ml^{-1}) were used for LRP/LR detection and anti-β-actin antibody (Chemicon) (1:5,000) for β-actin detection. After washing with TBS/0.05 % Tween 20 the blot was incubated for 1 hour with a peroxidase-conjugated secondary antibody (Sigma) (1:2,500). Detection was carried out by enhanced chemiluminescence (Western Lightning, NEN).

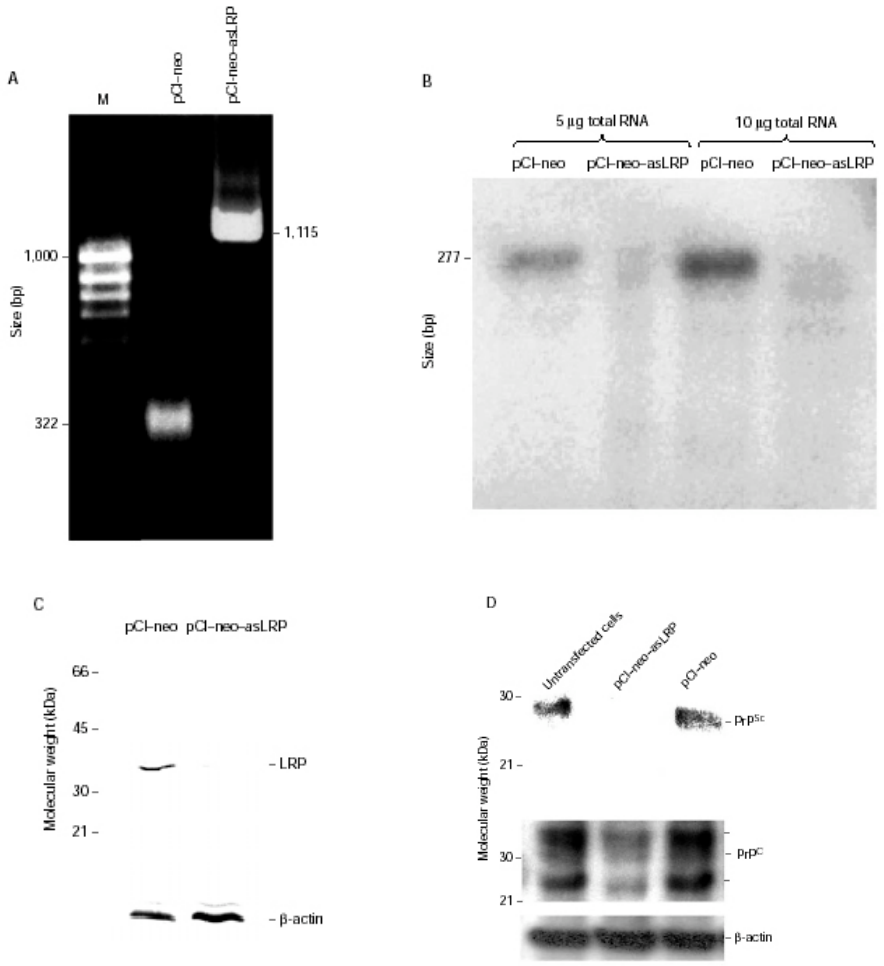

Fig.1 | Abolition of PrP^{Sc} propagation using laminin receptor precursor (LRP) antisense RNA. (**A**) Analysis by PCR with reverse transcription of total RNA extracts from transfected ScMNB cells. Oligodesoxythymidine-primed complementary DNA was amplified by PCR using specific primers for the pCI-neo plasmid. This gave a 322-bp cDNA fragment for the pCI-neo transfected cells and a 1,115-bp cDNA fragment for the pCI-neo-asLRP transfected cells. (**B**) A ribonuclease protection assay was carried out on total RNA from cells transfected with either pCI-neo or pCI-neo-asLRP; the RNA was then separated using a 5% acrylamide/ urea gel. 5 µg or 10 µg of total RNA was used, and in both cases the level of LRP messenger RNA was reduced by 80-85% in cells transfected with pCI-neo-asLRP (quantified by phosphorimaging). (**C**) Western blot analysis of cell lysates from pCI-neo and pCI-neo-asLRP-transfected ScMNB cells assayed 48 hours after transfection. LRP was detected using the polyclonal anti-LRP/LR antibody, W3. β-actin was detected using an anti β-actin antibody as loading control. (**D**) ScMNB cells were transfected with pCI-neo and pCI-neo-asLRP. The PrP^{Sc} content of ScMNB cells was analysed 72 h after transfection. The monoclonal anti-PrP antibody SAF70 was used for PrP^{Sc} detection and the SAF32 antibody were used for detection of PrP^{c}.

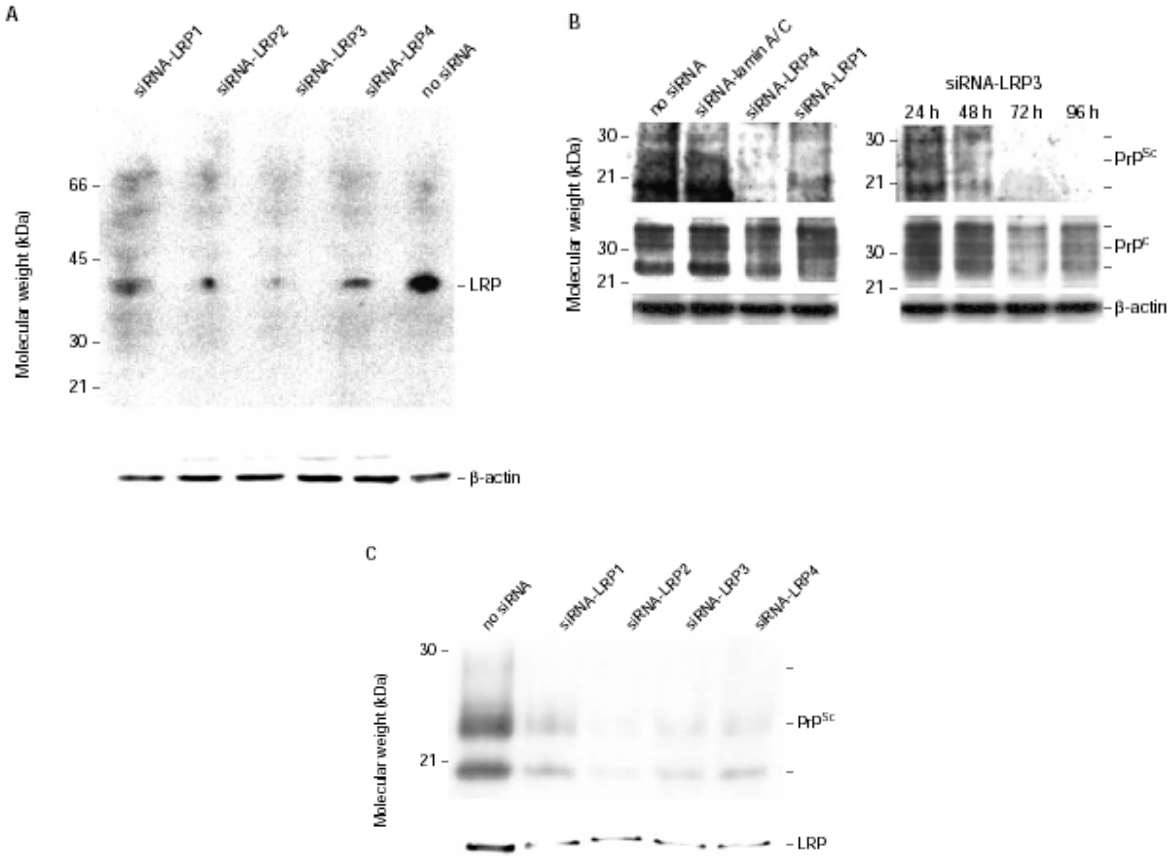

Fig.2 | Inhibition of PrP^{Sc} propagation using small interfering RNAs. (**A**) Western blot analysis of ScN2a cells transfected with small interfering RNAs (siRNAs). Cells were analyzed 72 h after transfection using the polyclonal anti-laminin receptor (LRP/LR) antibody W3. (**B**) The effect of siRNAs on PrP^{Sc} propagation was assayed 72 h after transfection (left panel). The time-dependent effect of siRNA-LRP3 on PrP^{Sc} propagation (right panel) was analyzed using the SAF70 antibody; PrP^{c} was detected with the SAF32 antibody. β-actin was detected using an anti β-actin antibody as a loading control. (**C**) Western blot analysis of siRNA-transfected ScGT1 cells at 72 h after transfection. The cells were analyzed using the monoclonal antibodies LR43512 (lower panel) and SAF84 (upper panel). All samples were normalized to equal protein concentrations.

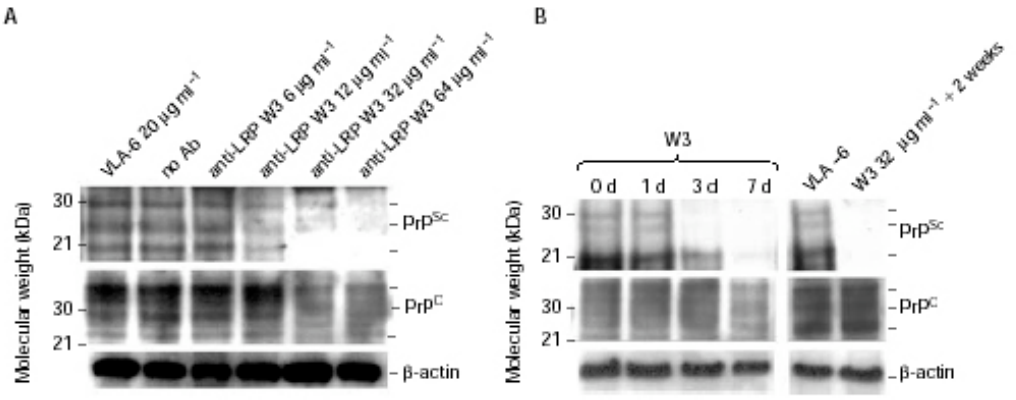

Fig.3 | The effect of the W3 anti-laminin receptor (LRP/LR) antibody on PrP^{Sc} propagation. (**A**) ScN2a cells were incubated with W3 at varying concentrations. The PrP^{Sc} content was determined after 72h incubation with W3. An anti-VLA-6 (integrin-type laminin receptor) antibody was used as a control. PrP^{Sc} was detected using the A7 polyclonal antibody; PrP^{c} was detected with the SAF32 antibody. (B) ScN2a cells were incubated with W3 at 32 µg ml^{-1} for varying durations. The last lane shows W3-treated ScN2a cells after an additional 2-week incubation without any antibody. PrP^{Sc} was detected with SAF70 antibody, PrP^{c} was detected with the SAF32 antibody. β-actin was detected using an anti-β-actin antibody as a loading control.

CHAPTER IV

Knock-down of the 37 kDa/ 67 kDa laminin receptor in mouse brain by transgenic expression of specific antisense LRP RNA

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Knock-down of the 37-kDa/67-kDa laminin receptor in mouse brain by transgenic expression of specific antisense LRP RNA. *Transgenic Research* **13**: 81-85

Abstract

The 37-kDa/67-kDa laminin receptor (LRP/LR) plays a major role in the propagation of PrP^{Sc}, the abnormal form of the prion protein. In order to ablate the expression of LRP/LR in mouse brain we generated transgenic mice ectopically expressing antisense LRP RNA in the brain under control of the neuron-specific enolase (NSE) promoter. Hemizygous transgenic mice TgN(NSEasLRP)2 showed a significant reduction of LRP/LR protein levels in hippocampal and cerebellar brain regions. These mice might act as powerful tools to investigate the role of the laminin receptor in scrapie pathogenesis.

Transmissible spongiform encephalopathies (TSEs) are neurodegenerative diseases thought to be caused by prions (*proteinaceous infectious particles*) which occur in humans and animals. The human TSEs include Creutzfeldt-Jakob-Disease (CJD), fatal familiar insomnia (FFI), Gerstmann-Sträussler-Scheinker syndrome (GSS) and Kuru, whereas bovine spongiform encephalopathy (BSE) affects cattle, chronic wasting disease (CWD) elk and deer, and scrapie sheep and goat, respectively (Lasmézas and Weiss, 2000; Weissmann and Aguzzi, 1997). The infectious agent, termed prion, is thought to consist of an abnormal protein as the main if not the sole infectious component. The key event in pathogenesis of prion diseases is the conversion of the cellular prion protein (PrP^{c}) to its homolog PrP^{Sc} (scrapie form of the prion protein). The latter represents an abnormally folded, infectious isoform of the host-encoded and naturally expressed PrP^{c} (Prusiner, 1998). PrP^{Sc} and its proteinase K resistant form PrP27-30, which is 142 amino acids in length (in case of hamster PrP), are present predominantly in brains or lymphoreticular organs of humans or animals suffering from prion diseases where they accumulate, sometimes in the form of amyloid plaques (McKinley *et al.*, 1991). Recently, we showed that the 37-kDa/67-kDa laminin receptor (LRP/LR), originally identified as an interactor for PrP^{c} (Rieger *et al.*, 1997) acts as the cellular receptor for the prion protein (Gauczynski *et al.*, 2001b; Hundt *et al.*, 2001) (for review: (Gauczynski *et al.*, 2001a; Leucht and Weiss, 2002)). More recently, we showed that the LRP/LR is required for PrP^{Sc}-propagation in prion-infected neuronal cells (Leucht *et al.*, 2003). The propagation of the protease resistant prion protein has been inhibited by transient transfection of short interfering RNAs specific for LRP/LR mRNA into scrapie-infected N2a and GT1 cells (Leucht *et al.*, 2003). Moreover, the transient expression of laminin receptor precursor (LRP) antisense-RNA interfered with PrP^{Sc} accumulation in ScMNB cells (Leucht *et al.*, 2003). The 37-kDa/67-kDa laminin receptor has two major isoforms, the mature 67-kDa laminin receptor and the 37-kDa laminin receptor precursor. Both forms have been shown to locate in the plasma membrane of neuronal cells (Gauczynski *et al.*, 2001b). However, four PrP-binding isoforms were identified in mouse brain, including a 60-kDa and a 220-kDa form (Simoneau *et al.*, 2003). Five genes of LRP/LR have been identified (Leucht *et al.*, 2003), therefore we aimed to establish transgenic mice that exhibit a lower level of LRP/LR by antisense RNA technology rather than producing a LRP/LR knock-out mouse.

Here we report on the generation of hemizygous transgenic mice that express LRP/LR antisense RNA under the control of the neuron-specific enolase (NSE) promoter from rat and show reduced LRP/LR levels in the cerebellum and hippocampus. This is the first example of a successful antisense RNA transgene expression ectopically in the brain employing the NSE-promoter.

The NSE promoter was amplified from the plasmid pNSElacZ by PCR using the primers 5´-GAAGATCTGAGCTCCTCCTCTGCTCGCCCAATC-3´ (forward) and 5´-GCGCCTCTCTTAAGGTAGCAGCGCGGGCGGGGGGT-3´ (reverse). The resulting 1.8 kb fragment was used to replace the CMV I.E. enhancer/ promoter in the plasmid pCI-neo-asLRP (Leucht *et al*., 2003) using the restriction enzymes *Bgl*II and *I-Ppo*-I. The resulting plasmid pCI-NSE-asLRP was linearized using the restriction enzyme *Bgl*II. The linearized plasmid was microinjected into pronuclei of mouse zygotes (B6D2F2) and then transferred into oviducts of pseudopregnant female NMRI mice.

The offspring were routinely screened by PCR to identify transgenic animals. The transgene copy number was evaluated by Southern blotting. Ten micrograms of genomic DNA from transgenic animals were digested with *Sac*I, analyzed on an 0.6 % agarose gel and blotted by capillary transfer to a positively charged Hybond-N+ membrane (Amersham). The membrane was probed with alpha-^{32}P random labeled fragments (Rediprime II, Amersham) derived from the *Bgl*II/*Bam*HI digested pCI-NSE-asLRP 4312 bp fragment. For quantitative analysis the obtained signals were measured by phosphor imaging and compared with blotted standard amounts of DNA derived from the plasmid pCI-NSE-asLRP.

The expression of the antisense RNA was monitored by RT-PCR using the specific primers 5'-AGAAGTTGGTCGTGAGGCAC-3' (forward) and 5'-TGTATCTTATCATGTCTGCTCGAAG-3' (reverse). The RT-PCR reaction was done in one step employing the Superscript One-Step RT-PCR kit (Invitrogen). For protein analysis homogenates from cortex, cerebellum and hippocampus were prepared in homogenization buffer (0.32 M sucrose, 10 mM HEPES pH 7.4, 2 mM EDTA plus protease inhibitors) from transgenic and control (non-transgenic littermates) animals. Four animals per transgenic line were analyzed. For the isolation of crude membranes the homogenates were centrifuged at 800g for 15 minutes. The supernatant was centrifuged at 120,000g for 90 minutes. The resulting pellet was resuspended in hypotonic lysis buffer (50 mM HEPES pH 7.4, 2 mM EDTA, 0.1% Triton X-100 plus protease inhibitors). Fifteen micrograms of each solubilized membrane preparation were subjected to SDS-PAGE. The gel was blotted onto a PVDF membrane (Millipore), blocked with 5% non-fat dry milk and probed with different antibodies diluted in TBST (0.05% Tween 20 in TBS). The proteins were visualized by enhanced chemiluminescence (Renaissance, NEN). Stripping of the blots was carried out using the Restore Western blot stripping buffer (Pierce).

For densitometric measurements the blots were scanned and the bands were quantified employing NIH-Image software. The intensity of all relevant bands was normalized with respect to the β-actin signal to circumvent loading inaccuracies.

We could establish four transgenic lines (out of 7 founder animals) harboring the LRP-antisense construct in their genome, which were analyzed by Southern blot (Figure 1). The genomes of mice TgN(NSEasLRP)2, TgN(NSEasLRP)3 and TgN(NSEasLRP)5 contain approximately the same transgene copy numbers (101-116), whereas TgN(NSEasLRP)4 have only about 16 copies in their genome (Figure 1).

We further analyzed transgene expression by RT-PCR (Figure 2 (A)) and monitored for a possible antisense-effect on LRP/LR mRNA expression by Western blotting (Figure 2 (B)). All analyzed mice showed transgene expression in distinct brain regions (Figure 2 (A), data not shown for TgN(NSEasLRP)3, TgN(NSEasLRP)4 and TgN(NSEasLRP)5). TgN(NSEasLRP)2 showed LRP/LR antisense expression in the cerebellum and the hippocampus and no or only marginal expression in the cortex (Figure 2 (A)). TgN(NSEasLRP)3-5 showed a weaker expression compared to TgN(NSEasLRP)2 in the hippocampus and the cerebellum (data not shown). We also observed a stronger antisense LRP mRNA expression in the cortex of TgN(NSEasLRP)3 and TgN(NSEasLRP)5 compared to TgN(NSEasLRP)2 (data not shown), possibly indicating that the transgene is susceptible to position efffects. These data demonstrate that the NSE promoter can be active in all examined brain tissues. However, the strong expression of antisense LRP mRNA predominantly in the hippocampus and cerebellum of TgN(NSEasLRP)2 was surprising since equal expression levels in all brain sections were exprected (Forss-Pett *et al.*, 1990). Since only weak or marginal activity of the NSE promoter was described in non-neuronal tissue (Mouse Genome Informatics (MGI) Accession ID: MGI: 1199209) we restricted our examinations to the central nervous system. Only TgN(NSEasLRP)2 showed a significant LRP/LR reduction in the brain (Figure 2 (B)), whereas no LRP/LR reduction was observed in the other transgenic mice (data not shown). Densitometric analysis revealed a reduction of ~60% in the hippocampus and ~20% in the cerebellum (Figure 2 (C)). No reduction was observed in the cortex (Figure 2 (C)) due to the lack of antisense RNA expression (Figure 2 (A)). The lack of LRP/LR reduction in TgN(NSEasLRP)3-5 is most likely due to the weaker expression of antisense LRP RNA in the examined brain tissues. The simultaneously measured PrP^c content showed no significant differences to control mice (Figure 2 (B)). This observation is in contrast with our recent finding that the PrP^c level is reduced in neuroblastoma cells expressing antisense LRP RNA (Leucht *et al.*, 2003). It can be explained by the fact that the complex mechanism of the regulation of PrP^c expression might be better compensated *in vivo* than in cultured cell lines. Transgenic mice did not show abnormal behavior compared to control mice. This is the first example of successful antisense transgenesis in mouse brain using the NSE-promoter. However, significant downregulation of the type

II glucocorticoid-receptor by antisense RNA in specific brain regions was achieved using the neurofilament L promoter (Pepin *et al*., 1992).

In summary, we showed that transgenic expression of an antisense construct derived from the LRP cDNA under the control of the NSE promoter is able to reduce the LRP/LR protein level in the cerebellum and hippocampus of transgenic mice. The cerebellum and the hippocampus represent brain regions where lesions occur in mice challenged with experimental scrapie (Fraser and Dickinson, 1967). Therefore, the transgenic mice will be used in scrapie infection studies to investigate if the accumulation of the abnormal prion protein is delayed or prevented in cerebellar and hippocampal brain regions due to a reduced level of LRP/LR. These mice might act as promising tools to elucidate the role of the LRP/LR in scrapie pathogenesis *in vivo*.

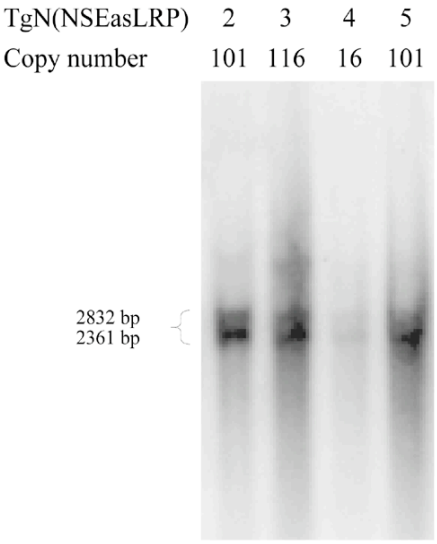

Figure 1. Southern blot analysis of 10 µg of *Sac*I digested genomic DNA derived from TgN(NSEasLRP) mice. The membrane was probed with alpha-^{32}P labeled fragments derived from pCI-NSE-asLRP. *SacI* digestion results in two major bands of 2832 and 2361 bp, respectively, due to tandem integration of the transgene.

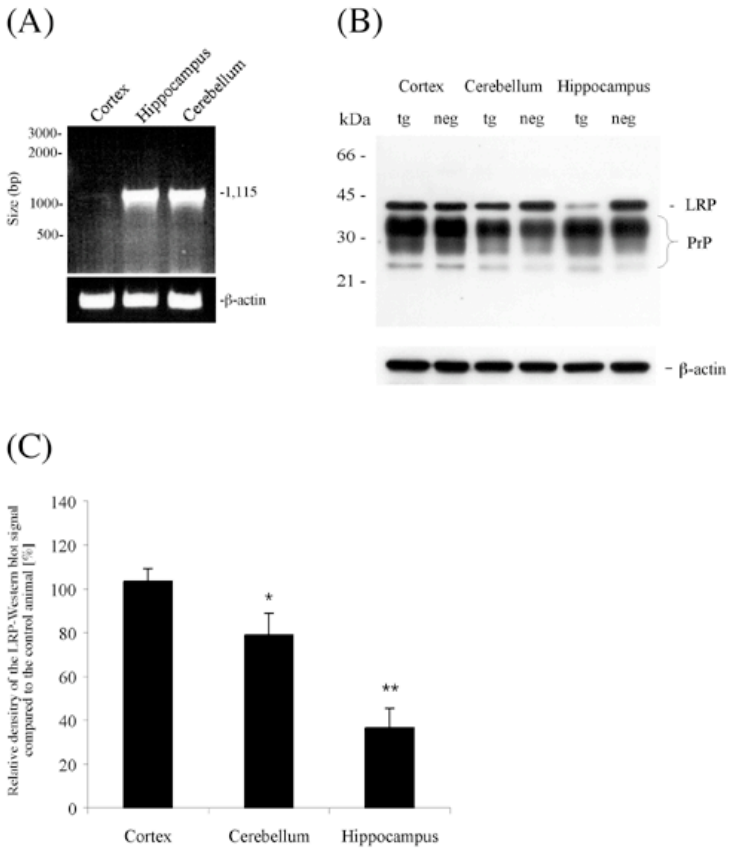

Figure 2. (A) The expression of LRP/LR antisense RNA in TgN(NSEasLRP)2 was monitored by a one-step RT-PCR reaction with primers specific for the transgene. This resulted in a 1115 bp DNA band detectable in cerebellum and hippocampus. (B) Representative Western blot of crude membrane preparation of cortical, cerebellar and hippocampal brain regions of TgN(NSEasLRP)2. The membrane was probed simultaneously with the monoclonal anti-PrP antibody SAF32 and the monoclonal anti-LRP/LR antibody 43506. As a loading control the stripped blot was incubated with a monoclonal anti-β-actin antibody. (C) Relative intensities of the LRP-bands in different brain regions compared to control brains. Four individual blots of four individual animals were analyzed. Student's *t*-test *p*-values: * <0.05, ** <0.005. We calculated the standard variation of the LRP/LR level in control animals to ± 9% by densitometrical measurements of Western blot signals (data not shown).

CHAPTER V

A trans-dominant negative 37 kDa/67 kDa laminin receptor mutant impairs PrP^{Sc} propagation in scrapie-infected neuronal cells

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The 37kDa/67kDa laminin receptor (LRP/LR) has been identified as a cell surface receptor for cellular and infectious prion proteins. Here, we show that a N-terminally truncated LRP mutant encompassing the extracellular domain of the LRP/LR (LRP102-295::FLAG) reduces the binding of (i) recombinant cellular huPrP to mouse neuroblastoma cells, (ii) infectious moPrP27-30 to BHK cells and (iii) interferes with the PrP^{Sc} propagation in scrapie-infected neuroblastoma cells ($N2aSc^{+}$). A cell free binding assay demonstrated the direct binding of the LRP102-295::FLAG mutant to both PrP^{c} and PrP^{Sc}. These results together with the finding that endogenous LRP levels remain unaffected by the expression of the mutant indicate that the secreted LRP102-295::FLAG mutant may act in a trans-dominant negative manner as a decoy by trapping PrP molecules. The LRP mutant might represent a potential therapeutic tool for the treatment of TSEs.

Introduction

Prion diseases are a group of fatal and transmissible neurodegenerative disorders (TSEs) which include scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt-Jakob disease (CJD) in humans (Aguzzi and Weissmann, 1998; Prusiner *et al.*, 1998; Weissmann, 1999). A hallmark of all prion diseases is the accumulation of an abnormal, proteinase-resistant isoform (PrP^{Sc}) of the cellular prion protein (PrP^{c}) which is a cell-surface glycosylphosphatidylinositol (GPI) anchored protein that is highly conserved among mammalian species (Schätzl *et al.*, 1995; Wopfner *et al.*, 1999). The fundamental step in the pathogenesis of TSEs is the conversion of the cellular PrP into the PrP^{Sc} isoform that involves a conformational change (Prusiner, 1994). We previously showed that the 37kDa/67kDa laminin receptor (LRP/LR) acts as the cell surface receptor for the cellular PrP (Gauczynski *et al.*, 2001b). The findings that (i) BSE prions become internalized by human enterocytes via the LRP/LR (Feraudet *et al.*, 2005) and (ii) the binding of mouse PrP27-30 to mammalian cells is dependent on LRP/LR (Gauczynski *et al.*, submitted) suggests that LRP/LR acts as a receptor for infectious prions. Heparan sulfate proteoglycanes (HSPGs) act as co-factors/co-receptors for PrP^{c} (Hundt *et al.*, 2001). Recently, we demonstrated that accumulation of PrP^{Sc} in scrapie-infected neuronal cells ($N2aSc^{+}$) can be prevented by (i) the transfection of a antisense LRP RNA-expression plasmid, (ii) transfection with small interfering RNAs (siRNAs) specific for LRP mRNA and (iii) incubation with the anti-LRP/LR antibody W3 (Leucht *et al.*, 2003).

Various isoforms corresponding to different maturation states of the non-integrin laminin receptor LRP/LR have been isolated from mouse brains and found to bind to PrP (Simoneau *et al.*, 2003). Therefore, a physiological role of the laminin receptor/PrP interaction in the brain and its relevance for transmissible spongiform encephalopathies is suggested.

At present, there is no approved therapy for prion diseases. Several anti-prion agents exist, including polyphenol inhibitors, anti-malaria drugs, antihistamines or phenothiazine derivates (Kocisko *et al.*, 2003). Chemically designed compounds such as heparan sulfate mimetics were shown to abolish prion

propagation (Adjou *et al*., 2003). Anti-PrP antibodies can antagonize prion propagation *in vitro* (Peretz *et al*., 2001) and *in vivo* (Heppner *et al*., 2001) which is probably due to a global effect on PrP trafficking and/or transconformation processes (Feraudet *et al*., 2005). A trans-dominant negative mutant approach targeting PrP^{c} showed that the accumulation of PrP^{Sc} was blocked, raising the possibility to render animals and humans resistant to prion diseases (Hölscher *et al*., 1998).

Since we have shown that a transmembrane deletion mutant of LRP/LR (LRPdelTMD::FLAG), which lacks the proposed transmembrane domain of amino acid residues 86-101 (Castronovo *et al*., 1991), was secreted to the medium and prevented PrP^{c} binding and internalization in BHK21 cells (Gauczynski *et al*., 2001b), in this study we investigated the effect of a N-terminally truncated LRP mutant (LRP102-295::FLAG), which represents only the extracellular domain of the LRP/LR, in normal and scrapie-infected neuroblastoma cells (N2a/N2aSc^{+}). Cell culture experiments exhibit the secretion of the LRP102-295::FLAG mutant into the medium of both N2a and N2aSc^{+} cells. We show that secretion of LRP102-295::FLAG mutant is able to significantly decrease the binding of recombinant PrP to N2a cells. Furthermore, expression of the LRP102-295::FLAG mutant led to a reduced PrP27-30 binding to BHK cells and a reduction of the PrP^{Sc} accumulation in chronically scrapie-infected mouse neuroblastoma cells. Furthermore, we show that the mutant binds to both PrP^{c} and PrP^{Sc}, and conclude that the N-terminally truncated LRP102-295::FLAG mutant might have a trans-dominant negative effect and might have potential for the development of a TSE therapy.

Results

Secretion of the LRP102-295::FLAG mutant reduces binding of GST::huPrP to N2a cells

Western blot analysis of the crude lysate and the supernatant of N2a cells transfected with pCIneo-LRP102-295::FLAG and pCIneo-LRP::FLAG, respectively, revealed that the LRP102-295::FLAG mutant is secreted to the medium of N2a cells, whereas LRP::FLAG is detectable only in the cell lysates of N2a cells (Figure 1a). The LRP102-295::FLAG mutant with a calculated molecular weight of approx. 22.3 kDa, migrated with approx. 32kDa on a SDS polyacrylamide gel. Recently, we showed that a transmembrane deletion mutant of LRP/LR (LRPdelTMD::FLAG) was secreted to the medium and prevented PrP^{c} binding and internalization in BHK21 cells (Gauczynski *et al*., 2001b). Therefore, the possibility that the N-terminally truncated LRP102-295::FLAG mutant has the potency to interfere with PrP^{c} binding by intercepting PrP molecules before entering the cell was investigated using a cell binding assay. Here, transfected cells were incubated with recombinant GST::huPrP. Owing to the fact, that N2a and N2aSc^{+} cells exhibit endogenous LRP on the cell surface, the anti-FLAG antibody M2 was used to discriminate between endogenous LRP, LRP102-295::FLAG and LRP::FLAG, respectively. Quantitative analysis of western blot signals (Figure 1b) revealed a reduction of approximately 60% of GST::huPrP binding to N2a cells expressing the LRP102-295::FLAG mutant compared to LRP::FLAG expressing N2a cells (Figure 1c). To investigate whether

the LRP102-295::FLAG mutant can act as a decoy, we analyzed whether the endognous LRP level is influenced upon LRP mutant expression. The fact that the expression of LRP102-295::FLAG did not affect endogenous LRP levels (Figure 1a, middle panel) suggests that LRP102-295::FLAG might act in a trans-dominant negative manner.

LRP102-295::FLAG reduces the binding of PrP27-30 to BHK cells

To investigate the potential inhibitory effect of the LRP102-295::FLAG mutant on the binding of moPrP27-30 to BHK21 cells, which express low levels of endogenous LRP, we transfected SFV-LRP::FLAG and SFV-LRP102-295::FLAG RNA, respectively, into BHK21 cells resulting in expression of both corresponding proteins (Figure 2a, upper panel). BHK21 cells expressing LRP::FLAG exhibit higher PrP27-30 binding compared to SFV-1 transfected cells (control), confirming an involvement of LRP/LR in the binding of PrP27-30 (Figure 2b, c and Gauczynski *et al.*, submitted). In contrast, expression of LRP102-295::FLAG reduced PrP27-30 binding to approximately 40% (Figure 2c), suggesting that the mutant traps PrP27-30 molecules and prevents their binding and internalization.

Expression of the LRP102-295::FLAG mutant interferes with the PrP^{Sc} propagation in $N2aSc^{+}$ cells

To investigate whether the LRP102-295::FLAG mutant also interferes with PrP^{Sc} propagation in scrapie-infected N2a cells, $N2aSc^{+}$ were transfected with pCIneo-LRP102-295::FLAG and pCIneo-LRP::FLAG, respectively, resulting in the expression of LRP102-295::FLAG and LRP::FLAG (Figure 3a, upper panel). Whereas the mutant was secreted to the medium (Figure 3a, lower panel), LRP::FLAG has been solely detected cell associated. Ninety hours post transfection, PrP^{Sc} levels were reduced up to approximately 35% (mean approximately 45%) (Figure 3b, c). In contrast, expression of LRP::FLAG showed no significant effect on PrP^{Sc} levels compared to pCIneo (mock) transfected cells (Figure 3c). Furthermore, we analyzed PrP^{c} levels in $N2aSc^{+}$ cells transfected with the LRP102-295::FLAG mutant and LRP::FLAG, respectively, and found that cellular PrP levels remain unaffected in the presence of both LRP102-295::FLAG and LRP::FLAG (data not shown). This finding was expected since the endogenous LRP levels remain unaffected by expression of LRP::FLAG or LRP102-295::FLAG (data not shown).

LRP102-295::FLAG binds to GST::PrP^{c} and PrP27-30 in a cell free binding assay

To prove whether the LRP102-295::FLAG mutant is able to bind directly PrP^{c} and PrP27-30, a cell-free binding assay was performed. (i) GST::huPrP-coupled (Figure 4a) and (ii) PrP^{Sc}-coupled immuno-magnetic beads (Figure 4b) were incubated with the supernatant and cell lysates of transfected N2a

cells. Expression and secretion of the LRP102-295::FLAG mutant and expression of LRP::FLAG, respectively, was tested prior to the binding experiment (data not shown). Western blot analysis of bound protein complexes revealed a distinct binding of the LRP102-295::FLAG mutant, expressed in the cells (CL) and secreted from these cells (SN), to recombinant GST::huPrP (Figure 4a) and PrP^{Sc} (Figure 4b), respectively, whereas supernatants of pCIneo (mock) transfected N2a cells showed no binding to recombinant GST::huPrP and PrP^{Sc} (Figure 4a).

Discussion

The fact that the prion protein is the central determinant in prion diseases has led to many efforts to find a strategy for the treatment of TSEs. A variety of anti-prion compounds have been reported, mostly targeting the prion protein itself and interfering with the conversion of PrP^{c} into the pathogenic isoform PrP^{Sc}. Identification of the 37kDa/67kDa laminin receptor (LRP/LR) as a cell surface receptor for the cellular (Gauczynski *et al*., 2001b) and the infectious prion protein (Morel *et al*., 2005) opened a further direction for the development of new TSE-therapeutics.

Trans-dominant negative mutants targeting PrP have been investigated for their therapeutic potential in prion diseases (Kaneko *et al*., 1997) *In vivo* studies with transgenic mice expressing PrP with either the Q167R or Q218K mutation alone or in combination with wild-type PrP showed that expression of the dominant-negative PrP strongly reduced PrP^{Sc} formation (Perrier *et al*., 2002). Moreover, these dominant-negative PrP mutants were not converted into PrP^{Sc}. The effect of a non-glycosylphosphatidylinositol (GPI)-anchored recombinant PrP with a Q218K (rPrP-Q218K) mutation revealed that rPrP-Q218K inhibits the PrP^{Sc} replication in $N2aSc^{+}$ cells and was more efficient than quinacrine (Kishida *et al*., 2004). Given the knowledge that these mutations (Q167R, Q218K) are naturally occurring polymorphic variants of PrP that render resistance to prion disease, gene therapy with transdominant negative mutants might be a promising tool in the therapy of TSEs.

In this manuscript, we investigate a trans-dominant negative LRP/LR mutant and its effect on PrP^{c}, PrP^{Sc} binding and PrP^{Sc} propagation as well as its ability to interact directly with PrP^{c} and PrP^{Sc} as a prerequisite for its decoy function. We further analyze the influence of the mutant on the endogenous LRP/LR levels to proof its possible trans-dominant negative effect.

Secretion of the LRP102-295::FLAG mutant interferes with the binding of GST::huPrP and PrP27-30

In order to investigate a possible therapeutic effect of a LRP-mutant encompassing only the extracellular domain of the LRP/LR (LRP102-295::FLAG), we employed mouse neuroblastoma (N2a) cells as an *in vitro* model. Due to the fact that mouse neuroblastoma cells exhibit endogenous LRP on the cell surface (Gauczynski *et al*., 2001b), a possible decoy effect of the LRP102-295::FLAG mutant

can be investigated in this cell system. Mapping analysis identified amino acid residues 161-179 as a direct PrP-binding site on LRP (Hundt *et al*., 2001). Therefore, this binding site is still intact in the LRP102-295::FLAG mutant, so that a binding to cellular PrP is conceivable upon secretion to the cell culture medium.

The N-terminally truncated LRP102-295::FLAG mutant comprises the ability to interfere with the binding of recombinant huPrP (Figure 1) and PrP27-30 (Figure 2) to mammalian cells. Binding of GST::huPrP to N2a cells is not totally blocked by the secretion of the LRP102-295::FLAG mutant, presumably due to the presence of functional LRP/LR receptor molecules on the surface of N2a cells capable to bind to PrP. Moreover, we did not observe an enhancement of the binding of recombinant GST::huPrP upon expression of LRP::FLAG, which might be explained by the relative high expression level of endogenous LRP in these cells (Figure 1). Upon transfection, LRP levels remain unaffected, suggesting that transfection did not influence the binding capacity of GST::huPrP in LRP::FLAG expressing cells. In contrast, BHK cells express only low levels of endogenous LRP (Figure 2). Nevertheless, addition of PrP27-30 to SFV1-transfected cells (control) resulted in a weak binding signal (Figure 2). The recent finding that sulfated glycans such as heparan sulfate act as a cellular receptor for purified infectious prions (Horonchik *et al*., 2005) might explain this result. Thus, the binding of PrP27-30 to SFV1-transfected BHK cells might represent binding to surface heparan sulfates or heparan sulfate proteoglycanes (HSPGs) and to endogenous LRP/LR. Recent experiments demonstrate that the binding of mouse PrP27-30 to LRP::FLAG expressing BHK cells can be blocked by preincubation of the cells with the LRP/LR specific antibody W3, demonstrating an LRP/LR-specific binding of the scrapie prion protein (Gauczynski *et al*., submitted).

In BHK cells, hyperexpression of full-length LRP::FLAG enhanced the binding of recombinant GST::huPrP compared to SFV1-transfected cells (Figure 2). These findings are consistent with the publication by Morel *et al*., showing that bovine prions are endocytosed by human enterocytes via the 37kDa/67kDa LRP/LR (Morel *et al*., 2005). In contrast, BHK cells expressing the LRP102-295::FLAG mutant revealed a significant reduced binding of PrP27-30 compared to LRP::FLAG hyperexpressing cells (Figure 2), demonstrating that the LRP102-295::FLAG mutant can interfere with the PrP27-30 binding *in vitro*. BHK cells reveal a weaker expression of the LRP102-295::FLAG mutant compared to N2a cells, which might be explained by an intracellular regulatory process within BHK cells. The observation that different LRP102-295::FLAG expression levels in N2a and BHK cells caused approximately 60% reduction of the PrP^c (N2a) and the PrP27-30 binding (BHK), respectively, might be due to different amounts of GST::huPrP (20 μg) and PrP27-30 (2 μg), respectively, utilized in the binding assays. Therefore, an approximately 10 fold reduced LRP102-295::FLAG expression level in BHK cells compared to N2a cells resulted in approximately the same reduction of PrP binding since also 10 fold less PrP27-30 was utilized in BHK cells compared to N2a cells. The fact that the secreted LRP102-295::FLAG mutant efficiently reduces GST::huPrP binding to

N2a cells (Figure 1 b), which encompass high levels of LRP (Figure 1 a), suggests that the mutant binds more efficiently to PrP as the endogenous LRP, confirming its trans-dominant negative capacity. Taken together, these results show that the LRP102-295::FLAG mutant interferes with the binding of both PrP^{c} and PrP27-30 to mammalian cells and confirm that LRP/LR acts as a receptor for cellular (Gauczynski *et al*., 2001b) and infectious prions (Gauczynski *et al*., submitted; Morel *et al*., 2005).

LRP102-295::FLAG hampers PrP^{Sc} propagation in neuroblastoma cells

We showed that expression of the LRP102-295::FLAG mutant hampers the PrP^{Sc} propagation in scrapie-infected neuronal cells ($N2aSc^{+}$) (Figure 3). Since we demonstrated that expression of the LRP102-295::FLAG mutant in BHK cells hampers PrP^{Sc} binding (Figure 2), we suppose that $N2aSc^{+}$ cells might loose PrP^{Sc} due to the trapping effect of the mutant in the extracellular space acting in a trans-dominant negative fashion and preventing PrP^{Sc} binding and internalization. In addition, considerable amounts of the mutant were detected cell associated possibly due to a trapping mechanism in the cytosol or an unspecific binding to the cell (Figure 1a, 3a). Although the mutant is capable to bind to PrP^{c} (Figure 4), intracellular expression of the mutant, however, did not influence PrP^{c} levels (data not shown), suggesting no interference of the LRP mutant with the cellular PrP metabolism.

We cannot exclude, however, that the intracellularly expressed LRP mutant might bind to PrP^{Sc} within the endocytic pathway trapping here PrP^{Sc} molecules and enhancing the trapping effect of the mutant in the extracellular space. Finally, the mutant might interfere with the PrP^{c}/PrP^{Sc} conversion process which occurs either at the cell surface or within endocytotic compartments via binding to PrP^{c} and PrP^{Sc}.

LRP102-295 ::FLAG might act in a trans-dominat negative fashion by trapping cellular and scrapie prion proteins

Mouse neuroblastoma (N2a) cells express LRP and PrP endogenously and *in vitro*-assays revealed a LRP/LR-dependent binding and -internalization of recombinant huPrP in mouse neuroblastoma cells (Gauczynski *et al*., 2001b). The LRP102-295::FLAG mutant is secreted to the extracellular space and might trap exogenously added recombinant huPrP, resulting in a reduced binding of huPrP to the cell surface, as shown in Figure 1. In scrapie-infected neuroblastoma cells, secreted LRP102-295::FLAG interferes with PrP^{Sc} propagation by trapping PrP^{Sc} molecules (Figure 3), circumventing their binding to the cell surface. The latter hypothesis was confirmed by our finding that secretion of the LRP102-295::FLAG mutant strongly reduced binding of PrP27-30 to BHK cells (Figure 2).

To support the model of a trans-dominant negative inhibition of the LRP102-295::FLAG mutant, the binding ability of the mutant to both PrP^{c} and PrP^{Sc} was investigated employing a cell-free binding assay. Incubation of LRP102-295::FLAG containing supernatants with immunomagnetic beads

coupled to recombinant huPrP and PrP27-30, respectively, revealed a direct binding of the LRP102-295::FLAG mutant to both huPrP and PrP27-30 (Figure 4). The direct binding of LRP::FLAG to PrP27-30, which we demonstrated in this manuscript for the first time, is supported by the finding that BSE prions and LRP/LR co-localize in early endosomes of human enterocytes (Morel *et al*., 2005). The proof of direct interactions between the LRP mutant and PrP^{c} and PrP27-30, respectively, suggests that the mutant might act in a trans-dominant negative manner as a decoy by trapping both PrP^{c} (Figure 5b) and PrP^{Sc} molecules (Figure 5c) and prevent their binding and internalization. The LRP mutant might also trap PrP^{Sc} molecules within the endocytic pathway and might interfere with the PrP^{c}-PrP^{Sc} conversion process either on the cell surface or within endocytic compartments.

In conclusion, the ability of the LRP102-295::FLAG mutant (i) to interfere with PrP^{c} and PrP^{Sc} binding and (ii) to reduce PrP^{Sc} propagation in scrapie propagating cells recommends the mutant which might act in a trans-dominant negative manner as a alternative promising tool for the treatment of prion diseases.

Materials and Methods

Recombinant pCIneo Plasmid Constructions. The PCR method was used to amplify the mouse laminin receptor (moLRP) using the pSVF1-LRP::FLAG (Gauczynski *et al*., 2002) plasmid DNA as template. NotI- and NheI- restriction sites were used to subclone the moLRP::FLAG into the pCIneo mammalian expression vector. To generate the pCIneo-LRP102-295::FLAG plasmid, the C-terminal part of the mouse laminin receptor was amplified by PCR using the pCIneo-moLRP::FLAG plasmid DNA as template. The correctness of LRP102-295::FLAG was proven by agarose gel electrophoresis and confirmed by dideoxy sequencing. The plasmid DNA pCIneo-LRP102-295::FLAG was amplified in *Escherichia coli* and purified by using a plasmid maxi kit (Qiagen) according to the manufactures instructions and stored at –20°C.

Recombinant pSFV1 Plasmid Constructions. The pSFV1-LRP::FLAG plasmid was constructed as described previously (Gauczynski *et al*., 2002). The LRP102-295::FLAG fragment was amplified by PCR using pSFV1-LRP::FLAG plasmid DNA as a template and subcloned into the pSFV1 vector via BamHI and SmaI restriction sites. The cloning was confirmed by dideoxy sequencing. The plasmid DNA LRP102-295::FLAG was amplified in *E.coli* and purified by using a plasmid maxi kit (Qiagen) according to the manufacturer's instructions.

Cell cultures. Mouse neuroblastoma cells (N2a) were grown in DMEM with GlutaMAX™I (Gibco) containing 10% fetal calf serum (Gibco), 1x MEM non-essential amino acids (Gibco), 100U/ml penicillin and 100µg/ml streptomycin (Gibco) at 37°C in 5% CO_2. Scrapie-infected mouse neuroblastoma cells ($N2aSc^{+}$) were provided by S. Lehmann. $N2aSc^{+}$ cells were cultured in DMEM with GlutaMAX™I containing 10% fetal calf serum, 1x MEM non-essential amino acids, 100 U/ml

penicillin, 100 µg/ml streptomycin and 0.5 mg/ml geneticin (Invitrogen) at 37°C in 5% CO_2. Baby hamster kidney cells (BHK21) were cultured in DMEM with GlutaMAX™ containing 10% fetal calf serum, 1x MEM non-essential amino acids, 100U/ml penicillin and 100µg/ml streptomycin at 37°C in 5% CO_2. During transfection cells were always cultured in their normal growth medium omitting penicillin and streptomycin (transfection medium).

***In vitro* transcription and cell transfections (SFV-System).** DNAs pSFV1, pSFV1-LRP::FLAG and pSFV1-LRP102-295::FLAG were linearized with SpeI following purification by phenol-chloroform extraction. Transcriptions were carried out in a total volume of 50 μl containing 1.5 μg linearized plasmid DNA, 10x SP6 transcription buffer (0.4 M Tris-HCl, pH 8.0 at 20°C; 60 mM $MgCl_2$; 100 mM dithiothreitol; 20 mM spermidine), 1 mM of each ATP, CTP and UTP, 500 μM of GTP, 1 mM of m7G(5')ppp(5')G, 50 units of RNasin and 50 units of SP6 RNA polymerase and incubated for 2 hours at 37°C. The correct length of the transcripts was verified by agarose gel electrophoresis.

Transfections of BHK21 cells using recombinant SFV1 RNAs were performed according to Gauczynski *et al*. (Gauczynski *et al.*, 2002). Transfection efficiency as determined by transfecting SFV3-lacZ RNA followed by X-gal staining was approximately 100%.

Cell transfections with pCIneo plasmids. The day before transfection, cells (N2a, N2aSc$^+$) were plated in normal growth medium in a 6-well tissue culture test plate to a confluency of 50%. On the day of transfection the normal growth medium was removed and replaced with 1 ml of transfection medium. Both N2a and N2aSc$^+$ cells were transfected with pCIneo, pCIneo-LRP::FLAG and pCIneo-LRP102-295::FLAG, respectively, using the Geneporter™2 transfection reagent (Peqlab) in accordance with the manufacturer's instructions. Transfection efficiency using pCIneo plasmids was approximately 60%.

Preparation of supernatants and cell lysates. 72-90 hours post transfection the supernatant and the crude cell lysate of N2a, N2aSc$^+$ and BHK21 cells were collected.

The supernatant was centrifuged for one minute at 40,000 g followed by protein concentration by overnight methanol-precipitation at –20°C. Precipitated proteins were centrifuged for 10 minutes at 40,000 g and pellets were resuspended in 5x SDS-loading buffer, boiled for 5 minutes and subjected to SDS-PAGE. Cells were washed with D-PBS (Invitrogen) and lyzed for 10 minutes at 4°C in lysis buffer (10 mM Tris/ HCl pH 7.5 containing 100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P40 and 0.05% sodium deoxycholat) and centrifuged for 5 minutes at 40,000 g to obtain the crude cell lysate that was mixed with 5x SDS-loading buffer, boiled for 5 minutes and applied on a polyacrylamide gel. For detection of PrP27-30 (N2aSc$^+$ cells) samples were digested with 20 μg/ml Proteinase K of total protein for 30 minutes at 37°C. The digestion was stopped by adding phenylmethylsulfonylfluoride (PMSF, 2 mM) for 5 minutes at room temperature and subsequently, PK-resistant proteins were precipitated by methanol at –20°C overnight. Samples were then centrifuged for 10 minutes at 40,000

g and pellets were resuspended in 5x SDS-loading buffer, boiled for 5 minutes and loaded on a 12% polyacrylamide (PAA) gel.

Western blotting. Proteins were separated by SDS-PAGE and blotted on a polyvinylidenedifluoride (PVDF) membrane (Peqlab). Membranes were blocked with a solution containing 20% horse serum in TBS/0.2% Tween20 for at least 1 hour, and incubated overnight with the appropriate primary antibody (diluted in 5% horse serum in TBS/0.2% Tween20) at 4°C followed by incubation with the suitable secondary antibody for 1 hour. For detection of FLAG-tagged proteins, the monoclonal anti-flag M2 antibody (1:5,000) was used. Endogenous LRP levels were detected using the LRP-specific single chain antibody N3 (1:1000) and the polyclonal anti-LRP antibody W3 (1:5000), respectively. Detection of PrP27-30 was carried out using anti-PrP antibodies A7 (1:5,000) and SAF83 (1:5,000), respectively. β-Actin was detected using the monoclonal anti-β-actin antibody (1:5,000). Blots were developed using an enhanced chemiluminescence system (Perkin Elmer) and exposed on chemiluminescense films (Hyperfilm, Amersham). Stripping of the membranes was carried out using stripping buffer (100 mM glycine, 1% SDS, 0.1% Nonidet P40; pH 2.2) followed by blocking as described and incubation with the sufficient antibody. Quantification of the Western Blot signals were performed by densitometric measurements using the NIH-software.

Generation of recombinant prion protein (GST::huPrP). Recombinant GST::huPrP 23-230 was generated as described previously (Gauczynski *et al.*, 2001b) and expressed in the baculovirus system. Proteins were purified to homogeneity as described for hamster GST::PrP fusions and dialyzed against PBS. Until usage recombinant GST::huPrP was stored at –20°C.

GST::huPrP cell binding assay. Seventy two hours post transfection, recombinant GST::huPrP (20 μg) was added to N2a cells. After 16-19 hours of incubation at 37°C in 5% CO_2, supernatants were collected. Cells were washed twice with D-PBS (Invitrogen) and cell lysates were prepared following western blot analysis.

Preparation of murine PrP27-30. PrP27-30 (PrP27-30 cell binding assay) was extracted from 20% mouse brain homogenate derived from 6PB4 (mouse-adapted BSE strain) infected mice (C.Lasmézas, Fontenay-aux-Roses, France). Brain homogenates were Proteinase K digested (20 μg/ml, 30 minutes, 37°C) followed by stopping the reaction with 2 mM PMSF (5 minutes, room temperature). After density gradient centrifugation (10% sarcosyl in 10 mM Tris/HCl pH 7.5) for 4 hours, the pellet was dried and sonified in 50 mM Hepes pH7.5. Different sonication steps were necessary to dissolve the pellet completely. To determine the PrP27-30 concentration a BCA protein assay (Perbio) was used.
For the cell-free binding assay PrP27-30 was isolated from 10% mouse brain homogenate derived from RML (mouse-adapted scrapie strain) and Proteinase K digested as described previously followed by a methanol precipitation. After centrifugation (40,000 g, 10 minutes) pellets were resuspended in 5x SDS-loading buffer, boiled for 5 minutes and subjected to SDS-PAGE.

PrP27-30 cell binding assay. Twenty-four hours post transfection, PrP27-30 (2 μg) was added to the culture growth medium of BHK21 cells and incubated for 16-19 hours at 37°C in 5% CO_2. Cells were washed twice with D-PBS (Invitrogen) and cell lysates were prepared as described and analyzed by western blot.

Cell-free binding assay (Dynabeads). Immunomagentic beads (Dynabead®ProteinG, Dynal Biotech; 50 μl per reaction) were coupled with polyclonal anti-PrP antibody A7 for 30 minutes at room temperature. For isolation of A7-coupled beads, the test tubes were placed in the Dynal Magnetic Particle Concentrator (MPC) for 2 minutes. During this time immunomagnet beads were attracted to the wall of the test tube. Supernatants were removed while the beads were held in place by the magnetic field of the Dynal MPC. Test tubes were removed from the magnet and beads were resuspended in 100 μl PBS and reinserted into the Dynal magnet. Binding of recombinant GST::huPrP (2 μg) and PrP27-30 (50 μg), respectively, to A7-coupled beads was performed for 1 hour at 4°C. Supernatant (3 ml) and crude cell lysate (400 μg), respectively, from transfected N2a cells was then added to the complex and incubated for 1 hour at 4°C. After each incubation step, PBS washing was carried out three times. Finally, beads were resuspended in 30 μl SDS-sample buffer and protein complexes were released from the immunomagnetic beads by incubation at 95°C for 5 minutes. Test tubes were placed into the MPC and supernatants were removed and subjected to SDS-PAGE. Immunomagnetic beads were washed three times in 250 mM sodium acetate pH 5.0 and were stored at 4°C for re-use.

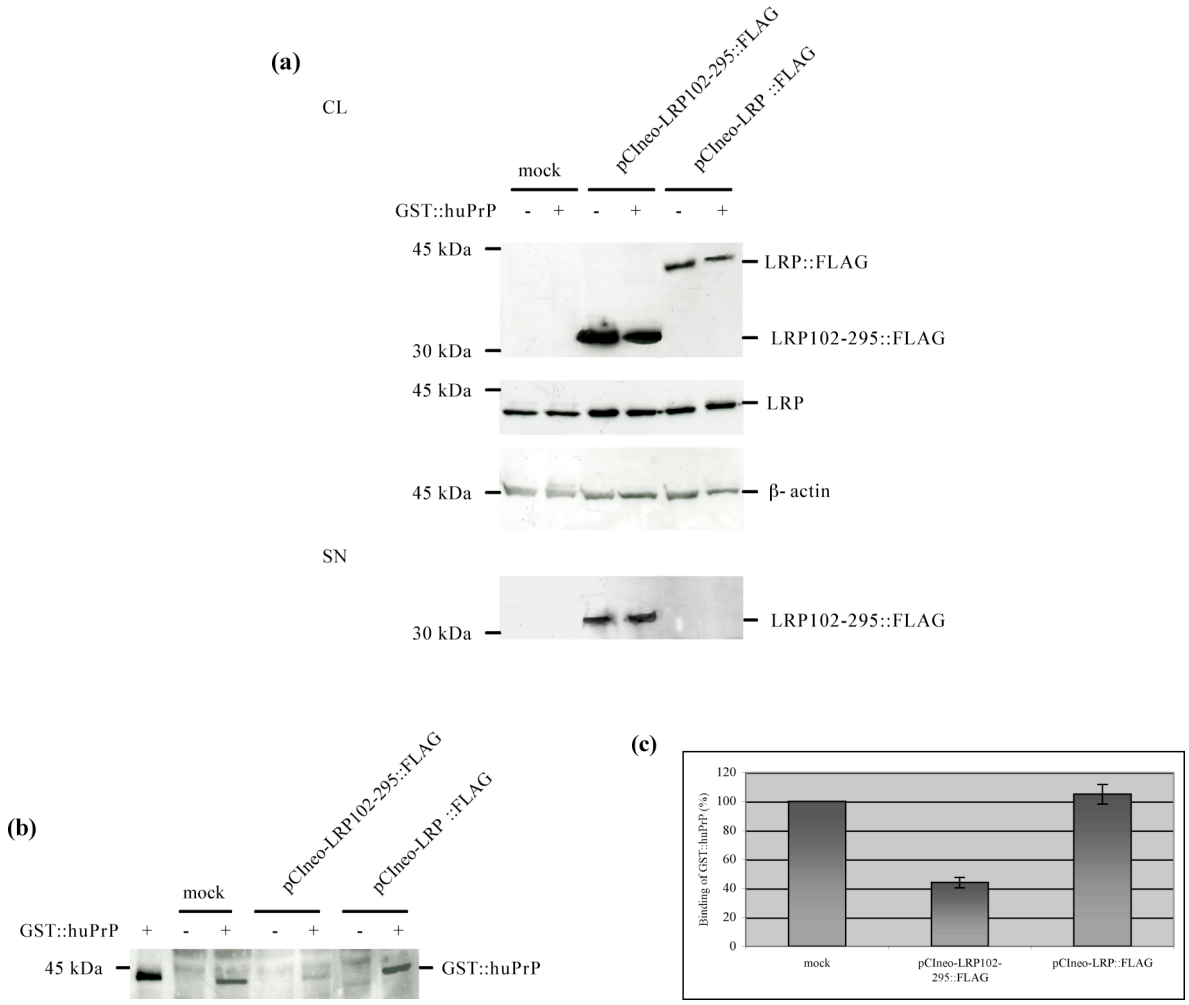

Figure 1. Effect of the LRP102-295::FLAG mutant on the binding of GST::huPrP to N2a cells. (a) Western blot of transfected N2a cells. Ninety hours post transfection supernatants (SN) and crude cell lysates (CL) were prepared and loaded on a 12% PAA gel. LRP102-295::FLAG and LRP::FLAG, respectively, were detected using monoclonal anti-flag M2 antibody. The blot was stripped and re-probed with the single chain antibody N3 to detect the cellular LRP/LR level. In the lower panel the blot was incubated with the monoclonal anti-β-actin antibody. Molecular weight markers in kilodalton (kDa) are indicated on the left. (b) The day after transfection 20 μg recombinant GST::huPrP was added to the cell culture medium and incubated overnight. Cell lysates were prepared and western blot analysis were performed using the anti-PrP 3B5 antibody. Figures were digitally arranged. (c) Densitometry analysis using the NIH IMAGE software. The results of three individual experiments are shown and expressed as a percentage of control levels ±s.e.m.

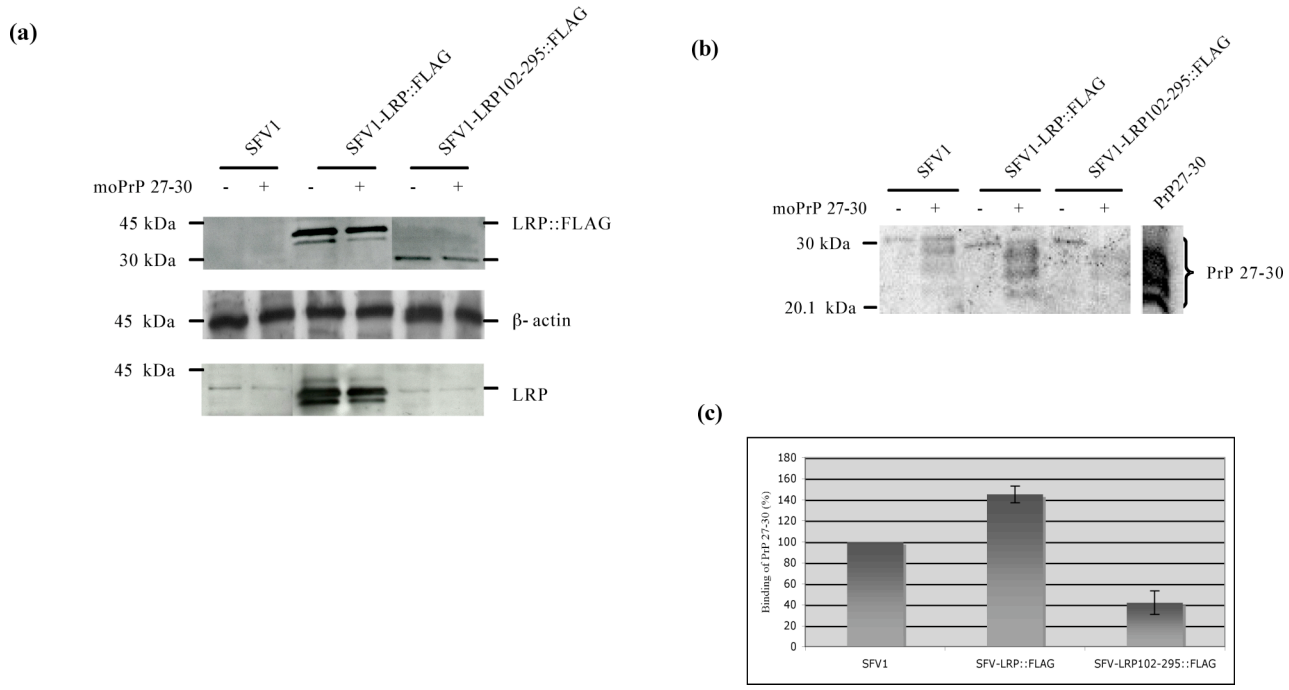

Figure 2. Effect of LRP102-295::FLAG mutant on the binding of PrP27-30 in BHK21 cells. (a) Cells were transfected with SFV1, SFV1-LRP::FLAG and SFV1-LRP102-295::FLAG, respectively, and cell lysates were analyzed by western blotting. Expression of LRP::FLAG and LRP102-295::FLAG was detected using the monoclonal anti-flag M2 antibody (upper panel). Endogenous LRP levels were detected using the polyclonal anti-LRP/LR antibody W3 (lower panel). β-actin was used as a loading control (middle panel). Molecular weight markers in kilodalton (kDa) are indicated on the left. (b) Twenty four hours post transfection, PrP27-30 was added to the cells and after overnight incubation lysates were analyzed by western blotting. PrP27-30 was detected using polyclonal anti-PrP A7 antibody. The three-band pattern represent the di-, mono- and unglycosylated form of PrP. As a positive control 5 μg purified PrP27-30 was loaded. Figures were digitally arranged. (c) Densitometry analysis using the NIH IMAGE software. The binding of PrP27-30 to SFV1-transfected BHK cells (control) was set to 100% to illustrate the effect of the LRP102-295::FLAG mutant. The results of three individual experiments are shown and expressed as a percentage of control levels ±s.e.m.

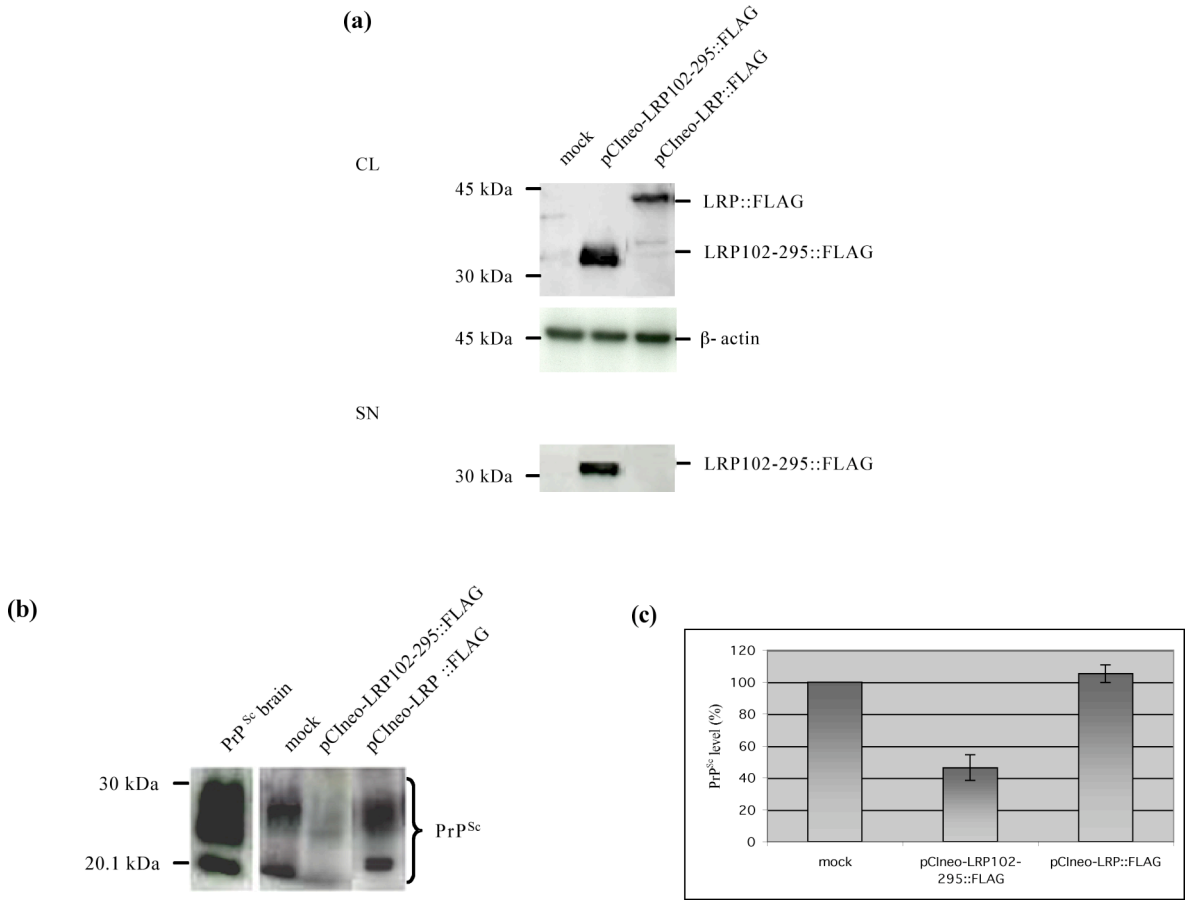

Figure 3. Influence of the LRP102-295::FLAG mutant expression on the PrP^{Sc} propagation in $N2aSc^{+}$ cells. (a) Representative western blot of transfected $N2aSc^{+}$. Ninety hours post transfection, supernatants (SN) and crude cell lysates (CL) were prepared. LRP102-295::FLAG and LRP::FLAG, respectively, were detected using monoclonal anti-flag M2 antibody. β-actin was used as a loading control. Molecular weight markers in kilodalton (kDa) are indicated on the left. (b) PK-digested cell lysates (150 μg) were subjected to SDS-PAGE and PrP^{Sc} was detected using SAF83 antibody. Figures were digitally arranged. (c) Densitometry analysis employing NIH IMAGE software. The results of four independent experiments are shown and expressed as a percentage of control levels ±s.e.m.

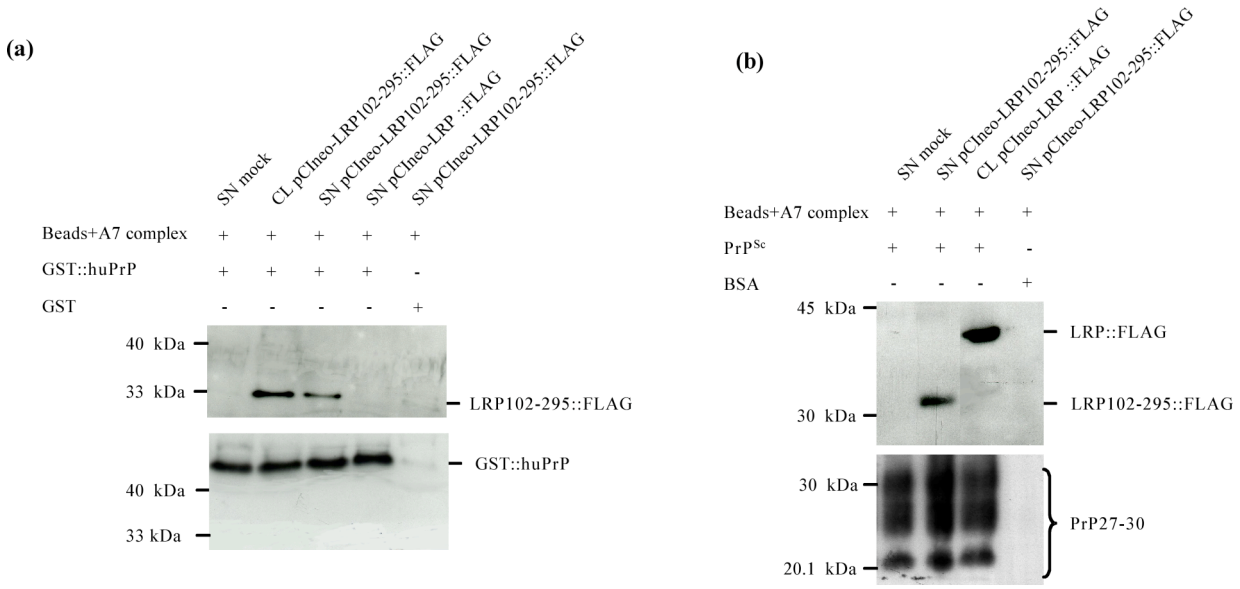

Figure 4. Cell-free binding assay of LRP102-295::FLAG mutant to GST::huPrP and PrP27-30. Polyclonal anti-PrP-antibody A7-coupled immunomagnetic beads were bound to (a) recombinant GST::huPrP and (b) to PrP27-30 and incubated with supernatants (SN) and cell lysates (CL) of transfected N2a cells. Expression and secretion of LRP102-295::FLAG mutant and expression of LRP::FLAG, respectively, was checked prior to the experiment. Loaded beads were isolated by using a magnetic field and bound proteins were released from the beads by a denaturation step at 95°C. Protein complexes were loaded on a 12% PAA gel and analyzed by western blotting. LRP102-295::FLAG and LRP::FLAG, respectively, were detected using monoclonal anti-flag M2 antibody (upper panels). The blots were stripped and re-probed with (a) monoclonal anti-PrP antibody 3B5 and (b) anti-PrP SAF83 antibody to detect the recombinant GST::huPrP and PrP27-30, respectively. Molecular weight markers in kilodalton (kDa) are indicated on the left.

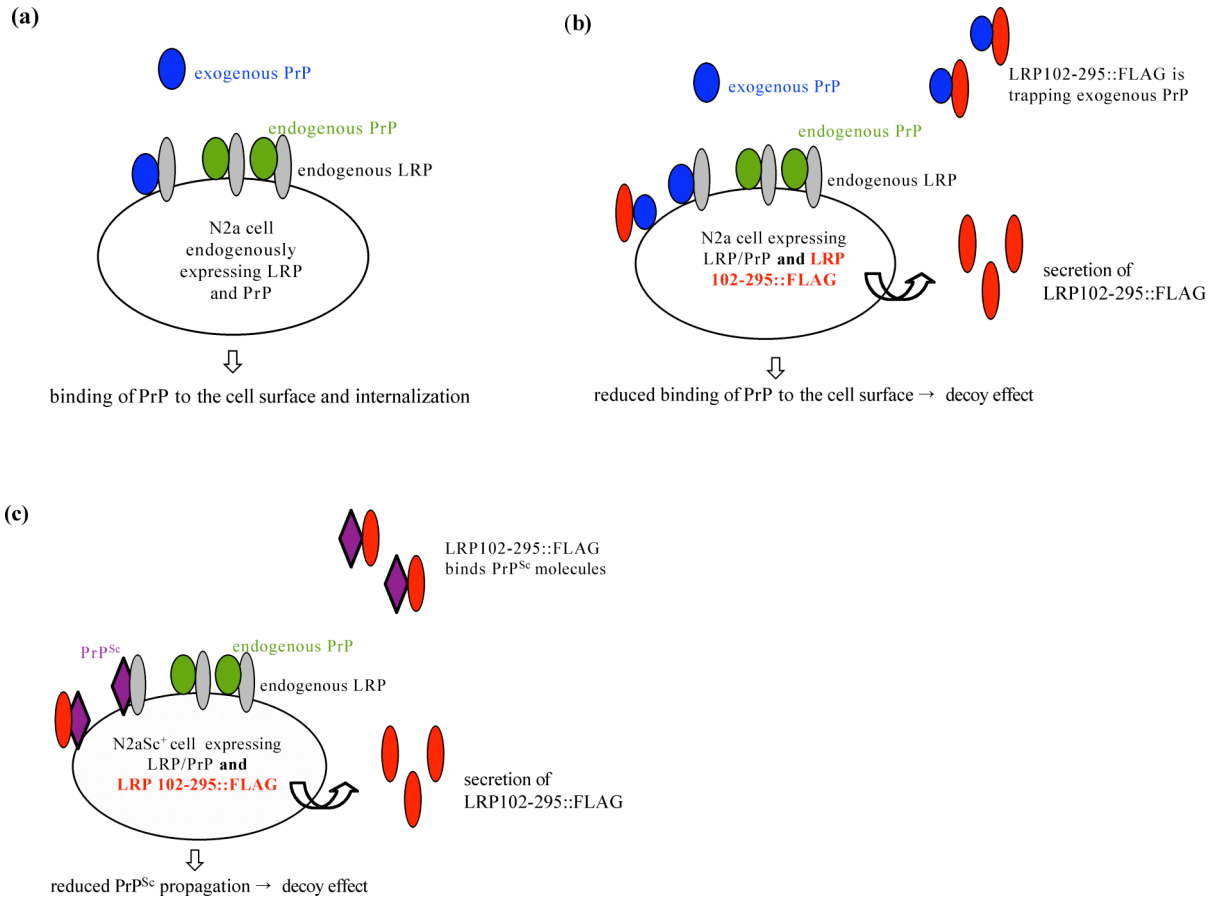

Figure 5. Model for the mode of action of the LRP 102-295::FLAG mutant. (a) Mouse neuroblastoma (N2a) cells express LRP/LR and PrP endogenously on the cell surface. Addition of recombinant exogeneous PrP (GST::huPrP) results in binding and internalization of PrP (Gauczynski *et al.*, 2001). (b) Upon transfection, the N-terminally truncated LRP 102-295::FLAG mutant, representing the extracellular domain of LRP/LR, secretes to the cell culture medium. Addition of exogenous PrP (GST::huPrP) results in a reduction of PrP binding to N2a cells due to the trapping of exogenous PrP by the LRP 102-295::FLAG mutant („decoy effect"). (c) Scrapie-infected mouse neuroblastoma ($N2aSc^+$) cells express LRP/LR and PrP on the cell surface. Following transfection, the LRP 102-295::FLAG mutant secretes to the cell culture medium. We hypothesize that the LRP 102-295::FLAG mutant is able to interfere with the PrP^{Sc} propagation by trapping free PrP^{Sc} molecules before entering the cell ("decoy effect"). The LRP mutant might in addition trap PrP^{Sc} molecules within compartments of the endocytic pathway and might hamper the PrP^{c}-PrP^{Sc} conversion process either on the cell surface or within endocytic compartments.

CHAPTER VI

Generation of transgenic mice expressing a LRP decoy mutant as a therapeutic *in vivo* approach in prion diseases

submitted as:

Vana K. and Weiss S. (2006)

Generation of transgenic mice expressing a LRP decoy mutant as a therapeutic *in vivo* approach in prion diseases. *Transgenic Res*.

Abstract

The 37kDa/67kDa laminin receptor (LRP/LR) was identified to act as a cell surface receptor for prion proteins. *In vitro* studies demonstrated that the LRP102-295::FLAG mutant reduced the binding of both recombinant cellular huPrP and infectious PrP27-30 and interfered with the PrP^{Sc} propagation in mouse neuronal cells. These results indicate that the LRP102-295::FLAG mutant may act in a trans-dominant negative manner as a decoy by trapping PrP molecules. In order to test the potential of the LRP mutant *in vivo* in respect of delay or prevention of prion disease, transgenic animals were generated expressing LRP102-295::FLAG ectopically in the brain. Animals showed no phenotype and transgene expression was detected in cortical and cerebellar brain regions. An intracerebral prion inoculation of these mice will prove, if the expression of the LRP102-295::FLAG mutant can impair the PrP^{Sc} accumulation in the brain and thus, can act as an alternative therapeutic tool in prion diseases.

Prion diseases are neurodegenerative disorders found in mammals, naturally in many ruminants (scrapie, BSE), deer (CWD) and mink (TME), as well as humans (Aguzzi and Weissmann, 1998; Prusiner *et al.*, 1998; Weissmann, 1999) and are characterized by the accumulation of an abnormal, partially protease-resistant isoform of the cellular prion protein (PrP^{c}). The infectious isoform of the prion protein (PrP^{Sc}) tend to aggregate in brains of affected humans and animals (McKinley *et al.*, 1991). All prion diseases show long incubation periods but are typically rapidly progressive. In the CNS, neuropathological changes, including spongiosis, astrogliosis and neuronal loss (Fraser, 1976), are prominent features of prion diseases.

The 37kDa/67kDa laminin receptor (LRP/LR) was identified as the cell surface receptor for cellular PrP (Gauczynski *et al.*, 2001b; Hundt *et al.*, 2001) and for infectious prions (Morel *et al.*, 2005). LRP/LR is implicated in a wide variety of biological processes including neuronal cell adhesion, differentiation, migration and neurite outgrowth and it has been shown that several isoforms of the LRP/LR are present in mouse brain (Simoneau *et al.*, 2003). Recently, we demonstrated that the LRP/LR is required for the prion propagation in scrapie-infected neuronal cells (Leucht *et al.*, 2003) and showed that secretion of LRP102-295::FLAG mutant, which represents the extracellular domain of the LRP/LR, led to a reduction of the PrP^{Sc} accumulation in chronically scrapie-infected neuroblastoma cells (Vana and Weiss, 2006). Moreover, the LRP102-295::FLAG mutant reduced the binding of PrP^{c} and PrP27-30 to cells, indicating that the LRP102-295::FLAG mutant may act in a trans-dominant negative manner as a decoy by trapping PrP molecules (Vana and Weiss, 2006). In addition, the finding that bovine prions are endocytosed in a LRP/LR dependent manner (Morel *et al.*, 2005) raised the possibility that the use of LRP/LR as a therapeutic target represent an alternative strategy in the development of a TSE treatment.

Here we report on the generation of hemizygous transgenic mice expressing the LRP102-295::FLAG mutant ectopically in mouse brain under the control of the rat neuron specific enolase (NSE) promoter to investigate the decoy effect of the LRP mutant *in vivo*. Assuming that the NSE promoter-containing construct is more efficient in various neuronal cell types than in non-neuronal tissues (Leucht *et al*., 2004) we concentrated on the analysis of distinct parts of the central nervous system (CNS). Previously, trans-dominant negative mutants targeting PrP (Q167R, Q218K) have been investigated for their therapeutic potential in prion diseases (Kaneko *et al*., 1997) and showed a strong effect on the reduction of PrP^{Sc} formation *in vivo* (Perrier *et al*., 2002). These studies encouraged us to use the trans-dominant negative LRP102-295::FLAG mutant of the prion protein receptor LRP/LR (Vana and Weiss, 2006) as a therapeutic approach *in vivo*.

*Nhe*I and *Not*I restriction sites were used to subclone the LRP102-295::FLAG (Vana and Weiss, 2006) into the pCIneo-NSE plasmid (Leucht *et al*., 2004) to generate the plasmid pCIneo-NSE-LRP102-295::FLAG. The pCIneo-NSE-LRP102-295::FLAG plasmid was digested using *Bgl*II and *BamH*I, purified by gel extraction (Genomed) following microinjection into pronuclei of mouse zygotes and transfer into oviducts of female recipient mice. The offspring were routinely screened by PCR using a specific LRP102-295::FLAG-specific primer pair to identify transgenic animals.
To analyze the expression of the LRP102-295::FLAG protein, cortical and cerebellar brain regions were homogenized using homogenization buffer (0.32 M sucrose, 10 mM HEPES pH 7.4, 2 mM EDTA plus protease inhibitors) and centrifuged at 750 x g for 15 minutes. Supernatants were subjected to SDS-PAGE followed by blotting the gel on a polyvinylidenedifluoride (PVDF) membrane (Peqlab). Membranes were blocked with a solution containing 20% horse serum in TBS/0.2% Tween20 and incubated with the monoclonal anti-flag M2 antibody (1:5000) and monoclonal anti-β-actin antibody (1:5000), respectively. Endogenous LRP and PrP levels were detected using the single chain anti-LRP antibody S18 (1:1000) and the anti-PrP antibody SAF32 (1:5000), respectively. Blots were developed using an enhanced chemiluminescence system (Perkin Elmer) and exposed on chemiluminescense films (Hyperfilm, Amersham). Stripping of the membranes was carried out using stripping buffer (100 mM glycine, 1% SDS, 0.1% Nonidet P40; pH 2.2).

Three transgenic lines (TgN(NSE_LRP102-295::FLAG)1, TgN(NSE_LRP102-295::FLAG) 2 and TgN(NSE_LRP102-295::FLAG) 3) were established (Figure 1 (A)) and the offspring were monitored for the expression of the LRP102-295::FLAG construct by PCR (Figure 1 (B), data not shown for TgN(NSE_LRP102-295::FLAG) 2 and TgN(NSE_LRP102-295::FLAG) 3). Transgenic LRP102-295::FLAG mice were compared with wild-type mice for variations in body size and body weight and showed no visible phenotype. Furthermore, behavioral screening tests were performed and resulted in

no obvious alterations. The expression of the LRP102-295::FLAG mutant in different brain areas was analyzed by western blotting. In TgN(NSE_LRP102-295::FLAG) 1 mice, transgene expression was detected in cortex and cerebellum (Figure 2, upper panel) to equal levels as expected (Forss Petter *et al*., 1990). In contrast, no expression of the LRP102-295::FLAG mutant was observed in non-transgenic littermates (data not shown). Analysis of endogenous PrP levels showed that expression of LRP102-295::FLAG in the brain of TgN(NSE_LRP102-295::FLAG) 1 mice resulted in a PrP^{c} upregulation in the cortical brain region compared to the cerebellum (Figure 2, middle panel). This is in contrast to our recent finding that the PrP^{c} level is unaltered upon expression of the LRP102-295::FLAG mutant in mouse neuronal cells (Vana and Weiss, 2006). This contradictory result might be explained by a complex regulatory mechanism of the PrP^{c} expression *in vivo*. In addition, due to the direct binding capacity of the LRP102-295::FLAG mutant to both PrP^{c} and PrP^{Sc} (Vana and Weiss, 2006), we cannot exclude that the mutant might trap cellular PrP molecules and thus, might enhance PrP synthesis.

Examination of endogenous LRP levels in TgN(NSE_LRP102-295::FLAG) 1 mice revealed no change in cortical and cerebellar brain areas upon expression of the LRP102-295::FLAG mutant (Figure 2, middle panel). This observation is consistent with our *in vitr*o results showing that the expression of LRP102-295::FLAG mutant did not affect endogenous LRP levels (Vana and Weiss, 2006). Intracerebral (i.c.) prion inoculation studies in these transgenic TgN(NSE_LRP102-295::FLAG) mice will verify, whether the LRP120-295::FLAG mutant has the ability to delay or prevent prion disease *in vivo*.

The development of transmissible spongiform encephalopathies in experimental animal models depends on two major factors: the intracerebral PrP^{Sc} accumulation and the existence of different strains of the agent. Previously, it was demonstrated that although intracerebellar prion injections gave the lowest yield of PrP27-30, the mice injected into the cerebellum had the shortest incubation period, reinforcing the proposal that only the strain of agent influence the pattern of distribution and the yield of PrP27-30 (Casaccia-Bonnefil *et al*., 1993). In addition, mouse-adapted scrapie-strains have been characterized showing clear late stage neuropathological differences (Cunningham *et al*., 2005). Nevertheless, *in vivo* prion infection studies will prove the hypothesis that the LRP mutant might act in a trans-dominant negative manner as a decoy by trapping PrP molecules and thus prevent their binding and internalization (Vana and Weiss, 2006).

In conclusion, the present manuscript showed successful transgenic LRP102-295::FLAG expression in specific mouse brain region using the NSE-promoter. Assuming that the CNS represent a major organ affected by prion diseases, the ectopic expression of a decoy LRP mutant in the brain might display an alternative strategy for the development of a TSE therapy.

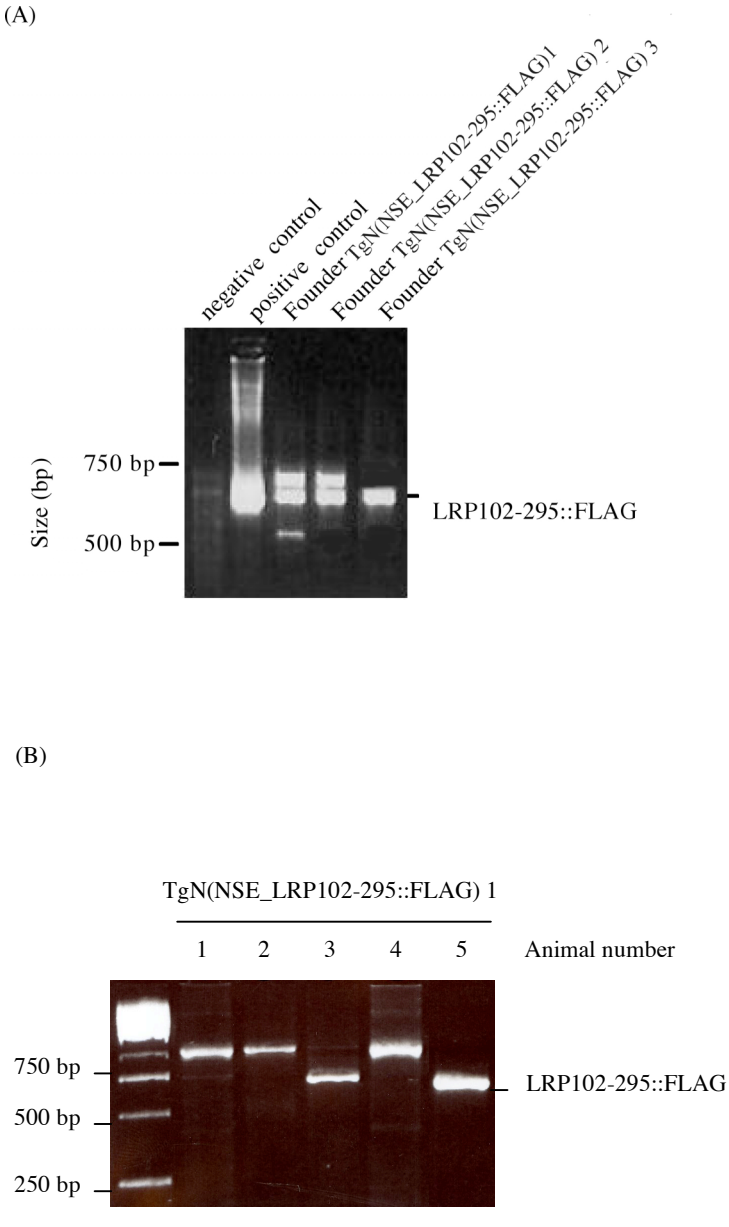

Figure 1. (A) PCR analysis of genomic DNA derived from founder animals of TgN(NSE_LRP102-295::FLAG) 1, TgN(NSE_LRP102-295::FLAG) 2 and TgN(NSE_LRP102-295::FLAG) 3. Genomic DNA was isolated from mouse tail samples and PCR was carried out using a specific primer pair for the transgen. This resulted in a 631bp DNA band detected on a 1.5% agarose gel. As a positive control, a vector containing LRP102-295::FLAG was used as a template for PCR reaction. Fragment size markers in basepairs (bp) are indicated on the left. (B) PCR analysis of genomic DNA derived from TgN(NSE_LRP102-295::FLAG) 1 offspring. Five animals were analyzed and two of them carried the LRP102-295::FLAG transgen as detected by the 631bp DNA band. Fragment size markers in basepairs (bp) are indicated on the left.

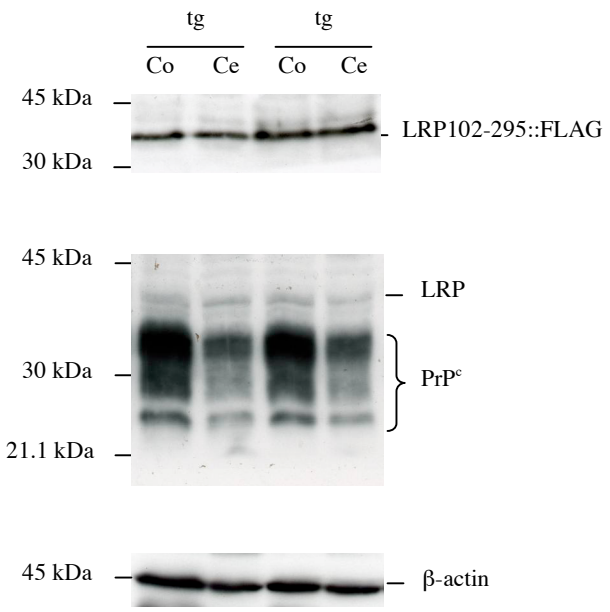

Figure 2. Representative Western blot of cortical (Co) and cerebellar (Ce) brain region preparations of TgN(NSE_LRP102-295::FLAG) 1 mice. LRP102-295::FLAG was detected using monoclonal anti-flag M2 antibody. The membrane was stripped and re-probed simultaneously with the single chain anti-LRP antibody S18 and the monoclonal anti-PrP antibody SAF32. β-Actin was used as a loading control. Molecular weight markers in kilodalton (kDa) are indicated on the left.

CHAPTER VII

Inhibition of PrP^{Sc} formation by LRP-specific siRNAs using a lentivirus-based RNAi delivery system

submitted as:

Vana K., Nikles D., Messow D. and Weiss S. (2006)

Inhibition of PrP^{Sc} formation by LRP-specific siRNAs using a lentivirus-based RNAi delivery system.

J.Virol.

The 37kDa/67kDa laminin receptor (LRP/LR) functions as a receptor for prions and represents a novel target for a therapeutic intervention in prion diseases. Here, we examined a therapeutic approach using lentivirus-based packaging of RNAi targeting the LRP mRNA. We report that VSV-G pseudotyped HIV-derived vectors expressing LRP-specific siRNAs strongly downregulate the cellular LRP level in different neuronal cell lines. In chronically scrapie-infected neuronal cells, we demonstrate that LRP downregulation leads to a reduction of prion-replication. Therefore, this study provides a promising therapeutic strategy for the treatment of prion diseases.

Introduction

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative disorders affecting animals and humans. TSEs are characterized by the accumulation of an "abnormal" pathogenic isoform (PrP^{Sc}) of the host-encoded cellular prion protein (PrP^{c}) in the brain of affected individuals (Prusiner *et al*., 1998). According to the "protein-only" hypothesis, the conversion of PrP^{c} to PrP^{Sc} is the pivotal event in the etiology of TSEs (Prusiner *et al*., 1990).

Since there is no efficient TSE-treatment available, great expenses have been driven to discover anti-prion drugs and to develop a vaccination against prion diseases. Due to possible side effects of drugs (e.g. toxicity, kidney damage, high blood pressure etc.) and the limitation of either passive or active immunization strategies, gene therapy approaches evolved.

Previously, experimental introduction of RNA was described as a system to interfere with the function of an endogenous gene later termed as RNA interference (RNAi). Long-lasting RNAi-mediated gene knockdown can be achieved using lentiviral vectors that express the siRNAs. Recently, in an animal model of amyotrophic lateral sclerosis (ALS), which is a neurological disease that attacks motor neurons, it has been shown that an intraspinal injection of lentiviral vectors led to a substantial delay in the onset and progression of the disease (Ralph *et al*., 2005; Raoul *et al*., 2005).). The advantage of lentiviral vectors lays in their ability to transduce dividing, non-dividing and terminally differentiated cells such as neurons (Blomer *et al*., 1997; Naldini *et al*., 1996b). Because neurons are the main target cells in prion diseases, the use of lentivirus-mediated RNAi displays a novel strategy to prolong disease or even prevent prion replication.

In order to evaluate a gene transfer therapeutic TSE strategy, we used lentiviral gene transfer, which has previously been reported to inhibit PrP^{Sc} formation in scrapie-infected neuroblastoma cells (Crozet *et al*., 2004).

In the present study, human immunodeficiency virus (HIV)-derived vectors that enable the expression of short hairpin RNA (shRNA) directed against the LRP mRNA were developed and characterized. Subsequently these vectors were applied onto scrapie-infected and non-infected mouse neuronal cells, respectively, and the effects on the cellular LRP levels were monitored. Furthermore, we examined the PrP^{Sc} level in scrapie-infected cells to investigate the influence of the LRP/LR downregulation on the

PrP^{Sc} propagation in neuronal cells. Our results demonstrate an efficient reduction of 37kDa/67kDa LRP/LR protein expression in neuronal cells upon both transient transfection of siRNA containing plasmids and genomic integration of lentiviral vectors. Downregulation of LRP gene expression in scrapie-infected N2a ($N2aSc^+$) cells resulted in a decrease of PrP^{Sc} levels in these cells, indicating an essential requirement of LRP/LR for prion propagation *in vitro*.

Materials and Methods

Cell lines. Mouse neuroblastoma (N2a) and 293FT cells were grown in DMEM with GlutaMAX™I (Invitrogen) supplemented with 10% fetal calf serum, 1x MEM non-essential amino acids, 100U/ml penicillin and 100µg/ml streptomycin (Invitrogen) at 37°C in 5% CO_2. 293FT cells are a fast-growing 293 cells that stably expresses the large T antigen of SV40. Scrapie infected mouse neuroblastoma cells ($N2aSc^+$) were provided by S.Lehmann. $N2aSc^+$ cells were cultured in DMEM with GlutaMAX™I containing 10% fetal calf serum, 1x MEM non-essential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.5 mg/ml geneticin (Invitrogen) at 37°C in 5% CO_2. Hypothalamic neuronal cells (GT1) were kindly provided by C.Lasmézas and were cultivated in OptiMEM (Invitrogen) containing 5% horse serum, 5% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen) at 37°C in 5% CO_2. HT1080 cells (human fibrosarcoma cells, ATCC No. CCL121) were cultivated in DMEM with GlutaMAX™I (Invitrogen) supplemented with 10% fetal calf serum, 1x MEM non-essential amino acids, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen) at 37°C in 5% CO_2.

During transfection all cells were cultured in their according growth medium omitting penicillin and streptomycin (transfection medium).

Generation of HIV-based lentiviral RNAi vectors. Using the BLOCK-it™ Lentiviral RNAi Expression System (Invitrogen), lentiviral RNAi vectors encoding siRNAs directed against the LRP mRNA were generated. Target sequences in the LRP mRNA for the RNAi were chosen according to the published guidelines (www.invitrogen.com). Double-stranded DNA-oligonucleotides were cloned into the pENTR/U6 vector (Invitrogen) to create an entry clone, which can be used to check the efficiency of gene silencing by transfection method. The replication-incompetent lentiviral vectors containing LRP mRNA-directed siRNAs were produced in 293FT cells according to the manufacturer's instructions. The viral vectors were concentrated (Burns *et al.*, 1993) and the vector titer of concentrated stocks was determined by crystal-violet staining of blasticidin selected colonies according to the manufacturer´s protocol.

Transfection. The day before transfection, cells were plated into their normal growth medium in a 6-well tissue culture test plate to a confluency of 50%. On the day of transfection the normal growth

medium was removed and replaced with 1 ml of transfection medium. Cells were transfected using the Geneporter™2 transfection reagent (Peqlab) in accordance with the manufacturer's instructions.

Virion production. VSV-G pseudotyped HIV-derived lentiviral vectors (LVV) expressing siRNAs directed against the LRP mRNA were produced in 293FT cells using BLOCK-iT™ Lentiviral RNAi Expression System (Invitrogen) according to the manufacturer's guidelines.

***In vitro* lentivirus transduction.** The day before transduction, target cells were plated into 6-well plates and were subsequently transduced with various multiplicities of infection (MOI) ranging from 0.1 to 10 in the presence of 6 μg/ml polybrene (Invitrogen). The culture media was changed 5 h to 24 h post transduction and cells were analysed 24 h to 72 h post transduction. To examine long-term influence of integrated siRNA coding sequences cells were treated with culture medium supplemented with 8 μg/ml blasticidin two days post transduction. Transduced cells were selected for 10 to 14 days in blasticidin containing culture medium to obtain stably transduced cell lines. Transduced cells were subsequently analyzed by Western Blot and preparation of genomic DNA as described below.

Preparation of cell lysates. Cells were washed with D-PBS (Invitrogen) and lyzed for 10 minutes at 4°C in lysis buffer (10 mM Tris/ HCl pH 7.5 containing 100 mM NaCl, 10 mM EDTA, 0.05% Nonidet P40 and 0.5% sodium deoxycholat) and centrifuged for 5 minutes at 14,000 g to obtain the crude cell lysate that was mixed with 5x SDS-loading buffer, boiled for 5 minutes and applied on a polyacrylamide gel. For detection of PrP^{Sc} samples were digested with 20 μg/ml Proteinase K of total protein for 30 minutes at 37°C. The digestion was stopped by adding phenylmethylsulfonylfluoride (2 mM) for 5 minutes at room temperature and subsequently, PK-resistant proteins were precipitated by methanol at –20°C. Then, samples were centrifuged for 10 minutes at 14000 g and the pellets were resuspended in 5x-SDS-loading buffer, boiled for 5 minutes and loaded on a 12% PAA gel.

Western blotting. Proteins were separated by SDS-PAGE and blotted on a polyvinylidenedifluoride (PVDF) membrane (Peqlab). Membranes were blocked with a solution containing 20% horse serum in TBS/ 0.1% Tween 20 for 3 hours, and incubated overnight with the appropriate primary antibody (diluted in 5% horse serum in TBS/ 0.1% Tween 20) at 4°C followed by incubation with the suitable secondary antibody (1:5,000) for 1 hour. Endogenous LRP levels were detected using the LRP-specific single chain antibody N3 (1:1,000) and the polyclonal anti-LRP-antibody W3 (1:5,000). Detection of PrP^{Sc} was carried out using anti-PrP antibody SAF83 (1:5,000). β-Actin was detected using the monoclonal anti-β-actin antibody (1:5,000). Blots were developed using an enhanced chemiluminescence system (Perkin Elmer) and exposed on chemiluminescence films (Hyperfilm, Amersham).

DNA preparation and subsequent PCR reaction. Genomic DNA was prepared using the DNeasy tissue kit (Qiagen) according to the manufacturer´s instructions. PCR reaction was carried out using 2 μg genomic DNA as template and the primer pair U6 forward (5´-GGACTATCATATGCTTACCG-

3´) and V5 reverse (5´-ACCGAGGAGAGGGTTAGGGAT -3´) to test for vector integration and universal ß-actin primer pair (forward 5´-CACACCTTCTACAATGAGCTG-3´, reverse 5´-GGAGGAGCAATGATCTTGATC-3´) to standardize samples, respectively. PCR cycles (1 min 95 °C, 2 min 55 °C, 2 min 72 °C; 30 cycles) were carried out using the taq DNA polymerase (Invitrogen) and according buffers and conditions. PCR products were subsequently analyzed on a 1.5% agarose/TBE gel.

RNA preparation and analysis. Total RNA out of cultured cells was prepared using the Trizol reagent (Invitrogen) according to the manufacturer´s instructions. Briefly, 1×10^6 cells were lyzed in 200 μl Trizol and 50 μl of chloroform was added. After centrifugation at full speed in an Eppifuge (4°C, 10 min) the upper phase was treated with one volume of isopropanol and pelleted RNA was washed with 70% of ethanol and air-dried to reveal 0.2-1 μg of total RNA. Subsequently, RNA was reverse transcribed into cDNA using the reverse transcriptase RT enzyme (Invitrogen). Reaction setup was carried out as described in the according manual using 0.1 μg of total RNA and a poly-dT primer for reverse transcription. The sample was incubated at 80 °C for 1 min and subsequently cooled down on ice. Then, 20 mM dNTPs (Promega), 5 units RT enzyme per reaction were added and the mixture was incubated at 37 °C for 1 h. For subsequent PCR reactions, 2 μl of the cDNA sample were used as template. PCR was carried out as described above for genomic DNA except the primer pair, which was LRP-specific (forw: 5´-ATG TCC GGA GCC CTT GAC GTC CTG-3´; rev:5´-GGA CCA CTC AGT GGT GGC TCC AAC C-3´).

Results

Vector-encoded siRNAs targeting the LRP mRNA repress the LRP synthesis in neuronal cells. In order to analyze the efficiency of several siRNA target sequences within the LRP mRNA transfections of siRNA-encoding vectors (pENTR) were performed using two different neuronal cell lines (Fig. 1). All of the three tested siRNAs repressed the LRP-synthesis in both mouse hypothalamic (GT1) and mouse neuroblastoma (N2a) cells 72 hours post transfection compared to control (pENTR-siRNA Lamin A/C) transfected cells (Fig. 1). In GT1 cells pENTR-siRNA 7 showed the strongest effect (Fig. 1a and b), whereas in N2a cells pENTR-siRNA 9 was most potent (Fig. 1c and d). In both cell lines the maximal reduction of the LRP level was approx. between 40-60%. In contrast, in scrapie-infected mouse neuroblastoma ($N2aSc^+$) cells LRP levels were reduced up to approx. 40% by pENTR-siRNA 4 and pENTR-siRNA 9 (Fig. 2a, upper panel and b). These results demonstrate that in different neuronal cell lines different siRNA target sequences are effective.

Vector-encoded LRP-specific siRNAs hamper PrP^{Sc} propagation in scrapie-infected cells. Recently, it has been shown that LRP-specific RNA oligonucleotide duplexes prevent PrP^{Sc} propagation in scrapie-infected neuronal cells (Leucht *et al.*, 2003). Due to the instability of RNA

duplexes, we generated lentiviral encoded siRNAs, which have the ability to integrate into the genome of the target cells and lead to a longlasting silencing effect. Therefore, we investigated whether vector-encoded LRP-specific siRNAs can interfere with the PrP^{Sc} propagation in scrapie-infected cells. In both $N2aSc^{+}$ and ScGT1 cells, seventy-two hours post transfection, the PrP^{Sc} levels were reduced (Fig. 2a and c, middle panels). Employing pENTR-siRNA 4 in $N2aSc^{+}$ cells we observed a downregulation of both LRP and PrP^{Sc} of approx. 60% (Fig. 2c). In ScGT1 cells, all three LRP-specific siRNAs showed an effect (Fig. 2c, middle panel and d). pENTR-siRNA Lamin A/C used as control showed no influence on the cellular LRP levels in both neuronal cell lines (Fig. 2). The downregulation of the PrP^{Sc} propagation correlated with the reduction of the cellular LRP level (Fig.2), confirming the requirement of LRP/LR for prion propagation.

Lentiviral-encoded LRP-specific siRNAs are integrated into the genome of neuronal cells. Virion particles expressing LRP-specific siRNAs (pLENTI) were produced by transfecting 293FT cells using the BLOCK-iT™ lentiviral expression system (Invitrogen). Viral supernatants were concentrated by ultracentrifugation (Burns *et al*., 1993; Naldini *et al*., 1996a) and vector titers were determined on HT1080 target cells by crystal violet staining ranging from 1 to 7×10^7 TU/ml.

To evaluate whether vector particles were able to efficiently integrate into the genome of target cells and express the LRP-specific siRNAs, N2a cells were transduced with virion preparations with a MOI of 1. As demonstrated by PCR analysis, the transduced cells express the LRP-specific siRNAs (Fig. 3a). In addition, transduction of HT1080 cells, which represent an important component in the titering step of the virus particles, revealed expression of LRP-specific siRNAs (data not shown). But, transduction of HT1080 cells with a MOI of 1 did not influence the LRP mRNA level in these cells (Fig. 3b), suggesting the necessity of a MOI greater than 1 to obtain visible RNAi effects.

Lentiviral particles expressing LRP-specific siRNAs downregulate the cellular LRP level in neuronal cells

To investigate the RNAi-potential of lentiviral particles expressing LRP-specific siRNAs, mouse neuronal cells were transduced with MOIs of 1, 5 and 10, respectively. Twenty-four hours post transduction, cell lysates of mouse neuroblastoma cells (N2a) were analyzed (Fig. 4a). Expression of both pLENTI-siRNA 9 and pLENTI-siRNA 7 resulted in a decrease of LRP levels, whereas the pLENTI-siRNA Lamin A/C (control) had no influence on the cellular LRP level (Fig. 4a). In mouse hypothalamic cells (GT1), the cell lysates were collected 4, 5 and 6 days post transduction and analyzed by western blot (Fig. 4b, upper panel). Four days post transduction, pLENTI-siRNA 7 resulted in an approx. 50-80% reduction of the LRP level (Fig. 4c). The LRP level is further reduced 5 days post transduction, reaching approx. 30-10% (Fig. 4c). More than 5 days post transduction, the LRP level reached a plateau and no further decrease of the LRP synthesis was observed (Fig. 4c). To

confirm the specificity of the effect of the LRP-pLENTI-siRNA 7, transduction was performed using a control siRNA (pLENTI-siRNA Lamin A/C). Western blot analysis revealed no change in the cellular LRP level after expression of pLENTI-siRNA Lamin A/C in GT1 cells (Fig. 4d, upper panel), suggesting a specific effect of siRNAs directed against the LRP mRNA.

Discussion

Transient transfection of PrP-specific siRNA duplexes in scrapie-infected neuroblastoma cells has been shown to trigger specific *Prn-p* gene silencing and caused a rapid loss of its PrPres content (Daude *et al*., 2003), suggesting a promising strategy for a therapy of prion diseases. For a non-transient expression of siRNAs within mammalian cells, several retroviral-based vectors have been developed (Brummelkamp *et al*., 2002; Devroe and Silver, 2002; Matta *et al*., 2003; Tomar *et al*., 2003) and showed high potential for a therapeutic application.

Lentivirus-mediated RNAi is a promising therapeutic tool to specifically knockdown disease-relevant genes as demonstrated in an ALS animal model (Ralph *et al*., 2005; Raoul *et al*., 2005). Lentivirus-based gene therapy has been developed for various human diseases such as Diabetes mellitus (Oh *et al*., 2005), Parkinson's disease (Lo Bianco *et al*., 2004) and Alzheimer's disease (Marr *et al*., 2003). In mouse models of amyloidosis, intracerebral injection of lentiviral vectors expressing neprilysin, which is the major Aβ-degrading enzyme in the brain, reduced amyloid-beta deposits by about 50% (Marr *et al*., 2003), suggesting a potential of gene transfer approaches for the development of alternative therapies. Thus, the ability of lentiviruses encoding siRNA to silence genes specifically provide a beneficial tool for a TSE therapy.

Recently, lentiviral gene transfer of PrP containing dominant negative mutations was performed and resulted in an inhibition of PrP^{Sc} formation in chronically scrapie-infected neuronal cells (Crozet *et al*., 2004). Furthermore, *in vivo* the distribution of lentiviral vectors using intracerebral and intravenous routes was examined, demonstrating a transduction of brain and spleen cells (Crozet *et al*., 2004).

In vitro, the 37kDa/67kDa LRP/LR has been shown to act as the cell surface receptor for PrP (Gauczynski *et al*., 2001b; Morel *et al*., 2005) and has been found to be present in the murine central nervous system in different maturation states (Simoneau *et al*., 2003). In addition, it was reported that that the 67kDa LR is the major receptor form in adult rat brain and spinal cord (Baloui *et al*., 2004). Furthermore, it was demonstrated that the distribution of LR correlated well with that reported for laminin-1 but also with brain regions classically associated with prion-related neurodegeneration (Baloui *et al*., 2004). The 37kDa LRP is thought to be the precursor of the mature 67kDa laminin receptor (LR) (Buto *et al*., 1998), but the exact molecular structure of the 67kDa LR remain unknown so far.

Moreover, it has been shown that LRP/LR is required for PrP^{Sc} propagation in scrapie-infected neuroblastoma cells (Leucht *et al*., 2003). In natural scrapie, the gastrointestinal tract is considered to

be the major route of infection (Hadlow *et al*., 1982; Pattison *et al*., 1972). The involvement of the 37kDa/67kDa laminin receptor (LRP/LR) in the endocytosis of prions has been recently investigated using human enterocytes, which are epithelial cells of the intestine and seem to act as antigen-presenting cells (Morel *et al*., 2005). Immunofluorescence analysis revealed that LRP/LR specifically mediates the endocytosis of bovine PrP^{Sc} into human enterocytes. Interestingly, scrapie prions were not found to be internalized, suggesting a specific uptake of bovine BSE prions by human enterocytes (Morel *et al*., 2005). Taken together, these studies encouraged us to assess a further therapeutic approach for prion diseases targeting LRP/LR.

In this study, we used an *in vitro* system to investigate whether lentivirus-based RNAi directed against the 37kDa/67kDa LRP mRNA is able to interfere with the PrP^{Sc} propagation in scrapie-infected neuronal cells due to a downregulation of LRP/LR expression. Assuming that not all siRNAs directed against a target gene are equally effective in suppressing target gene expression (Harborth *et al*., 2003), in a pre-experiment we confirmed in both non-infected N2a and GT1 cells that all three siRNAs were active and suppressed the cellular LRP synthesis. We observed that in both cell lines a different siRNA showed the greatest effect, namely pENTR-siRNA 7 in GT1 cells and pENTR-siRNA 9 in N2a cells, respectively. Interestingly, in GT1 cells all three siRNAs result in a comparable LRP downregulation, whereas in N2a cells a strong variation in the RNAi effect was detected. Both cell lines are of neuronal origin and vary in the RNAi response, pointing out the importance of testing an assortment of siRNAs to discover the one with the maximal silencing effect.

In prion diseases, the accumulation of the abnormal, infectious isoform of the cellular prion protein is the pivotal step. In the present study, we used the RNAi technology to target the prion protein receptor LRP/LR to interfere with the PrP^{Sc} propagation in chronically scrapie-infected neuronal cells. Our results demonstrate that in both $N2aSc^{+}$ and ScGT1 cells lentiviral siRNAs targeting the LRP mRNA are able to reduce the PrP^{Sc} level in these cells and PrP^{Sc} reduction correlates with the LRP/LR downregulation, suggesting a LRP/LR-dependent PrP^{Sc} propagation. In contrast, a siRNA targeting Lamin A/C showed no effect on both cellular LRP and PrP^{Sc} levels.

Since there is no LRP/LR-knockout mouse available, RNAi directed against LRP mRNA may provide a potential tool to study the role of LRP/LR *in vivo*. To further enlighten the role of LRP/LR in prion diseases, injection of recombinant LRP-specific RNA interference (RNAi) lentiviral particles into mice using an intracerebral (i.c.) or intraperitoneal (i.p.) route is projected. Subsequent prion inoculation in these mice will prove whether the knockdown of LRP/LR by RNAi might prolong the onset or even prevent prion disease.

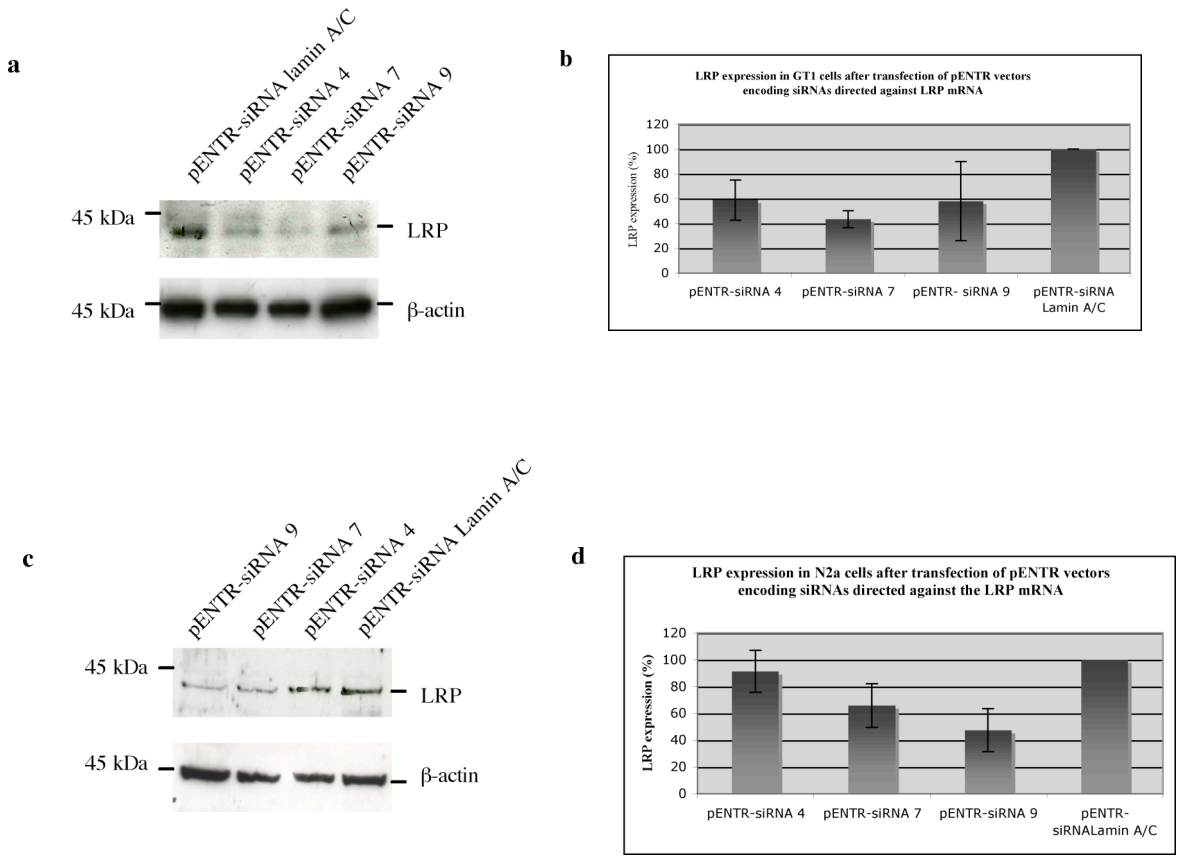

FIG. 1. Effect of pENTR vectors encoding siRNAs directed against the LRP mRNA in neuronal cells. (a) Western blot of transfected GT1 cells. Seventy-two hours post transfection crude cell lysates were analyzed using the single chain anti-LRP antibody N3 (upper panel). The blot was stripped and re-probed with the monoclonal anti-β-actin antibody (lower panel). Molecular weight markers in kilodalton (kDa) are indicated on the left. (b) Densitometry analysis using the NIH IMAGE software. The results of three individual experiments are shown and expressed as a percentage of control levels ±s.e.m. (c) Western blot of transfected N2a cells. The effect of LRP-specific siRNAs on cellular LRP levels was assayed 65 hours post transfection. Endogenous LRP was detected using the single chain anti-LRP antibody N3 (upper panel). β-actin was used as loading control (lower panel). Molecular weight markers in kilodalton (kDa) are indicated on the left. (d) Densitometry analysis using the NIH IMAGE software. The experiment was performed six times and the results are shown as a percentage of control levels ±s.e.m.

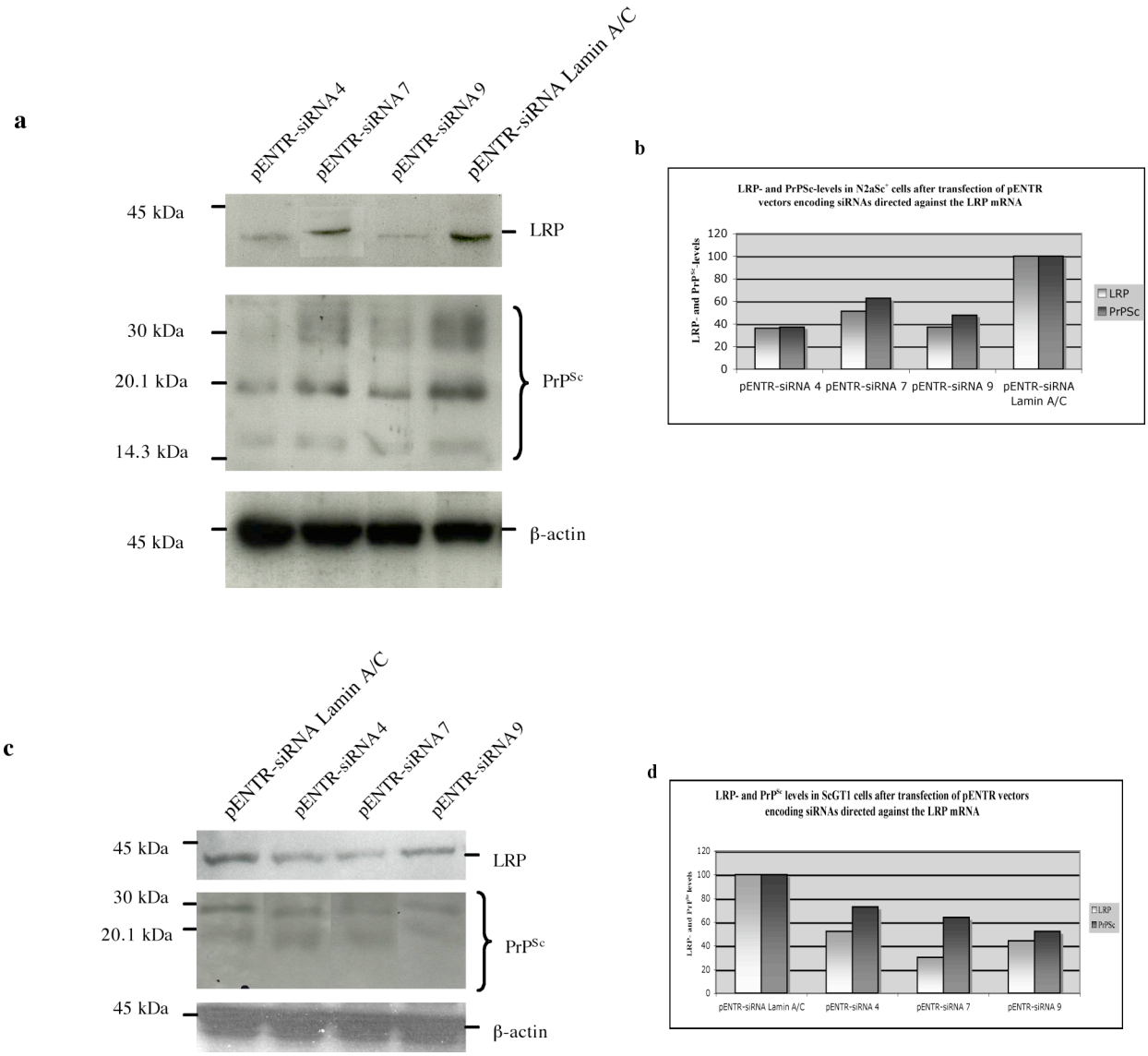

FIG. 2. Influence of pENTR vectors encoding LRP-specific siRNAs on the PrP^{Sc} propagation in scrapie-infected neuronal cells. (a) Western blot of transfected $N2aSc^{+}$ cells. Seventy-two hours post transfection crude cell lysates were prepared and subjected to SDS-PAGE. Cellular LRP was detected using the single chain anti-LRP antibody N3 (upper panel). The blot was stripped and re-probed with the monoclonal anti-β-actin antibody (lower panel). PK-digested cell lysates were analyzed using anti-PrP SAF83 antibody (middle panel). Molecular weight markers in kilodalton (kDa) are indicated on the left. (b) Densitometry analysis using the NIH IMAGE software. (c) Western blot of transfected ScGT1 cells. The effect of LRP-specific siRNAs on cellular LRP levels was assayed using the single chain anti-LRP antibody N3 (upper panel). PrP^{Sc} was detected using the SAF83 antibody (middle panel). β-actin was used as loading control (lower panel). Molecular weight markers in kilodalton (kDa) are indicated on the left. (d) Densitometry analysis using the NIH IMAGE software.

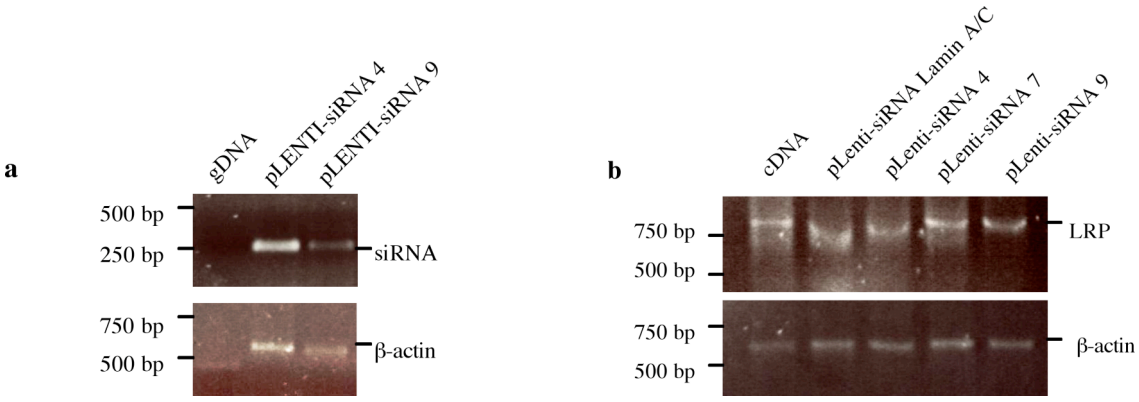

FIG. 3. Detection of the integration of lentiviral vectors expressing LRP-specific siRNAs and their effect on the mRNA level. (a) PCR analysis of transduced mouse neuroblastoma cells. N2a cells were transduced with virion preparations with a MOI of 1 and genomic DNA was prepared. PCR reaction was carried out and PCR products were analyzed on an agarose/TBE gel. The expression of the LRP-specific siRNAs was tested using the primer pair U6 forward and V5 reverse, giving a band of 250 bp (upper panel). To standardize samples, a β-actin primer pair was used resulting in a band of 600 bp (lower panel). The specificity of the PCR signal for the siRNAs was proven using genomic DNA (gDNA) of non transduced N2a cells as a template for the PCR reaction. (b) mRNA levels in HT1080 cells after transduction with lentiviral vectors expressing siRNAs directed against the LRP mRNA. HT1080 cells were transduced with a MOI of 1 and were selected for 5 days in blasticidin containing medium following isolation of total cellular RNA. The cDNA resulting from the reverse transcription was amplified in a PCR reaction using a LRP-specific primer pair. PCR products were separated on an agarose/ TBE gel and visualized using ethidium bromide staining. The LRP-specific signal resulted in a 800 bp pair band (upper panel). As a control, cDNA from non transduced HT1080 cells was used. To standardize samples, a β-actin primer pair was used resulting in a band of 600 bp (lower panel).

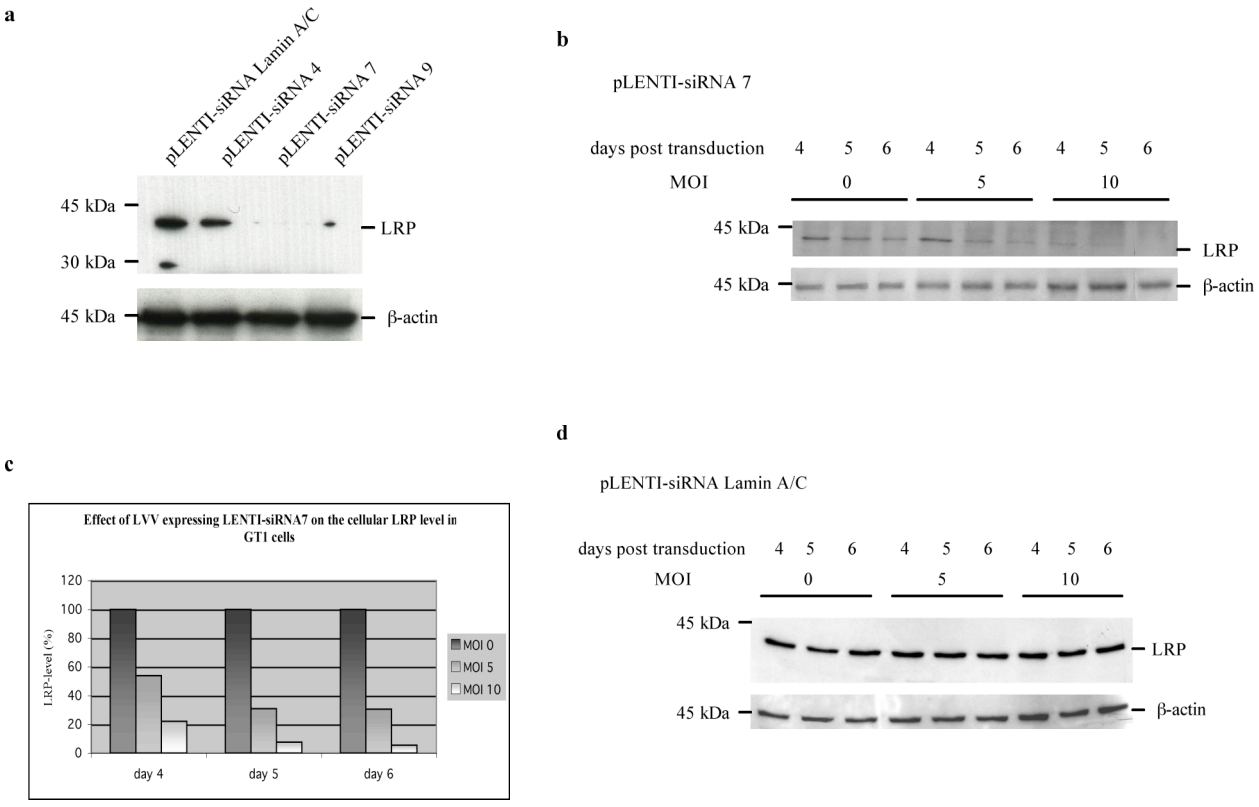

FIG. 4. Effect of lentiviral vectors expressing LRP-specific siRNAs in neuronal cells. (a) Western blot of N2a cells transduced with virion particles (MOI=10) expressing LRP-specific siRNA 4, 7, 9 and control siRNA Lamin A/C, respectively (upper panel). Cells lysates were analyzed one day post transduction using the anti-LRP-antibody W3. As a loading control β-actin was used (lower panel). Molecular weight markers in kilodalton (kDa) are indicated on the left. (b) Western blot of GT1 cells transduced with pLENTI-siRNA 7. Cells were transduced using MOI of 0, 5 and 10, respectively. Four, five and six days post transduction crude cell lysates were prepared and analyzed using the single chain anti-LRP antibody N3 (upper panel). The blot was stripped and re-probed with the monoclonal anti-β-actin antibody (lower panel). Molecular weight markers in kilodalton (kDa) are indicated on the left. (c) Densitometry analysis using the NIH IMAGE software. (d) Western blot of GT1 cells transduced with pLENTI-Lamin A/C. Four, five and six days post transduction crude cell lysates were prepared and subjected to SDS-PAGE. The effect on cellular LRP levels was assayed using the single chain anti-LRP antibody N3 (upper panel). β-actin was used as loading control (lower panel). Molecular weight markers in kilodalton (kDa) are indicated on the left.

CHAPTER VIII

References

Adjou, K.T., Demaimay, R., Lasmezas, C., Deslys, J.P., Seman, M. and Dormont, D. (1995) MS-8209, a new amphotericin B derivative, provides enhanced efficacy in delaying hamster scrapie. *Antimicrob Agents Chemother*, **39**, 2810-2812.

Adjou, K.T., Simoneau, S., Sales, N., Lamoury, F., Dormont, D., Papy-Garcia, D., Barritault, D., Deslys, J.P. and Lasmezas, C.I. (2003) A novel generation of heparan sulfate mimetics for the treatment of prion diseases. *J Gen Virol*, **84**, 2595-2603.

Aguzzi, A. and Polymenidou, M. (2004) Mammalian prion biology: one century of evolving concepts. *Cell*, **116**, 313-327.

Aguzzi, A. and Weissmann, C. (1998) Prion diseases. *Haemophilia*, **4**, 619-627.

Almer, G., Hainfellner, J.A., Brucke, T., Jellinger, K., Kleinert, R., Bayer, G., Windl, O., Kretzschmar, H.A., Hill, A., Sidle, K., Collinge, J. and Budka, H. (1999) Fatal familial insomnia: a new Austrian family. *Brain*, **122 (Pt 1)**, 5-16.

Alper, T., Cramp, W.A., Haig, D.A. and Clarke, M.C. (1967) Does the agent of scrapie replicate without nucleic acid? *Nature*, **214**, 764-766.

Ardini, E., Pesole, G., Tagliabue, E., Magnifico, A., Castronovo, V., Sobel, M.E., Colnaghi, M.I. and Menard, S. (1998) The 67-kDa laminin receptor originated from a ribosomal protein that acquired a dual function during evolution. *Mol Biol Evol*, **15**, 1017-1025.

Arnold, J.E., Tipler, C., Laszlo, L., Hope, J., Landon, M. and Mayer, R.J. (1995) The abnormal isoform of the prion protein accumulates in late-endosome-like organelles in scrapie-infected mouse brain. *J Pathol*, **176**, 403-411.

Auth, D. and Brawerman, G. (1992) A 33-kDa polypeptide with homology to the laminin receptor: component of translation machinery. *Proc Natl Acad Sci U S A*, **89**, 4368-4372.

Baker, H.E., Poulter, M., Crow, T.J., Frith, C.D., Lofthouse, R. and Ridley, R.M. (1991) Aminoacid polymorphism in human prion protein and age at death in inherited prion disease. *Lancet*, **337**, 1286.

Baloui, H., von Boxberg, Y., Vinh, J., Weiss, S., Rossier, J., Nothias, F. and Stettler, O. (2004) Cellular prion protein/laminin receptor: distribution in adult central nervous system and characterization of an isoform associated with a subtype of cortical neurons. *Eur J Neurosci*, **20**, 2605-2616.

Baron, G.S. and Caughey, B. (2003) Effect of glycosylphosphatidylinositol anchor-dependent and -independent prion protein association with model raft membranes on conversion to the protease-resistant isoform. *J Biol Chem*, **278**, 14883-14892.

Barret, A., Tagliavini, F., Forloni, G., Bate, C., Salmona, M., Colombo, L., De Luigi, A., Limido, L., Suardi, S., Rossi, G., Auvre, F., Adjou, K.T., Sales, N., Williams, A., Lasmezas, C. and Deslys, J.P. (2003) Evaluation of quinacrine treatment for prion diseases. *J Virol*, **77**, 8462-8469.

Basler, K., Oesch, B., Scott, M., Westaway, D., Walchli, M., Groth, D.F., McKinley, M.P., Prusiner, S.B. and Weissmann, C. (1986) Scrapie and cellular PrP isoforms are encoded by the same chromosomal gene. *Cell*, **46**, 417-428.

Behrens, A., Brandner, S., Genoud, N. and Aguzzi, A. (2001) Normal neurogenesis and scrapie pathogenesis in neural grafts lacking the prion protein homologue Doppel. *EMBO Rep*, **2**, 347-352.

Behrens, A., Genoud, N., Naumann, H., Rulicke, T., Janett, F., Heppner, F.L., Ledermann, B. and Aguzzi, A. (2002) Absence of the prion protein homologue Doppel causes male sterility. *Embo J*, **21**, 3652-3658.

Belichenko, P.V., Miklossy, J., Belser, B., Budka, H. and Celio, M.R. (1999) Early destruction of the extracellular matrix around parvalbumin-immunoreactive interneurons in Creutzfeldt-Jakob disease. *Neurobiol Dis*, **6**, 269-279.

Benito-Leon, J. (2004) Combined quinacrine and chlorpromazine therapy in fatal familial insomnia. *Clin Neuropharmacol*, **27**, 201-203.

Bertoni, J.M., Brown, P., Goldfarb, L.G., Rubenstein, R. and Gajdusek, D.C. (1992) Familial Creutzfeldt-Jakob disease (codon 200 mutation) with supranuclear palsy. *Jama*, **268**, 2413-2415.

Bertsch, U., Winklhofer, K.F., Hirschberger, T., Bieschke, J., Weber, P., Hartl, F.U., Tavan, P., Tatzelt, J., Kretzschmar, H.A. and Giese, A. (2005) Systematic identification of antiprion drugs by high-throughput screening based on scanning for intensely fluorescent targets. *J Virol*, **79**, 7785-7791.

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.

Blomer, U., Naldini, L., Kafri, T., Trono, D., Verma, I.M. and Gage, F.H. (1997) Highly efficient and sustained gene transfer in adult neurons with a lentivirus vector. *J Virol*, **71**, 6641-6649.

Boelle, P.Y., Cesbron, J.Y. and Valleron, A.J. (2004) Epidemiological evidence of higher susceptibility to vCJD in the young. *BMC Infect Dis*, **4**, 26.

Bolard, J. (1986) Interaction of polyene antibiotics with membrane lipids: physicochemical studies of the molecular basis of selectivity. *Drugs Exp Clin Res*, **12**, 613-618.

Bolton, D.C., McKinley, M.P. and Prusiner, S.B. (1982) Identification of a protein that purifies with the scrapie prion. *Science*, **218**, 1309-1311.

Bonifacino, J.S. and Weissman, A.M. (1998) Ubiquitin and the control of protein fate in the secretory and endocytic pathways. *Annu Rev Cell Dev Biol*, **14**, 19-57.

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.

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.

Booth, C. and Potten, C.S. (2000) Gut instincts: thoughts on intestinal epithelial stem cells. *J Clin Invest*, **105**, 1493-1499.

Bosque, P.J. and Prusiner, S.B. (2000) Cultured cell sublines highly susceptible to prion infection. *J Virol*, **74**, 4377-4386.

Bosque, P.J., Ryou, C., Telling, G., Peretz, D., Legname, G., DeArmond, S.J. and Prusiner, S.B. (2002) Prions in skeletal muscle. *Proc Natl Acad Sci U S A*, **99**, 3812-3817.

Botto, L., Masserini, M., Cassetti, A. and Palestini, P. (2004) Immunoseparation of Prion protein-enriched domains from other detergent-resistant membrane fractions, isolated from neuronal cells. *FEBS Lett*, **557**, 143-147.

Bounhar, Y., Zhang, Y., Goodyer, C.G. and LeBlanc, A. (2001) Prion protein protects human neurons against Bax-mediated apoptosis. *J Biol Chem*, **276**, 39145-39149.

Bragason, B.T. and Palsdottir, A. (2005) Interaction of PrP with NRAGE, a protein involved in neuronal apoptosis. *Mol Cell Neurosci*, **29**, 232-244.

Brown, D.R., Qin, K., Herms, J.W., Madlung, A., Manson, J., Strome, R., Fraser, P.E., Kruck, T., von Bohlen, A., Schulz-Schaeffer, W., Giese, A., Westaway, D. and Kretzschmar, H. (1997) The cellular prion protein binds copper in vivo. *Nature*, **390**, 684-687.

Brown, D.R., Schmidt, B., Groschup, M.H. and Kretzschmar, H.A. (1998) Prion protein expression in muscle cells and toxicity of a prion protein fragment. *Eur J Cell Biol*, **75**, 29-37.

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, H.R., Goller, N.L., Rudelli, R.D., Merz, G.S., Wolfe, G.C., Wisniewski, H.N. and Robakis, N.K. (1990) The mRNA encoding the scrapie agent protein is present in a variety of non-neuronal cells. *Acta Neuropathol (Berl)*, **80**, 1-6.

Brown, L.R. and Harris, D.A. (2003) Copper and zinc cause delivery of the prion protein from the plasma membrane to a subset of early endosomes and the Golgi. *J Neurochem*, **87**, 353-363.

Brummelkamp, T.R., Bernards, R. and Agami, R. (2002) Stable suppression of tumorigenicity by virus-mediated RNA interference. *Cancer Cell*, **2**, 243-247.

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.

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.

Burger, D. and Hartsough, G.R. (1965) Encephalopathy of mink. II. Experimental and natural transmission. *J Infect Dis*, **115**, 393-399.

Burns, J.C., Friedmann, T., Driever, W., Burrascano, M. and Yee, J.K. (1993) Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc Natl Acad Sci U S A*, **90**, 8033-8037.

Buschmann, A., Pfaff, E., Reifenberg, K., Muller, H.M. and Groschup, M.H. (2000) Detection of cattle-derived BSE prions using transgenic mice overexpressing bovine PrP(C). *Arch Virol Suppl*, 75-86.

Buto, S., Tagliabue, E., Ardini, E., Magnifico, A., Ghirelli, C., van den Brule, F., Castronovo, V., Colnaghi, M.I., Sobel, M.E. and Menard, S. (1998) Formation of the 67-kDa laminin receptor by acylation of the precursor. *J Cell Biochem*, **69**, 244-251.

Calzolai, L., Lysek, D.A., Perez, D.R., Guntert, P. and Wuthrich, K. (2005) Prion protein NMR structures of chickens, turtles, and frogs. *Proc Natl Acad Sci U S A*, **102**, 651-655.

Campana, V., Sarnataro, D. and Zurzolo, C. (2005) The highways and byways of prion protein trafficking. *Trends Cell Biol*, **15**, 102-111.

Carota, A., Pizzolato, G.P., Gailloud, P., Macchi, G., Fasel, J., Le Floch, J. and Cardone, F. (1996) A panencephalopathic type of Creutzfeldt-Jakob disease with selective lesions of the thalamic nuclei in 2 Swiss patients. *Clin Neuropathol*, **15**, 125-134.

Casaccia-Bonnefil, P., Kascsak, R.J., Fersko, R., Callahan, S. and Carp, R.I. (1993) Brain regional distribution of prion protein PrP27-30 in mice stereotaxically microinjected with different strains of scrapie. *J Infect Dis*, **167**, 7-12.

Castronovo, V., Taraboletti, G. and Sobel, M.E. (1991) Functional domains of the 67-kDa laminin receptor precursor. *J Biol Chem*, **266**, 20440-20446.

Caughey, B., Kocisko, D.A., Raymond, G.J. and Lansbury, P.T., Jr. (1995) Aggregates of scrapie-associated prion protein induce the cell-free conversion of protease-sensitive prion protein to the protease-resistant state. *Chem Biol*, **2**, 807-817.

Caughey, B. and Race, R.E. (1992) Potent inhibition of scrapie-associated PrP accumulation by congo red. *J Neurochem*, **59**, 768-771.

Caughey, B. and Raymond, G.J. (1991) The scrapie-associated form of PrP is made from a cell surface precursor that is both protease- and phospholipase-sensitive. *J Biol Chem*, **266**, 18217-18223.

Caughey, B. and Raymond, G.J. (1993) Sulfated polyanion inhibition of scrapie-associated PrP accumulation in cultured cells. *J Virol*, **67**, 643-650.

Caughey, W.S., Raymond, L.D., Horiuchi, M. and Caughey, B. (1998) Inhibition of protease-resistant prion protein formation by porphyrins and phthalocyanines. *Proc Natl Acad Sci U S A*, **95**, 12117-12122.

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.

Chandler, R.L. (1961) Encephalopathy in mice produced by inoculation with scrapie brain material. *Lancet*, **1**, 1378-1379.

Chen, Y., Maguire, T., Hileman, R.E., Fromm, J.R., Esko, J.D., Linhardt, R.J. and Marks, R.M. (1997) Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. *Nat Med*, **3**, 866-871.

Chernoff, Y.O., Lindquist, S.L., Ono, B., Inge-Vechtomov, S.G. and Liebman, S.W. (1995) Role of the chaperone protein Hsp104 in propagation of the yeast prion-like factor [psi+]. *Science*, **268**, 880-884.

Chesebro, B., Race, R., Wehrly, K., Nishio, J., Bloom, M., Lechner, D., Bergstrom, S., Robbins, K., Mayer, L., Keith, J.M. and *et al*. (1985) Identification of scrapie prion protein-specific mRNA in scrapie-infected and uninfected brain. *Nature*, **315**, 331-333.

Cohen, F.E., Pan, K.M., Huang, Z., Baldwin, M., Fletterick, R.J. and Prusiner, S.B. (1994) Structural clues to prion replication. *Science*, **264**, 530-531.

Cohen, F.E. and Prusiner, S.B. (1998) Pathologic conformations of prion proteins. *Annu Rev Biochem*, **67**, 793-819.

Coitinho, A.S., Roesler, R., Martins, V.R., Brentani, R.R. and Izquierdo, I. (2003) Cellular prion protein ablation impairs behavior as a function of age. *Neuroreport*, **14**, 1375-1379.

Collinge, J. (1996) New diagnostic tests for prion diseases. *N Engl J Med*, **335**, 963-965.

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

Collinge, J. and Palmer, M.S. (1994) Molecular genetics of human prion diseases. *Philos Trans R Soc Lond B Biol Sci*, **343**, 371-378.

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

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.

Coschigano, P.W. and Magasanik, B. (1991) The URE2 gene product of Saccharomyces cerevisiae plays an important role in the cellular response to the nitrogen source and has homology to glutathione s-transferases. *Mol Cell Biol*, **11**, 822-832.

Courchesne, W.E. and Magasanik, B. (1988) Regulation of nitrogen assimilation in Saccharomyces cerevisiae: roles of the URE2 and GLN3 genes. *J Bacteriol*, **170**, 708-713.

Cousens, S.N., Vynnycky, E., Zeidler, M., Will, R.G. and Smith, P.G. (1997) Predicting the CJD epidemic in humans. *Nature*, **385**, 197-198.

Coustou, V., Deleu, C., Saupe, S. and Begueret, J. (1997) The protein product of the het-s heterokaryon incompatibility gene of the fungus Podospora anserina behaves as a prion analog. *Proc Natl Acad Sci U S A*, **94**, 9773-9778.

Cox, B.S., Tuite, M.F. and McLaughlin, C.S. (1988) The psi factor of yeast: a problem in inheritance. *Yeast*, **4**, 159-178.

Creutzfeldt, H.G. (1920) Über eine eigenartige Erkrankung des Zentralnervensystems. *Vorläufige Mitteilung. Z. f. d. ges. Neurol. und Psych., 1-18.*

Crozet, C., Flamant, F., Bencsik, A., Aubert, D., Samarut, J. and Baron, T. (2001) Efficient transmission of two different sheep scrapie isolates in transgenic mice expressing the ovine PrP gene. *J Virol*, **75**, 5328-5334.

Crozet, C., Lin, Y.L., Mettling, C., Mourton-Gilles, C., Corbeau, P., Lehmann, S. and Perrier, V. (2004) Inhibition of PrPSc formation by lentiviral gene transfer of PrP containing dominant negative mutations. *J Cell Sci*, **117**, 5591-5597.

Cuillé, J. and Chelle, C. (1936) La maladie dite tremblante du mouton est-elle inoculable? *C. R. Acad. Sci. Paris*, **203**, 1552-1554.

Cunningham, C., Deacon, R.M., Chan, K., Boche, D., Rawlins, J.N. and Perry, V.H. (2005) Neuropathologically distinct prion strains give rise to similar temporal profiles of behavioral deficits. *Neurobiol Dis*, **18**, 258-269.

Daude, N., Marella, M. and Chabry, J. (2003) Specific inhibition of pathological prion protein accumulation by small interfering RNAs. *J Cell Sci*, **116**, 2775-2779.

DebBurman, S.K., Raymond, G.J., Caughey, B. and Lindquist, S. (1997) Chaperone-supervised conversion of prion protein to its protease-resistant form. *Proc Natl Acad Sci U S A*, **94**, 13938-13943.

Delay, J., Deniker, P. and Harl, J.M. (1952) [Therapeutic use in psychiatry of phenothiazine of central elective action (4560 RP).]. *Ann Med Psychol (Paris)*, **110**, 112-117.

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

Demaimay, R., Adjou, K.T., Beringue, V., Demart, S., Lasmezas, C.I., Deslys, J.P., Seman, M. and Dormont, D. (1997) Late treatment with polyene antibiotics can prolong the survival time of scrapie-infected animals. *J Virol*, **71**, 9685-9689.

Demaimay, R., Chesebro, B. and Caughey, B. (2000) Inhibition of formation of protease-resistant prion protein by Trypan Blue, Sirius Red and other Congo Red analogs. *Arch Virol Suppl*, 277-283.

Devroe, E. and Silver, P.A. (2002) Retrovirus-delivered siRNA. *BMC Biotechnol*, **2**, 15.

Diarra-Mehrpour, M., Arrabal, S., Jalil, A., Pinson, X., Gaudin, C., Pietu, G., Pitaval, A., Ripoche, H., Eloit, M., Dormont, D. and Chouaib, S. (2004) Prion protein prevents human breast carcinoma cell line from tumor necrosis factor alpha-induced cell death. *Cancer Res*, **64**, 719-727.

Diringer, H. and Ehlers, B. (1991) Chemoprophylaxis of scrapie in mice. *J Gen Virol*, **72 (Pt 2)**, 457-460.

Dlouhy, S.R., Hsiao, K., Farlow, M.R., Foroud, T., Conneally, P.M., Johnson, P., Prusiner, S.B., Hodes, M.E. and Ghetti, B. (1992) Linkage of the Indiana kindred of Gerstmann-Straussler-Scheinker disease to the prion protein gene. *Nat Genet*, **1**, 64-67.

Doh-ura, K., Tateishi, J., Sasaki, H., Kitamoto, T. and Sakaki, Y. (1989) Pro----leu change at position 102 of prion protein is the most common but not the sole mutation related to Gerstmann-Straussler syndrome. *Biochem Biophys Res Commun*, **163**, 974-979.

Donne, D.G., Viles, J.H., Groth, D., Mehlhorn, I., James, T.L., Cohen, F.E., Prusiner, S.B., Wright, P.E. and Dyson, H.J. (1997) Structure of the recombinant full-length hamster prion protein PrP(29-231): the N terminus is highly flexible. *Proc Natl Acad Sci U S A*, **94**, 13452-13457.

Donofrio, G., Heppner, F.L., Polymenidou, M., Musahl, C. and Aguzzi, A. (2005) Paracrine inhibition of prion propagation by anti-PrP single-chain Fv miniantibodies. *J Virol*, **79**, 8330-8338.

Dormont, D. (2003) Approaches to prophylaxis and therapy. *Br Med Bull*, **66**, 281-292.

Douville, P.J. and Carbonetto, S. (1992) Genetic linkage analysis in recombinant inbred mice of P40, a putative clone for the high-affinity laminin receptor. *Mamm Genome*, **3**, 438-446.

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.

Edenhofer, F., Rieger, R., Famulok, M., Wendler, W., Weiss, S. and Winnacker, E.L. (1996) Prion protein PrPc interacts with molecular chaperones of the Hsp60 family. *J Virol*, **70**, 4724-4728.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature*, **411**, 494-498.

Enari, M., Flechsig, E. and Weissmann, C. (2001) Scrapie prion protein accumulation by scrapie-infected neuroblastoma cells abrogated by exposure to a prion protein antibody. *Proc Natl Acad Sci U S A*, **98**, 9295-9299.

Enright, A.J., Van Dongen, S. and Ouzounis, C.A. (2002) An efficient algorithm for large-scale detection of protein families. *Nucleic Acids Res*, **30**, 1575-1584.

Erickson, R.P., Lai, L.W. and Grimes, J. (1993) Creating a conditional mutation of Wnt-1 by antisense transgenesis provides evidence that Wnt-1 is not essential for spermatogenesis. *Dev Genet*, **14**, 274-281.

Fantini, J., Garmy, N., Mahfoud, R. and Yahi, N. (2002) Lipid rafts: structure, function and role in HIV, Alzheimers and prion diseases. *Expert Rev Mol Med*, **2002**, 1-22.

Farquhar, C., Dickinson, A. and Bruce, M. (1999) Prophylactic potential of pentosan polysulphate in transmissible spongiform encephalopathies. *Lancet*, **353**, 117.

Farquhar, C.F. and Dickinson, A.G. (1986) Prolongation of scrapie incubation period by an injection of dextran sulphate 500 within the month before or after infection. *J Gen Virol*, **67 (Pt 3)**, 463-473.

Feng, X., Eide, F.F., Jiang, H. and Reder, A.T. (2004) Adeno-associated viral vector-mediated ApoE expression in Alzheimer's disease mice: low CNS immune response, long-term expression, and astrocyte specificity. *Front Biosci*, **9**, 1540-1546.

Feraudet, C., Morel, N., Simon, S., Volland, H., Frobert, Y., Creminon, C., Vilette, D., Lehmann, S. and Grassi, J. (2005) Screening of 145 anti-PrP monoclonal antibodies for their capacity to inhibit PrPSc replication in infected cells. *J Biol Chem*, **280**, 11247-11258.

Fischer, M., Rulicke, T., Raeber, A., Sailer, A., Moser, M., Oesch, B., Brandner, S., Aguzzi, A. and Weissmann, C. (1996) Prion protein (PrP) with amino-proximal deletions restoring susceptibility of PrP knockout mice to scrapie. *Embo J*, **15**, 1255-1264.

Forss-Petter, S., Danielson, P.E., Catsicas, S., Battenberg, E., Price, J., Nerenberg, M. and Sutcliffe, J.G. (1990) Transgenic mice expressing beta-galactosidase in mature neurons under neuron-specific enolase promoter control. *Neuron*, **5**, 187-197.

Fraser, H. and Dickinson, A.G. (1967) Distribution of experimentally induced scrapie lesions in the brain. *Nature*, **216**, 1310-1311.

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

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. and Zigas, V. (1957) Degenerative disease of the central nervous system in New Guinea; the endemic occurrence of kuru in the native population. *N Engl J Med*, **257**, 974-978.

Gauczynski, S., Hundt, C., Leucht, C. and Weiss, S. (2001a) Interaction of prion proteins with cell surface receptors, molecular chaperones, and other molecules. *Adv Protein Chem*, **57**, 229-272.

Gauczynski, S., Krasemann, S., Bodemer, W. and Weiss, S. (2002) Recombinant human prion protein mutants huPrP D178N/M129 (FFI) and huPrP+9OR (fCJD) reveal proteinase K resistance. *J Cell Sci*, **115**, 4025-4036.

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. (2001b) The 37-kDa/67-kDa laminin receptor acts as the cell-surface receptor for the cellular prion protein. *Embo J*, **20**, 5863-5875.

Gerstmann, J., Straussler, E. and Scheinker, I. (1936) Über eine eigenartige hereditär-familiäre Erkrankung des Zentralnervensystems. Zugleich ein Beitrag zur Frage des vorzeitigen lokalen Alterns. *Zeitschrift für die gesamte Neurologie und Psychiatrie*, **154**, 736-762.

Ghani, A.C., Ferguson, N.M., Donnelly, C.A., Hagenaars, T.J. and Anderson, R.M. (1998) Epidemiological determinants of the pattern and magnitude of the vCJD epidemic in Great Britain. *Proc Biol Sci*, **265**, 2443-2452.

Ghetti, B., Dlouhy, S.R., Giaccone, G., Bugiani, O., Frangione, B., Farlow, M.R. and Tagliavini, F. (1995) Gerstmann-Straussler-Scheinker disease and the Indiana kindred. *Brain Pathol*, **5**, 61-75.

Gibbs, C.J., Jr. and Bolis, C.L. (1997) Normal isoform of amyloid protein (PrP) in brains of spawning salmon. *Mol Psychiatry*, **2**, 146-147.

Gibbs, C.J., Jr., Gajdusek, D.C., Asher, D.M., Alpers, M.P., Beck, E., Daniel, P.M. and Matthews, W.B. (1968) Creutzfeldt-Jakob disease (spongiform encephalopathy): transmission to the chimpanzee. *Science*, **161**, 388-389.

Gilch, S., Winklhofer, K.F., Groschup, M.H., Nunziante, M., Lucassen, R., Spielhaupter, C., Muranyi, W., Riesner, D., Tatzelt, J. and Schätzl, H.M. (2001) Intracellular re-routing of prion protein prevents propagation of PrP(Sc) and delays onset of prion disease. *Embo J*, **20**, 3957-3966.

Goldfarb, L.G., Brown, P., Haltia, M., Cathala, F., McCombie, W.R., Kovanen, J., Cervenakova, L., Goldin, L., Nieto, A., Godec, M.S. and *et al*. (1992) Creutzfeldt-Jakob disease cosegregates with the codon 178Asn PRNP mutation in families of European origin. *Ann Neurol*, **31**, 274-281.

Goldfarb, L.G., Brown, P., McCombie, W.R., Goldgaber, D., Swergold, G.D., Wills, P.R., Cervenakova, L., Baron, H., Gibbs, C.J., Jr. and Gajdusek, D.C. (1991) Transmissible familial Creutzfeldt-Jakob disease associated with five, seven, and eight extra octapeptide coding repeats in the PRNP gene. *Proc Natl Acad Sci U S A*, **88**, 10926-10930.

Goldfarb, L.G., Korczyn, A.D., Brown, P., Chapman, J. and Gajdusek, D.C. (1990a) Mutation in codon 200 of scrapie amyloid precursor gene linked to Creutzfeldt-Jakob disease in Sephardic Jews of Libyan and non-Libyan origin. *Lancet*, **336**, 637-638.

Goldfarb, L.G., Mitrova, E., Brown, P., Toh, B.K. and Gajdusek, D.C. (1990b) Mutation in codon 200 of scrapie amyloid protein gene in two clusters of Creutzfeldt-Jakob disease in Slovakia. *Lancet*, **336**, 514-515.

Goldgaber, D., Goldfarb, L.G., Brown, P., Asher, D.M., Brown, W.T., Lin, S., Teener, J.W., Feinstone, S.M., Rubenstein, R., Kascsak, R.J. and *et al*. (1989) Mutations in familial Creutzfeldt-Jakob disease and Gerstmann-Straussler-Scheinker's syndrome. *Exp Neurol*, **106**, 204-206.

Goni, F., Knudsen, E., Schreiber, F., Scholtzova, H., Pankiewicz, J., Carp, R., Meeker, H.C., Rubenstein, R., Brown, D.R., Sy, M.S., Chabalgoity, J.A., Sigurdsson, E.M. and Wisniewski, T. (2005) Mucosal vaccination delays or prevents prion infection via an oral route. *Neuroscience*, **133**, 413-421.

Gossert, A.D., Bonjour, S., Lysek, D.A., Fiorito, F. and Wuthrich, K. (2005) Prion protein NMR structures of elk and of mouse/elk hybrids. *Proc Natl Acad Sci U S A*, **102**, 646-650.

Graner, E., Mercadante, A.F., Zanata, S.M., Forlenza, O.V., Cabral, A.L., Veiga, S.S., Juliano, M.A., Roesler, R., Walz, R., Minetti, A., Izquierdo, I., Martins, V.R. and Brentani, R.R. (2000) Cellular prion protein binds laminin and mediates neuritogenesis. *Brain Res Mol Brain Res*, **76**, 85-92.

Griffith, J.S. (1967) Self-replication and scrapie. *Nature*, **215**, 1043-1044.

Hadlow, W.J. (1959) Scrapie and kuru. *Lancet*, **2**, 289-290.

Hadlow, W.J., Kennedy, R.C. and Race, R.E. (1982) Natural infection of Suffolk sheep with scrapie virus. *J Infect Dis*, **146**, 657-664.

Haik, S., Brandel, J.P., Salomon, D., Sazdovitch, V., Delasnerie-Laupretre, N., Laplanche, J.L., Faucheux, B.A., Soubrie, C., Boher, E., Belorgey, C., Hauw, J.J. and Alperovitch, A. (2004) Compassionate use of quinacrine in Creutzfeldt-Jakob disease fails to show significant effects. *Neurology*, **63**, 2413-2415.

Hake, L.E. and Richter, J.D. (1994) CPEB is a specificity factor that mediates cytoplasmic polyadenylation during Xenopus oocyte maturation. *Cell*, **79**, 617-627.

Haltia, M., Kovanen, J., Goldfarb, L.G., Brown, P. and Gajdusek, D.C. (1991) Familial Creutzfeldt-Jakob disease in Finland: epidemiological, clinical, pathological and molecular genetic studies. *Eur J Epidemiol*, **7**, 494-500.

Hanson, R.P., Eckroade, R.J., Marsh, R.F., Zu Rhein, G.M., Kanitz, C.L. and Gustafson, D.P. (1971) Susceptibility of mink to sheep scrapie. *Science*, **172**, 859-861.

Hara, H., Monsonego, A., Yuasa, K., Adachi, K., Xiao, X., Takeda, S., Takahashi, K., Weiner, H.L. and Tabira, T. (2004) Development of a safe oral Abeta vaccine using recombinant adeno-associated virus vector for Alzheimer's disease. *J Alzheimers Dis*, **6**, 483-488.

Haraguchi, T., Fisher, S., Olofsson, S., Endo, T., Groth, D., Tarentino, A., Borchelt, D.R., Teplow, D., Hood, L., Burlingame, A. and *et al*. (1989) Asparagine-linked glycosylation of the scrapie and cellular prion proteins. *Arch Biochem Biophys*, **274**, 1-13.

Harborth, J., Elbashir, S.M., Vandenburgh, K., Manninga, H., Scaringe, S.A., Weber, K. and Tuschl, T. (2003) Sequence, chemical, and structural variation of small interfering RNAs and short hairpin RNAs and the effect on mammalian gene silencing. *Antisense Nucleic Acid Drug Dev*, **13**, 83-105.

Harris, D.A. (2003) Trafficking, turnover and membrane topology of PrP. *Br Med Bull*, **66**, 71-85.

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.

Hawthorne, D.C. and Mortimer, R.K. (1968) Genetic mapping of nonsense suppressors in yeast. *Genetics*, **60**, 735-742.

Hegde, R.S., Mastrianni, J.A., Scott, M.R., DeFea, K.A., Tremblay, P., Torchia, M., DeArmond, S.J., Prusiner, S.B. and Lingappa, V.R. (1998) A transmembrane form of the prion protein in neurodegenerative disease. *Science*, **279**, 827-834.

Hegde, R.S., Tremblay, P., Groth, D., DeArmond, S.J., Prusiner, S.B. and Lingappa, V.R. (1999) Transmissible and genetic prion diseases share a common pathway of neurodegeneration. *Nature*, **402**, 822-826.

Heppner, F.L., Musahl, C., Arrighi, I., Klein, M.A., Rulicke, T., Oesch, B., Zinkernagel, R.M., Kalinke, U. and Aguzzi, A. (2001) Prevention of scrapie pathogenesis by transgenic expression of anti-prion protein antibodies. *Science*, **294**, 178-182.

Hill, A.F., Desbruslais, M., Joiner, S., Sidle, K.C., Gowland, I., Collinge, J., Doey, L.J. and Lantos, P. (1997) The same prion strain causes vCJD and BSE. *Nature*, **389**, 448-450, 526.

Hölscher, C., Delius, H. and Burkle, A. (1998) Overexpression of nonconvertible PrPc delta114-121 in scrapie-infected mouse neuroblastoma cells leads to trans-dominant inhibition of wild-type PrP(Sc) accumulation. *J Virol*, **72**, 1153-1159.

Hooper, N.M. (1999) Detergent-insoluble glycosphingolipid/cholesterol-rich membrane domains, lipid rafts and caveolae (review). *Mol Membr Biol*, **16**, 145-156.

Hope, J., Morton, L.J., Farquhar, C.F., Multhaup, G., Beyreuther, K. and Kimberlin, R.H. (1986) The major polypeptide of scrapie-associated fibrils (SAF) has the same size, charge distribution and N-terminal protein sequence as predicted for the normal brain protein (PrP). *Embo J*, **5**, 2591-2597.

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.

Horonchik, L., Tzaban, S., Ben-Zaken, O., Yedidia, Y., Rouvinski, A., Papy-Garcia, D., Barritault, D., Vlodavsky, I. and Taraboulos, A. (2005) Heparan sulfate is a cellular receptor for purified infectious prions. *J Biol Chem*, **280**, 17062-17067.

Hsiao, K., Baker, H.F., Crow, T.J., Poulter, M., Owen, F., Terwilliger, J.D., Westaway, D., Ott, J. and Prusiner, S.B. (1989) Linkage of a prion protein missense variant to Gerstmann-Straussler syndrome. *Nature*, **338**, 342-345.

Hsiao, K., Dlouhy, S.R., Farlow, M.R., Cass, C., Da Costa, M., Conneally, P.M., Hodes, M.E., Ghetti, B. and Prusiner, S.B. (1992) Mutant prion proteins in Gerstmann-Straussler-Scheinker disease with neurofibrillary tangles. *Nat Genet*, **1**, 68-71.

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.

Hundt, C. and Weiss, S. (2004) The prion-like protein Doppel fails to interact with itself, the prion protein and the 37 kDa/67 kDa laminin receptor in the yeast two-hybrid system. *Biochim Biophys Acta*, **1689**, 1-5.

Ingrosso, L., Ladogana, A. and Pocchiari, M. (1995) Congo red prolongs the incubation period in scrapie-infected hamsters. *J Virol*, **69**, 506-508.

Jackers, P., Clausse, N., Fernandez, M., Berti, A., Princen, F., Wewer, U., Sobel, M.E. and Castronovo, V. (1996a) Seventeen copies of the human 37 kDa laminin receptor precursor/p40 ribosome-associated protein gene are processed pseudogenes arisen from retropositional events. *Biochim Biophys Acta*, **1305**, 98-104.

Jackers, P., Minoletti, F., Belotti, D., Clausse, N., Sozzi, G., Sobel, M.E. and Castronovo, V. (1996b) Isolation from a multigene family of the active human gene of the metastasis-associated multifunctional protein 37LRP/p40 at chromosome 3p21.3. *Oncogene*, **13**, 495-503.

Jackson, T., Ellard, F.M., Ghazaleh, R.A., Brookes, S.M., Blakemore, W.E., Corteyn, A.H., Stuart, D.I., Newman, J.W. and King, A.M. (1996) Efficient infection of cells in culture by type O foot-and-mouth disease virus requires binding to cell surface heparan sulfate. *J Virol*, **70**, 5282-5287.

Jakob, A. (1921) Über eigenartige Erkrankungen des Zentralnervensystems mit bemerkenswerten anatomischen Befunden (spastische Pseudosklerose-Encephalomyelopathie mit dissemierten Degenerationsherden). *Vorläufige Mitteilung. Z. Ges. Neurol. Psychiatr*, **64**, 147-228.

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

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.

Katsuki, M., Sato, M., Kimura, M., Yokoyama, M., Kobayashi, K. and Nomura, T. (1988) Conversion of normal behavior to shiverer by myelin basic protein antisense cDNA in transgenic mice. *Science*, **241**, 593-595.

Kimberlin, R.H. and Walker, C.A. (1983) The antiviral compound HPA-23 can prevent scrapie when administered at the time of infection. *Arch Virol*, **78**, 9-18.

Kirby, L., Birkett, C.R., Rudyk, H., Gilbert, I.H. and Hope, J. (2003) In vitro cell-free conversion of bacterial recombinant PrP to PrPres as a model for conversion. *J Gen Virol*, **84**, 1013-1020.

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., 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.

Kishida, H., Sakasegawa, Y., Watanabe, K., Yamakawa, Y., Nishijima, M., Kuroiwa, Y., Hachiya, N.S. and Kaneko, K. (2004) Non-glycosylphosphatidylinositol (GPI)-anchored recombinant prion protein with dominant-negative mutation inhibits PrPSc replication in vitro. *Amyloid*, **11**, 14-20.

Klamt, F., Dal-Pizzol, F., Conte da Frota, M.J., Walz, R., Andrades, M.E., da Silva, E.G., Brentani, R.R., Izquierdo, I. and Fonseca Moreira, J.C. (2001) Imbalance of antioxidant defense in mice lacking cellular prion protein. *Free Radic Biol Med*, **30**, 1137-1144.

Klatzo, I., Gajdusek, D.C. and Zigas, V. (1959) Pathology of Kuru. *Lab Invest*, **8**, 799-847.

Knaus, K.J., Morillas, M., Swietnicki, W., Malone, M., Surewicz, W.K. and Yee, V.C. (2001) Crystal structure of the human prion protein reveals a mechanism for oligomerization. *Nat Struct Biol*, **8**, 770-774.

Koch, C.A., Anderson, D., Moran, M.F., Ellis, C. and Pawson, T. (1991) SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. *Science*, **252**, 668-674.

Kocisko, D.A., Baron, G.S., Rubenstein, R., Chen, J., Kuizon, S. and Caughey, B. (2003) New inhibitors of scrapie-associated prion protein formation in a library of 2000 drugs and natural products. *J Virol*, **77**, 10288-10294.

Korth, C., May, B.C., Cohen, F.E. and Prusiner, S.B. (2001) Acridine and phenothiazine derivatives as pharmacotherapeutics for prion disease. *Proc Natl Acad Sci U S A*, **98**, 9836-9841.

Korth, C., Stierli, B., Streit, P., Moser, M., Schaller, O., Fischer, R., Schulz-Schaeffer, W., Kretzschmar, H., Raeber, A., Braun, U., Ehrensperger, F., Hornemann, S., Glockshuber, R., Riek, R., Billeter, M., Wuthrich, K. and Oesch, B. (1997) Prion (PrPSc)-specific epitope defined by a monoclonal antibody. *Nature*, **390**, 74-77.

Krasemann, S., Groschup, M.H., Harmeyer, S., Hunsmann, G. and Bodemer, W. (1996) Generation of monoclonal antibodies against human prion proteins in PrP0/0 mice. *Mol Med*, **2**, 725-734.

Kretzschmar, H.A. (1993) Human prion diseases (spongiform encephalopathies). *Arch Virol Suppl*, **7**, 261-293.

Kretzschmar, H.A., Prusiner, S.B., Stowring, L.E. and DeArmond, S.J. (1986) Scrapie prion proteins are synthesized in neurons. *Am J Pathol*, **122**, 1-5.

Kretzschmar, H.A., Tings, T., Madlung, A., Giese, A. and Herms, J. (2000) Function of PrP(C) as a copper-binding protein at the synapse. *Arch Virol Suppl*, 239-249.

Kurschner, C. and Morgan, J.I. (1995) The cellular prion protein (PrP) selectively binds to Bcl-2 in the yeast two-hybrid system. *Brain Res Mol Brain Res*, **30**, 165-168.

Lacroute, F. (1971) Non-Mendelian mutation allowing ureidosuccinic acid uptake in yeast. *J Bacteriol*, **106**, 519-522.

Laine, J., Marc, M.E., Sy, M.S. and Axelrad, H. (2001) Cellular and subcellular morphological localization of normal prion protein in rodent cerebellum. *Eur J Neurosci*, **14**, 47-56.

Landowski, T.H., Dratz, E.A. and Starkey, J.R. (1995) Studies of the structure of the metastasis-associated 67 kDa laminin binding protein: fatty acid acylation and evidence supporting dimerization of the 32 kDa gene product to form the mature protein. *Biochemistry*, **34**, 11276-11287.

Lansbury, P.T., Jr. and Caughey, B. (1995) The chemistry of scrapie infection: implications of the 'ice 9' metaphor. *Chem Biol*, **2**, 1-5.

Lasmezas, C. and Weiss, S. (2000) in Microbial Foodborne Diseases. Mechanisms of Pathogenicity and Toxin Synthesis (eds Cary JW., Linz JE. & Bhatnagar D.), Technomic, Lancaster, PA USA. 495-537.

Lasmezas, C.I., Deslys, J.P., Demaimay, R., Adjou, K.T., Hauw, J.J. and Dormont, D. (1996) Strain specific and common pathogenic events in murine models of scrapie and bovine spongiform encephalopathy. *J Gen Virol*, **77 (Pt 7)**, 1601-1609.

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, K.S., Linden, R., Prado, M.A., Brentani, R.R. and Martins, V.R. (2003) Towards cellular receptors for prions. *Rev Med Virol*, **13**, 399-408.

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.

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.

Leucht, C., Vana, K., Renner-Muller, I., Dormont, D., Lasmezas, C.I., Wolf, E. and Weiss, S. (2004) Knock-down of the 37-kDa/67-kDa laminin receptor in mouse brain by transgenic expression of specific antisense LRP RNA. *Transgenic Res*, **13**, 81-85.

Leucht, C. and Weiss, S. (2002) in BSE- Wahnsinn und Wirklichkeit (eds Brehm M. & Müller M.), Nova Acta Leopoldina, Halle, Germany. **87**, 39-54.

Lewis, D.L., Hagstrom, J.E., Loomis, A.G., Wolff, J.A. and Herweijer, H. (2002) Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. *Nat Genet*, **32**, 107-108.

Liao, Y.C., Lebo, R.V., Clawson, G.A. and Smuckler, E.A. (1986) Human prion protein cDNA: molecular cloning, chromosomal mapping, and biological implications. *Science*, **233**, 364-367.

Lin, X. (2004) Functions of heparan sulfate proteoglycans in cell signaling during development. *Development*, **131**, 6009-6021.

Liu, T., Li, R., Wong, B.S., Liu, D., Pan, T., Petersen, R.B., Gambetti, P. and Sy, M.S. (2001) Normal cellular prion protein is preferentially expressed on subpopulations of murine hemopoietic cells. *J Immunol*, **166**, 3733-3742.

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.

Lo Bianco, C., Schneider, B.L., Bauer, M., Sajadi, A., Brice, A., Iwatsubo, T. and Aebischer, P. (2004) Lentiviral vector delivery of parkin prevents dopaminergic degeneration in an alpha-synuclein rat model of Parkinson's disease. *Proc Natl Acad Sci U S A*, **101**, 17510-17515.

Lopez Garcia, 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.

Ludwig, G.V., Kondig, J.P. and Smith, J.F. (1996) A putative receptor for Venezuelan equine encephalitis virus from mosquito cells. *J Virol*, **70**, 5592-5599.

Lugaresi, E., Medori, R., Montagna, P., Baruzzi, A., Cortelli, P., Lugaresi, A., Tinuper, P., Zucconi, M. and Gambetti, P. (1986) Fatal familial insomnia and dysautonomia with selective degeneration of thalamic nuclei. *N Engl J Med*, **315**, 997-1003.

Lysek, D.A., Schorn, C., Nivon, L.G., Esteve-Moya, V., Christen, B., Calzolai, L., von Schroetter, C., Fiorito, F., Herrmann, T., Guntert, P. and Wuthrich, K. (2005) Prion protein NMR structures of cats, dogs, pigs, and sheep. *Proc Natl Acad Sci U S A*, **102**, 640-645.

Ma, J. and Lindquist, S. (2001) Wild-type PrP and a mutant associated with prion disease are subject to retrograde transport and proteasome degradation. *Proc Natl Acad Sci U S A*, **98**, 14955-14960.

Ma, J. and Lindquist, S. (2002) Conversion of PrP to a self-perpetuating PrPSc-like conformation in the cytosol. *Science*, **298**, 1785-1788.

Ma, J., Wollmann, R. and Lindquist, S. (2002) Neurotoxicity and neurodegeneration when PrP accumulates in the cytosol. *Science*, **298**, 1781-1785.

Magri, G., Clerici, M., Dall'Ara, P., Biasin, M., Caramelli, M., Casalone, C., Giannino, M.L., Longhi, R., Piacentini, L., Della Bella, S., Gazzuola, P., Martino, P.A., Pollera, C., Puricelli, M., Servida, F., Crescio, I., Boasso, A., Ponti, W. and Poli, G. (2005) Decrease in pathology and progression of scrapie after immunisation with synthetic prion protein peptides in hamsters. *Vaccine*, **23**, 2862-2868.

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.

Makrides, S., Chitpatima, S.T., Bandyopadhyay, R. and Brawerman, G. (1988) Nucleotide sequence for a major messenger RNA for a 40 kilodalton polypeptide that is under translational control in mouse tumor cells. *Nucleic Acids Res*, **16**, 2349.

Mange, A., Nishida, N., Milhavet, O., McMahon, H.E., Casanova, D. and Lehmann, S. (2000) Amphotericin B inhibits the generation of the scrapie isoform of the prion protein in infected cultures. *J Virol*, **74**, 3135-3140.

Marella, M., Lehmann, S., Grassi, J. and Chabry, J. (2002) Filipin prevents pathological prion protein accumulation by reducing endocytosis and inducing cellular PrP release. *J Biol Chem*, **277**, 25457-25464.

Marr, R.A., Rockenstein, E., Mukherjee, A., Kindy, M.S., Hersh, L.B., Gage, F.H., Verma, I.M. and Masliah, E. (2003) Neprilysin gene transfer reduces human amyloid pathology in transgenic mice. *J Neurosci*, **23**, 1992-1996.

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

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 Hanson, R.P. (1979) In: Prusiner SB, Hadlow WJ (eds). 1979. Slow transmissible diseases of the nervous system. New York: Academic Press. 451-460.

Martins, V.R., Graner, E., Garcia-Abreu, J., de Souza, S.J., Mercadante, A.F., Veiga, S.S., Zanata, S.M., Neto, V.M. and Brentani, R.R. (1997) Complementary hydropathy identifies a cellular prion protein receptor. *Nat Med*, **3**, 1376-1382.

Masison, D.C. and Wickner, R.B. (1995) Prion-inducing domain of yeast Ure2p and protease resistance of Ure2p in prion-containing cells. *Science*, **270**, 93-95.

Masters, C.L., Gajdusek, D.C. and Gibbs, C.J., Jr. (1981) Creutzfeldt-Jakob disease virus isolations from the Gerstmann-Straussler syndrome with an analysis of the various forms of amyloid plaque deposition in the virus-induced spongiform encephalopathies. *Brain*, **104**, 559-588.

Mastrianni, J.A., Capellari, S., Telling, G.C., Han, D., Bosque, P., Prusiner, S.B. and DeArmond, S.J. (2001) Inherited prion disease caused by the V210I mutation: transmission to transgenic mice. *Neurology*, **57**, 2198-2205.

Mastrianni, J.A., Nixon, R., Layzer, R., Telling, G.C., Han, D., DeArmond, S.J. and Prusiner, S.B. (1999) Prion protein conformation in a patient with sporadic fatal insomnia. *N Engl J Med*, **340**, 1630-1638.

Matta, H., Hozayev, B., Tomar, R., Chugh, P. and Chaudhary, P.M. (2003) Use of lentiviral vectors for delivery of small interfering RNA. *Cancer Biol Ther*, **2**, 206-210.

Mayer, R.J., Landon, M., Laszlo, L., Lennox, G. and Lowe, J. (1992) Protein processing in lysosomes: the new therapeutic target in neurodegenerative disease. *Lancet*, **340**, 156-159.

McGowan, J.P. (1922) Scrapie in sheep. *Scott.J.Agric*, **5**, 365-375.

McKinley, M.P., Meyer, R.K., Kenaga, L., Rahbar, F., Cotter, R., Serban, A. and Prusiner, S.B. (1991) Scrapie prion rod formation in vitro requires both detergent extraction and limited proteolysis. *J Virol*, **65**, 1340-1351.

McLean, C.A., Storey, E., Gardner, R.J., Tannenberg, A.E., Cervenakova, L. and Brown, P. (1997) The D178N (cis-129M) "fatal familial insomnia" mutation associated with diverse clinicopathologic phenotypes in an Australian kindred. *Neurology*, **49**, 552-558.

Medori, R., Tritschler, H.J., LeBlanc, A., Villare, F., Manetto, V., Chen, H.Y., Xue, R., Leal, S., Montagna, P., Cortelli, P. and *et al.* (1992) Fatal familial insomnia, a prion disease with a mutation at codon 178 of the prion protein gene. *N Engl J Med*, **326**, 444-449.

Meggio, F., Negro, A., Sarno, S., Ruzzene, M., Bertoli, A., Sorgato, M.C. and Pinna, L.A. (2000) Bovine prion protein as a modulator of protein kinase CK2. *Biochem J*, **352 Pt 1**, 191-196.

Merlini, G., Ascari, E., Amboldi, N., Bellotti, V., Arbustini, E., Perfetti, V., Ferrari, M., Zorzoli, I., Marinone, M.G., Garini, P. and *et al.* (1995) Interaction of the anthracycline 4'-iodo-4'-deoxydoxorubicin with amyloid fibrils: inhibition of amyloidogenesis. *Proc Natl Acad Sci U S A*, **92**, 2959-2963.

Michelitsch, M.D. and Weissman, J.S. (2000) A census of glutamine/asparagine-rich regions: implications for their conserved function and the prediction of novel prions. *Proc Natl Acad Sci U S A*, **97**, 11910-11915.

Miller, M.W., Williams, E.S., McCarty, C.W., Spraker, T.R., Kreeger, T.J., Larsen, C.T. and Thorne, E.T. (2000) Epizootiology of chronic wasting disease in free-ranging cervids in Colorado and Wyoming. *J Wildl Dis*, **36**, 676-690.

Moelling, K., Schulze, T. and Diringer, H. (1989) Inhibition of human immunodeficiency virus type 1 RNase H by sulfated polyanions. *J Virol*, **63**, 5489-5491.

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.

Morel, E., Andrieu, T., Casagrande, F., Gauczynski, S., Weiss, S., Grassi, J., Rousset, M., Dormont, D. and Chambaz, J. (2005) Bovine Prion Is Endocytosed by Human Enterocytes via the 37 kDa/67 kDa Laminin Receptor. *Am J Pathol*, **167**, 1033-1042.

Morel, E., Fouquet, S., Chateau, D., Yvernault, L., Frobert, Y., Pincon-Raymond, M., Chambaz, J., Pillot, T. and Rousset, M. (2004) The cellular prion protein PrPc is expressed in human enterocytes in cell-cell junctional domains. *J Biol Chem*, **279**, 1499-1505.

Moudjou, M., Frobert, Y., Grassi, J. and La Bonnardiere, C. (2001) Cellular prion protein status in sheep: tissue-specific biochemical signatures. *J Gen Virol*, **82**, 2017-2024.

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

Nagayama, M., Shinohara, Y., Furukawa, H. and Kitamoto, T. (1996) Fatal familial insomnia with a mutation at codon 178 of the prion protein gene: first report from Japan. *Neurology*, **47**, 1313-1316.

Naldini, L., Blomer, U., Gage, F.H., Trono, D. and Verma, I.M. (1996a) Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc Natl Acad Sci U S A*, **93**, 11382-11388.

Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F.H., Verma, I.M. and Trono, D. (1996b) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science*, **272**, 263-267.

Naslavsky, N., Stein, R., Yanai, A., Friedlander, G. and Taraboulos, A. (1997) Characterization of detergent-insoluble complexes containing the cellular prion protein and its scrapie isoform. *J Biol Chem*, **272**, 6324-6331.

Nichols, B.J. (2002) A distinct class of endosome mediates clathrin-independent endocytosis to the Golgi complex. *Nat Cell Biol*, **4**, 374-378.

Nieto, A., Goldfarb, L.G., Brown, P., McCombie, W.R., Trapp, S., Asher, D.M. and Gajdusek, D.C. (1991) Codon 178 mutation in ethnically diverse Creutzfeldt-Jakob disease families. *Lancet*, **337**, 622-623.

Nikles, D., Bach, P., Boller, K., Merten, C.A., Montrasio, F., Heppner, F.L., Aguzzi, A., Cichutek, K., Kalinke, U. and Buchholz, C.J. (2005) Circumventing tolerance to the prion protein (PrP): vaccination with PrP-displaying retrovirus particles induces humoral immune responses against the native form of cellular PrP. *J Virol*, **79**, 4033-4042.

Nunziante, M., Gilch, S. and Schätzl, H.M. (2003) Essential role of the prion protein N terminus in subcellular trafficking and half-life of cellular prion protein. *J Biol Chem*, **278**, 3726-3734.

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 *et al.* (1985) A cellular gene encodes scrapie PrP 27-30 protein. *Cell*, **40**, 735-746.

Oh, T.K., Li, M.Z. and Kim, S.T. (2005) Gene therapy for diabetes mellitus in rats by intramuscular injection of lentivirus containing insulin gene. *Diabetes Res Clin Pract*.

Osterhout, D.J., Wolven, A., Wolf, R.M., Resh, M.D. and Chao, M.V. (1999) Morphological differentiation of oligodendrocytes requires activation of Fyn tyrosine kinase. *J Cell Biol*, **145**, 1209-1218.

Otto, M., Cepek, L., Ratzka, P., Doehlinger, S., Boekhoff, I., Wiltfang, J., Irle, E., Pergande, G., Ellers-Lenz, B., Windl, O., Kretzschmar, H.A., Poser, S. and Prange, H. (2004) Efficacy of flupirtine on cognitive function in patients with CJD: A double-blind study. *Neurology*, **62**, 714-718.

Padovani, A., D'Alessandro, M., Parchi, P., Cortelli, P., Anzola, G.P., Montagna, P., Vignolo, L.A., Petraroli, R., Pocchiari, M., Lugaresi, E. and Gambetti, P. (1998) Fatal familial insomnia in a new Italian kindred. *Neurology*, **51**, 1491-1494.

Paitel, E., Fahraeus, R. and Checler, F. (2003) Cellular prion protein sensitizes neurons to apoptotic stimuli through Mdm2-regulated and p53-dependent caspase 3-like activation. *J Biol Chem*, **278**, 10061-10066.

Paitel, E., Sunyach, C., Alves da Costa, C., Bourdon, J.C., Vincent, B. and Checler, F. (2004) Primary cultured neurons devoid of cellular prion display lower responsiveness to staurosporine through the control of p53 at both transcriptional and post-transcriptional levels. *J Biol Chem*, **279**, 612-618.

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.

Paltrinieri, S., Comazzi, S., Spagnolo, V., Rondena, M., Ponti, W. and Ceciliani, F. (2004) Bovine Doppel (Dpl) and prion protein (PrP) expression on lymphoid tissue and circulating leukocytes. *J Histochem Cytochem*, **52**, 1639-1645.

Pan, K.M., Baldwin, M., Nguyen, J., Gasset, M., Serban, A., Groth, D., Mehlhorn, I., Huang, Z., Fletterick, R.J., Cohen, F.E. and *et al.* (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.

Pan, T., Wong, B.S., Liu, T., Li, R., Petersen, R.B. and Sy, M.S. (2002) Cell-surface prion protein interacts with glycosaminoglycans. *Biochem J*, **368**, 81-90.

Panegyres, P.K., Toufexis, K., Kakulas, B.A., Cernevakova, L., Brown, P., Ghetti, B., Piccardo, P. and Dlouhy, S.R. (2001) A new PRNP mutation (G131V) associated with Gerstmann-Straussler-Scheinker disease. *Arch Neurol*, **58**, 1899-1902.

Papassotiropoulos, A., Wollmer, M.A., Aguzzi, A., Hock, C., Nitsch, R.M. and de Quervain, D.J. (2005) The prion gene is associated with human long-term memory. *Hum Mol Genet*, **14**, 2241-2246.

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.

Parkin, E.T., Turner, A.J. and Hooper, N.M. (1999) Amyloid precursor protein, although partially detergent-insoluble in mouse cerebral cortex, behaves as an atypical lipid raft protein. *Biochem J*, **344 Pt 1**, 23-30.

Patel, M., Yanagishita, M., Roderiquez, G., Bou-Habib, D.C., Oravecz, T., Hascall, V.C. and Norcross, M.A. (1993) Cell-surface heparan sulfate proteoglycan mediates HIV-1 infection of T-cell lines. *AIDS Res Hum Retroviruses*, **9**, 167-174.

Pattison, I.C.H. (1965) Experiments with scrapie wih special reference to the nature of the agent and the pathology of disease. In: Slow, latent and temperate virus infections, NINDB Monogr., ed. DC Gajdusek, CJ Gibbs, MP Alpers, 2: 249-57. Washington DC: US Gov. Print. Off.

Pattison, I.H., Hoare, M.N., Jebbett, J.N. and Watson, W.A. (1972) Spread of scrapie to sheep and goats by oral dosing with foetal membranes from scrapie-affected sheep. *Vet Rec*, **90**, 465-468.

Pattison, I.H. and Millson, G.C. (1961) Scrapie produced experimentally in goats with special reference to the clinical syndrome. *J Comp Pathol*, **71**, 101-109.

Pauly, P.C. and Harris, D.A. (1998) Copper stimulates endocytosis of the prion protein. *J Biol Chem*, **273**, 33107-33110.

Peoc'h, K., Serres, C., Frobert, Y., Martin, C., Lehmann, S., Chasseigneaux, S., Sazdovitch, V., Grassi, J., Jouannet, P., Launay, J.M. and Laplanche, J.L. (2002) The human "prion-like" protein Doppel is expressed in both Sertoli cells and spermatozoa. *J Biol Chem*, **277**, 43071-43078.

Pepin, M.C., Pothier, F. and Barden, N. (1992) Impaired type II glucocorticoid-receptor function in mice bearing antisense RNA transgene. *Nature*, **355**, 725-728.

Peretz, D., Williamson, R.A., Kaneko, K., Vergara, J., Leclerc, E., Schmitt-Ulms, G., Mehlhorn, I.R., Legname, G., Wormald, M.R., Rudd, P.M., Dwek, R.A., Burton, D.R. and Prusiner, S.B. (2001) Antibodies inhibit prion propagation and clear cell cultures of prion infectivity. *Nature*, **412**, 739-743.

Perrier, V., Kaneko, K., Safar, J., Vergara, J., Tremblay, P., DeArmond, S.J., Cohen, F.E., Prusiner, S.B. and Wallace, A.C. (2002) Dominant-negative inhibition of prion replication in transgenic mice. *Proc Natl Acad Sci U S A*, **99**, 13079-13084.

Pocchiari, M., Schmittinger, S. and Masullo, C. (1987) Amphotericin B delays the incubation period of scrapie in intracerebrally inoculated hamsters. *J Gen Virol*, **68 (Pt 1)**, 219-223.

Priola, S.A. and Caughey, B. (1994) Inhibition of scrapie-associated PrP accumulation. Probing the role of glycosaminoglycans in amyloidogenesis. *Mol Neurobiol*, **8**, 113-120.

Priola, S.A., Raines, A. and Caughey, W.S. (2000) Porphyrin and phthalocyanine antiscrapie compounds. *Science*, **287**, 1503-1506.

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

Prusiner, S.B. (1982) Research on scrapie. *Lancet*, **2**, 494-495.

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

Prusiner, S.B. (1994) Molecular biology and genetics of prion diseases. *Philos Trans R Soc Lond B Biol Sci*, **343**, 447-463.

Prusiner, S.B. (1998) Prions. *Proc Natl Acad Sci U S A*, **95**, 13363-13383.

Prusiner, S.B., Bolton, D.C., Groth, D.F., Bowman, K.A., Cochran, S.P. and McKinley, M.P. (1982) Further purification and characterization of scrapie prions. *Biochemistry*, **21**, 6942-6950.

Prusiner, S.B., Cochran, S.P., Groth, D.F., Downey, D.E., Bowman, K.A. and Martinez, H.M. (1982b) Measurement of the scrapie agent using an incubation time interval assay. *Ann Neurol*, **11**, 353-358.

Prusiner, S.B., Gajdusek, C. and Alpers, M.P. (1982c) Kuru with incubation periods exceeding two decades. *Ann Neurol*, **12**, 1-9.

Prusiner, S.B., Groth, D., Serban, A., Stahl, N. and Gabizon, R. (1993) Attempts to restore scrapie prion infectivity after exposure to protein denaturants. *Proc Natl Acad Sci U S A*, **90**, 2793-2797.

Prusiner, S.B., Scott, M., Foster, D., Pan, K.M., Groth, D., Mirenda, C., Torchia, M., Yang, S.L., Serban, D., Carlson, G.A. and *et al*. (1990) Transgenetic studies implicate interactions between homologous PrP isoforms in scrapie prion replication. *Cell*, **63**, 673-686.

Prusiner, S.B., Scott, M.R., DeArmond, S.J. and Cohen, F.E. (1998) Prion protein biology. *Cell*, **93**, 337-348.

Ralph, G.S., Radcliffe, P.A., Day, D.M., Carthy, J.M., Leroux, M.A., Lee, D.C., Wong, L.F., Bilsland, L.G., Greensmith, L., Kingsman, S.M., Mitrophanous, K.A., Mazarakis, N.D. and Azzouz, M. (2005) Silencing mutant SOD1 using RNAi protects against neurodegeneration and extends survival in an ALS model. *Nat Med*, **11**, 429-433.

Rao, N.C., Barsky, S.H., Terranova, V.P. and Liotta, L.A. (1983) Isolation of a tumor cell laminin receptor. *Biochem Biophys Res Commun*, **111**, 804-808.

Raoul, C., Abbas-Terki, T., Bensadoun, J.C., Guillot, S., Haase, G., Szulc, J., Henderson, C.E. and Aebischer, P. (2005) Lentiviral-mediated silencing of SOD1 through RNA interference retards disease onset and progression in a mouse model of ALS. *Nat Med*, **11**, 423-428.

Ravilochan, K., Seetharam, W. and Dinakar, I. (1983) Vocal cord paralysis in neuraxial malformation. *Indian J Pediatr*, **50**, 557-559.

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.

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.

Robakis, N.K., Devine-Gage, E.A., Jenkins, E.C., Kascsak, R.J., Brown, W.T., Krawczun, M.S. and Silverman, W.P. (1986) Localization of a human gene homologous to the PrP gene on the p arm of chromosome 20 and detection of PrP-related antigens in normal human brain. *Biochem Biophys Res Commun*, **140**, 758-765.

Romanov, V., Sobel, M.E., pinto da Silva, P., Menard, S. and Castronovo, V. (1994) Cell localization and redistribution of the 67 kD laminin receptor and alpha 6 beta 1 integrin subunits in response to laminin stimulation: an immunogold electron microscopy study. *Cell Adhes Commun*, **2**, 201-209.

Rossi, D., Cozzio, A., Flechsig, E., Klein, M.A., Rulicke, T., Aguzzi, A. and Weissmann, C. (2001) Onset of ataxia and Purkinje cell loss in PrP null mice inversely correlated with Dpl level in brain. *Embo J*, **20**, 694-702.

Rostand, K.S. and Esko, J.D. (1997) Microbial adherence to and invasion through proteoglycans. *Infect Immun*, **65**, 1-8.

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.

Sanftner, L.M., Sommer, J.M., Suzuki, B.M., Smith, P.H., Vijay, S., Vargas, J.A., Forsayeth, J.R., Cunningham, J., Bankiewicz, K.S., Kao, H., Bernal, J., Pierce, G.F. and Johnson, K.W. (2005) AAV2-mediated gene delivery to monkey putamen: evaluation of an infusion device and delivery parameters. *Exp Neurol*, **194**, 476-483.

Sarnataro, D., Campana, V., Paladino, S., Stornaiuolo, M., Nitsch, L. and Zurzolo, C. (2004) PrP(C) association with lipid rafts in the early secretory pathway stabilizes its cellular conformation. *Mol Biol Cell*, **15**, 4031-4042.

Sato, M., Kinoshita, K., Kaneda, Y., Saeki, Y., Iwamatsu, A. and Tanaka, K. (1996) Analysis of nuclear localization of laminin binding protein precursor p40 (LBP/p40). *Biochem Biophys Res Commun*, **229**, 896-901.

Scaravilli, F., Cordery, R.J., Kretzschmar, H., Gambetti, P., Brink, B., Fritz, V., Temlett, J., Kaplan, C., Fish, D., An, S.F., Schulz-Schaeffer, W.J. and Rossor, M.N. (2000) Sporadic fatal insomnia: a case study. *Ann Neurol*, **48**, 665-668.

Schmitt-Ulms, G., Legname, G., Baldwin, M.A., Ball, H.L., Bradon, N., Bosque, P.J., Crossin, K.L., Edelman, G.M., DeArmond, S.J., Cohen, F.E. and Prusiner, S.B. (2001) Binding of neural cell adhesion molecules (N-CAMs) to the cellular prion protein. *J Mol Biol*, **314**, 1209-1225.

Schätzl, H.M., Da Costa, M., Taylor, L., Cohen, F.E. and Prusiner, S.B. (1995) Prion protein gene variation among primates. *J Mol Biol*, **245**, 362-374.

Scott, M., Foster, D., Mirenda, C., Serban, D., Coufal, F., Walchli, M., Torchia, M., Groth, D., Carlson, G., DeArmond, S.J. and *et al*. (1989) Transgenic mice expressing hamster prion protein produce species-specific scrapie infectivity and amyloid plaques. *Cell*, **59**, 847-857.

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.

Shaked, G.M., Engelstein, R., Avraham, I., Kahana, E. and Gabizon, R. (2003) Dimethyl sulfoxide delays PrP sc accumulation and disease symptoms in prion-infected hamsters. *Brain Res*, **983**, 137-143.

Shieh, M.T., WuDunn, D., Montgomery, R.I., Esko, J.D. and Spear, P.G. (1992) Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. *J Cell Biol*, **116**, 1273-1281.

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.

Shyng, S.L., Huber, M.T. and Harris, D.A. (1993) A prion protein cycles between the cell surface and an endocytic compartment in cultured neuroblastoma cells. *J Biol Chem*, **268**, 15922-15928.

Shyng, S.L., Moulder, K.L., Lesko, A. and Harris, D.A. (1995) The N-terminal domain of a glycolipid-anchored prion protein is essential for its endocytosis via clathrin-coated pits. *J Biol Chem*, **270**, 14793-14800.

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

Sigurdsson, B. (1954) Rida-a chronic encephalitis of sheep-with general remarks on infections which develop slowly and some of their special characteristics. *Br. Vet. J.*, **110**, 341-354.

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.

Simons, K. and Ikonen, E. (1997) Functional rafts in cell membranes. *Nature*, **387**, 569-572.

Sioud, M. and Sorensen, D.R. (2003) Cationic liposome-mediated delivery of siRNAs in adult mice. *Biochem Biophys Res Commun*, **312**, 1220-1225.

Snow, A.D., Sekiguchi, R.T., Nochlin, D., Kalaria, R.N. and Kimata, K. (1994) Heparan sulfate proteoglycan in diffuse plaques of hippocampus but not of cerebellum in Alzheimer's disease brain. *Am J Pathol*, **144**, 337-347.

Sofer, A. and Futerman, A.H. (1995) Cationic amphiphilic drugs inhibit the internalization of cholera toxin to the Golgi apparatus and the subsequent elevation of cyclic AMP. *J Biol Chem*, **270**, 12117-12122.

Solforosi, L., Criado, J.R., McGavern, D.B., Wirz, S., Sanchez-Alavez, M., Sugama, S., DeGiorgio, L.A., Volpe, B.T., Wiseman, E., Abalos, G., Masliah, E., Gilden, D., Oldstone, M.B., Conti, B. and Williamson, R.A. (2004) Cross-linking cellular prion protein triggers neuronal apoptosis in vivo. *Science*, **303**, 1514-1516.

Sondheimer, N. and Lindquist, S. (2000) Rnq1: an epigenetic modifier of protein function in yeast. *Mol Cell*, **5**, 163-172.

Sonnichsen, B., De Renzis, S., Nielsen, E., Rietdorf, J. and Zerial, M. (2000) Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. *J Cell Biol*, **149**, 901-914.

Sorensen, D.R., Leirdal, M. and Sioud, M. (2003) Gene silencing by systemic delivery of synthetic siRNAs in adult mice. *J Mol Biol*, **327**, 761-766.

Sparkes, R.S., Simon, M., Cohn, V.H., Fournier, R.E., Lem, J., Klisak, I., Heinzmann, C., Blatt, C., Lucero, M., Mohandas, T. and *et al*. (1986) Assignment of the human and mouse prion protein genes to homologous chromosomes. *Proc Natl Acad Sci U S A*, **83**, 7358-7362.

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

Stahl, N., Baldwin, M.A., Teplow, D.B., Hood, L., Gibson, B.W., Burlingame, A.L. and Prusiner, S.B. (1993) Structural studies of the scrapie prion protein using mass spectrometry and amino acid sequencing. *Biochemistry*, **32**, 1991-2002.

Stahl, N., Borchelt, D.R., Hsiao, K. and Prusiner, S.B. (1987) Scrapie prion protein contains a phosphatidylinositol glycolipid. *Cell*, **51**, 229-240.

Stahl, N. and Prusiner, S.B. (1991) Prions and prion proteins. *Faseb J*, **5**, 2799-2807.

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.

Stewart, R.S., Drisaldi, B. and Harris, D.A. (2001) A transmembrane form of the prion protein contains an uncleaved signal peptide and is retained in the endoplasmic Reticulum. *Mol Biol Cell*, **12**, 881-889.

Summerford, C. and Samulski, R.J. (1998) Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J Virol*, **72**, 1438-1445.

Sunyach, C., Jen, A., Deng, J., Fitzgerald, K.T., Frobert, Y., Grassi, J., McCaffrey, M.W. and Morris, R. (2003) The mechanism of internalization of glycosylphosphatidylinositol-anchored prion protein. *Embo J*, **22**, 3591-3601.

Supattapone, S., Wille, H., Uyechi, L., Safar, J., Tremblay, P., Szoka, F.C., Cohen, F.E., Prusiner, S.B. and Scott, M.R. (2001) Branched polyamines cure prion-infected neuroblastoma cells. *J Virol*, **75**, 3453-3461.

Tagliavini, F., McArthur, R.A., Canciani, B., Giaccone, G., Porro, M., Bugiani, M., Lievens, P.M., Bugiani, O., Peri, E., Dall'Ara, P., Rocchi, M., Poli, G., Forloni, G., Bandiera, T., Varasi, M., Suarato, A., Cassutti, P., Cervini, M.A., Lansen, J., Salmona, M. and Post, C. (1997) Effectiveness of anthracycline against experimental prion disease in Syrian hamsters. *Science*, **276**, 1119-1122.

Tanji, K., Saeki, K., Matsumoto, Y., Takeda, M., Hirasawa, K., Doi, K. and Onodera, T. (1995) Analysis of PrPc mRNA by in situ hybridization in brain, placenta, uterus and testis of rats. *Intervirology*, **38**, 309-315.

Taraboulos, A., Scott, M., Semenov, A., Avrahami, D., Laszlo, L. and Prusiner, S.B. (1995) Cholesterol depletion and modification of COOH-terminal targeting sequence of the prion protein inhibit formation of the scrapie isoform. *J Cell Biol*, **129**, 121-132.

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.

Thackray, A.M., Klein, M.A., Aguzzi, A. and Bujdoso, R. (2002) Chronic subclinical prion disease induced by low-dose inoculum. *J Virol*, **76**, 2510-2517.

Thormar, H. (1971) Slow infections of the central nervous system. II. *Z Neurol*, **199**, 151-166.

Tilly, G., Chapuis, J., Vilette, D., Laude, H. and Vilotte, J.L. (2003) Efficient and specific down-regulation of prion protein expression by RNAi. *Biochem Biophys Res Commun*, **305**, 548-551.

Tio, P.H., Jong, W.W. and Cardosa, M.J. (2005) Two dimensional VOPBA reveals laminin receptor (LAMR1) interaction with dengue virus serotypes 1, 2 and 3. *Virol J*, **2**, 25.

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.

Tomar, R.S., Matta, H. and Chaudhary, P.M. (2003) Use of adeno-associated viral vector for delivery of small interfering RNA. *Oncogene*, **22**, 5712-5715.

Tsai, B., Ye, Y. and Rapoport, T.A. (2002) Retro-translocation of proteins from the endoplasmic reticulum into the cytosol. *Nat Rev Mol Cell Biol*, **3**, 246-255.

Turk, E., Teplow, D.B., Hood, L.E. and Prusiner, S.B. (1988) Purification and properties of the cellular and scrapie hamster prion proteins. *Eur J Biochem*, **176**, 21-30.

Tuschl, T. (2001) RNA interference and small interfering RNAs. *Chembiochem*, **2**, 239-245.

Tuzi, N.L., Gall, E., Melton, D. and Manson, J.C. (2002) Expression of doppel in the CNS of mice does not modulate transmissible spongiform encephalopathy disease. *J Gen Virol*, **83**, 705-711.

van der Goot, F.G. and Harder, T. (2001) Raft membrane domains: from a liquid-ordered membrane phase to a site of pathogen attack. *Semin Immunol*, **13**, 89-97.

Vana, K. and Weiss, S. (2006) A trans-dominant negative 37kDa/67kDa laminin receptor muutant impairs PrPSc propagation in scrapie-infected neuronal cells. *J Mol Biol.*, **358**(1), 57-66

Vassallo, N. and Herms, J. (2003) Cellular prion protein function in copper homeostasis and redox signalling at the synapse. *J Neurochem*, **86**, 538-544.

Vey, M., Pilkuhn, S., Wille, H., Nixon, R., DeArmond, S.J., Smart, E.J., Anderson, R.G., Taraboulos, A. and Prusiner, S.B. (1996) Subcellular colocalization of the cellular and scrapie prion proteins in caveolae-like membranous domains. *Proc Natl Acad Sci U S A*, **93**, 14945-14949.

Vilotte, J.L., Soulier, S., Essalmani, R., Stinnakre, M.G., Vaiman, D., Lepourry, L., Da Silva, J.C., Besnard, N., Dawson, M., Buschmann, A., Groschup, M., Petit, S., Madelaine, M.F., Rakatobe, S., Le Dur, A., Vilette, D. and Laude, H. (2001) Markedly increased susceptibility to natural sheep scrapie of transgenic mice expressing ovine prp. *J Virol*, **75**, 5977-5984.

Walther, W. and Stein, U. (2000) Viral vectors for gene transfer: a review of their use in the treatment of human diseases. *Drugs*, **60**, 249-271.

Wang, K.S., Kuhn, R.J., Strauss, E.G., Ou, S. and Strauss, J.H. (1992) High-affinity laminin receptor is a receptor for Sindbis virus in mammalian cells. *J Virol*, **66**, 4992-5001.

Wang, L.H., Rothberg, K.G. and Anderson, R.G. (1993) Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. *J Cell Biol*, **123**, 1107-1117.

Weissmann, C. (1994) The prion connection: now in yeast? *Science*, **264**, 528-530.

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

Weissmann, C. and Aguzzi, A. (1997) Bovine spongiform encephalopathy and early onset variant Creutzfeldt-Jakob disease. *Curr Opin Neurobiol*, **7**, 695-700.

Weissmann, C. and Aguzzi, A. (1999) Perspectives: neurobiology. PrP's double causes trouble. *Science*, **286**, 914-915.

Weissmann, C. and Flechsig, E. (2003) PrP knock-out and PrP transgenic mice in prion research. *Br Med Bull*, **66**, 43-60.

Wells, G.A., Scott, A.C., Johnson, C.T., Gunning, R.F., Hancock, R.D., Jeffrey, M., Dawson, M. and Bradley, R. (1987) A novel progressive spongiform encephalopathy in cattle. *Vet Rec*, **121**, 419-420.

White, A.R., Enever, P., Tayebi, M., Mushens, R., Linehan, J., Brandner, S., Anstee, D., Collinge, J. and Hawke, S. (2003) Monoclonal antibodies inhibit prion replication and delay the development of prion disease. *Nature*, **422**, 80-83.

Whyte, S.M., Sylvester, I.D., Martin, S.R., Gill, A.C., Wopfner, F., Schätzl, 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. (1988) Bovine spongiform encephalopathy. *Vet Rec*, **122**, 614.

Will, R.G. (1999) The transmission of prions to humans. *Acta Paediatr Suppl*, **88**, 28-32.

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.

Williams, E.S. and Miller, M.W. (2002) Chronic wasting disease in deer and elk in North America. *Rev Sci Tech*, **21**, 305-316.

Williams, E.S. and Young, S. (1980) Chronic wasting disease of captive mule deer: a spongiform encephalopathy. *J Wildl Dis*, **16**, 89-98.

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

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.

Windl, O., Dempster, M., Estibeiro, P. and Lathe, R. (1995) A candidate marsupial PrP gene reveals two domains conserved in mammalian PrP proteins. *Gene*, **159**, 181-186.

Winklhofer, K.F. and Tatzelt, J. (2000) Cationic lipopolyamines induce degradation of PrPSc in scrapie-infected mouse neuroblastoma cells. *Biol Chem*, **381**, 463-469.

Wong, B.S., Liu, T., Paisley, D., Li, R., Pan, T., Chen, S.G., Perry, G., Petersen, R.B., Smith, M.A., Melton, D.W., Gambetti, P., Brown, D.R. and Sy, M.S. (2001) Induction of HO-1 and NOS in doppel-expressing mice devoid of PrP: implications for doppel function. *Mol Cell Neurosci*, **17**, 768-775.

Wopfner, F., Weidenhofer, G., Schneider, R., von Brunn, A., Gilch, S., Schwarz, T.F., Werner, T. and Schätzl, H.M. (1999) Analysis of 27 mammalian and 9 avian PrPs reveals high conservation of flexible regions of the prion protein. *J Mol Biol*, **289**, 1163-1178.

Wyatt, J.M. (1990) Spongiform encephalopathy in a cat. *Vet Rec*, **126**, 513.

Yamada, M., Itoh, Y., Fujigasaki, H., Naruse, S., Kaneko, K., Kitamoto, T., Tateishi, J., Otomo, E., Hayakawa, M., Tanaka, J. and *et al*. (1993) A missense mutation at codon 105 with codon 129 polymorphism of the prion protein gene in a new variant of Gerstmann-Straussler-Scheinker disease. *Neurology*, **43**, 2723-2724.

Yedidia, Y., Horonchik, L., Tzaban, S., Yanai, A. and Taraboulos, A. (2001) Proteasomes and ubiquitin are involved in the turnover of the wild-type prion protein. *Embo J*, **20**, 5383-5391.

Yehiely, F., Bamborough, P., Da Costa, M., Perry, B.J., Thinakaran, G., Cohen, F.E., Carlson, G.A. and Prusiner, S.B. (1997) Identification of candidate proteins binding to prion protein. *Neurobiol Dis*, **3**, 339-355.

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 Garcia, 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.

Zanata, S.M., Lopes, M.H., Mercadante, A.F., Hajj, G.N., Chiarini, L.B., Nomizo, R., Freitas, A.R., Cabral, A.L., Lee, K.S., Juliano, M.A., de Oliveira, E., Jachieri, S.G., Burlingame, A., Huang, L., Linden, R., Brentani, R.R. and Martins, V.R. (2002) Stress-inducible protein 1 is a cell surface ligand for cellular prion that triggers neuroprotection. *Embo J*, **21**, 3307-3316.

Zhang, J., Wu, X., Qin, C., Qi, J., Ma, S., Zhang, H., Kong, Q., Chen, D., Ba, D. and He, W. (2003) A novel recombinant adeno-associated virus vaccine reduces behavioral impairment and beta-amyloid plaques in a mouse model of Alzheimer's disease. *Neurobiol Dis*, **14**, 365-379.

Zhang, Y., Boado, R.J. and Pardridge, W.M. (2003a) In vivo knockdown of gene expression in brain cancer with intravenous RNAi in adult rats. *J Gene Med*, **5**, 1039-1045.

Zhang, Y., Schlachetzki, F. and Pardridge, W.M. (2003b) Global non-viral gene transfer to the primate brain following intravenous administration. *Mol Ther*, **7**, 11-18.

Abbreviations

1-character code for amino acids

A	alanine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
K	lysine
L	leucine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
Y	tyrosine

3-character code for amino acids

Asn	asparagine
Cys	cysteine
Leu	leucine
Met	methionine
Phe	phenylalanine
Ser	serine
Thr	threonine
Val	valine

%	percentage
°C	degree Celsius
37kDa LRP	37kDa laminin receptor precursor
67kDa LR	67kDa laminin receptor
[α-^{32}P]-UTP	uridine 5´-alpha 32P triphosphate
aa	amino acid
AAV	adeno associated virus
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
ApoEe4	apolipoprotein E type epsilon 4
approx.	approximately
asLRP	antisense laminin receptor precursor
BBB	blood-brain barrier
BSE	bovine spongiform encephalopathie
cAMP	cyclic adenosinemonophosphat
CaCo-2/TC7	human colon carcinoma cells
cDNA	complementary deoxyribonucleic acid
CHO	chinese ovary hamster cells

CJD (sCJD, vCJD, fCJD, iCJD)	Creutzfeldt-Jakob disease (sporadic, variant, familiar, iatrogenic form of CJD)
CLD	caveolae-like domain
CNS	central nervous system
Cos-7	african green monkey kidney cells
CPEB	cytoplasmic polyadenylation element binding protein
C-terminal	carboxyterminal
Cu^{2+}	copper (II) ion
CWD	chronic wasting disease
Dicer-RDE-1	Dicer-RNAi deficient-1
DIG	detergent insoluble glycosphingolipid-rich membrane domain
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
Dpl	doppel protein
DPG_2-Fe^{3+}	deuteroporphyrin IX 2,4-bis-(ethylene glycol) iron (III)
DY	drowsy (TME prion strain)
ds	double-stranded
DS500	dextran sulfate 500
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmatic reticulum
EU	European Union
EUE	exotic ungulate encephalopathy
FFI	fatal familial insomnia
FGF	fibroblast growth factor
FSE	feline spongiform encephalopathy
GAG	glycosaminoglycan
GPI	glycosyl phosphatidyl inositol
Grb2	growth factor receptor-bound protein-2
GSS	Gerstmann-Straussler-Scheinker syndrome
GST	gluthation-S-transferase
GT1	hypothalamic neuronal cells
h	hour
HEPES	2-(4-(2-Hydroxyethyl)-1piperazinyl) ethanesulfonic acid sodium salt

hGH	human growth hormon
hGNH	human gonadotropin
HM	heparansulfate mimetic
HPA23	heteropolyanion 23
Hsp	heat shock protein
HSPG	heparansulfate proteoglycan
HSV	herpes simplex virus
HY	hyper (TME prion strain)
IDX	4´-iodo-4´-deoxy-doxorubicin
kb	kilobase
kDa	kilodalton
M	molar
mM	millimolar
MCF-7	human breast adenocarcinoma cells
μg	microgram
mg	milligram
ml	milliliter
mRNA	messenger RNA
N2a	mouse neuroblastoma cells
N2aSc$^+$	scrapie-infected mouse neuroblastoma cells
NaCl	sodiumchloride
NMR	nuclear magnetic resonance
NSE	neuron specific enolase
N-terminal	aminoterminal
PAA	polyacrylamide
PAMAM	polyamidoamide
PCR	polymerase chain reaction
PcTS	phtalocyanine tetrasulfonate
PEI	polyethyleneimine
PK	proteinase K
PIL	pegylated immunoliposomes
PKA	proteinkinase A
PPI	polypropyleneimine
Prn-d	prion protein gene
Prn-p	prion protein dublet
Prn-p$^{-/-}$/ *Prn-p*$^{0/0}$	PrP knock out

PrP	prion protein
^{Ctm}PrP/ ^{Ntm}PrP	transmembrane forms of the prion protein
PrP 27-30	protease-resistent core of the prion protein
PrP^{c}	cellular prion protein
PrPres	protease-resistant form of the prion protein
PrP^{Sc}	pathogenic isoform of the cellular prion protein
PS	pentosan polysulfate
PSE	primate spongiform encephalopathy
PVDF	polyvinylidenedifluoride
rER	rough endoplasmatic reticulum
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RT-PCR	reverse transcriptase polymerase chain reaction
SAF	scrapie associated fibril
scFv	single chain variable fragment
ScGT1	scrapie-infected hypothalamic neuronal cells
ScMNB	scrapie-infected mouse neuroblastoma cells
SDS	sodiumdodecylsulfate
SDS-PAGE	SDS polyacrylamide gelelectrophoresis
sFI	sporadic familial insomnia
shRNA	short hairpin RNA
SIFT	scanning for intensely fluorescent targets
siRNA	small interfering RNA
STI1	stress-inducible protein-1
Sup35p*	prion form of Sup35p
TBE	tris-borate-EDTA
TBS	tris buffered saline
TBST	tris buffered saline/ tween 20
tg	trangenic/ transgene
TGF-β	transforming growth factor-β
TME	transmissible mink encephalopathy
TMPP-Fe^{3+}	*meso*-tetra(4-*N*-methylpyridyl) porphine iron (III)
TNF	tumor necrosis factor
TSE	transmissible spongiform encephalopathy
UK	United Kingdom

Ure2p*	prion form of Ure2p
US/ USA	United States of America
V_H, V_L	variable region immunoglobulin heavy, light chain
VEE	Venezuelan equine encephalitis virus

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