Dissertation

Zur Erlangung des Doktorgrades

Der Fakultät für Chemie und Pharmazie

Der Ludwig-Maximilians-Universität München

The 37kDa/67kDa Laminin Receptor LRP/LR

as a molecular target in neurodegenerative diseases

Heike Pflanz

aus

Lüneburg

2009

Erklärung

Diese Dissertation wurde im Sinne von §13 Abs. 3 der Promotionsordnung vom 29. Januar 1998 von Prof. Dr. Stefan Weiss betreut.

Ehrenwörtliche Versicherung

Diese Dissertation wurde selbständig, ohne unerlaubte Hilfe erarbeitet.

München, am 30.03.2009

_____

Heike Pflanz

Dissertation eingereicht am 30. März 2009

1. Gutachter: Prof. Dr. Stefan Weiss

2. Gutachter: Prof. Dr. Dr. Hans A. Kretzschmar

Mündliche Prüfung am 15. Mai 2009

Die vorliegende Arbeit wurde in der Zeit von Mai 2006 bis März 2009 im Labor von Prof. Dr. Stefan Weiss am Genzentrum der Ludwig-Maximilians-Universität München angefertigt.

Im Verlauf dieser Arbeit wurden folgende Originalpublikationen veröffentlicht bzw. eingereicht:

Nikles, D., Vana, K., Gauczynski, S., Knetsch, H., **Ludewigs, H.** and Weiss, S., Subcellular localization of prion proteins and the 37kDa/67kDa laminin receptor fused to fluorescent proteins. *Biochim Biophys Acta* 2008. 5: 335-340.

Pflanz, H., Vana, K., Mitteregger, G., Pace, C. Messow, D., Sedlaczek, C., Nikles, D., Kretzschmar, H. A. and Weiss, S. F., Microinjection of lentiviral vectors expressing small interfering RNAs directed against laminin receptor precursor mRNA prolongs the pre-clinical phase in scrapie-infected mice. *J Gen Virol* 2009. 1: 269-274.

Pflanz, H., Vana, K., Mitteregger, G., Renner-Müller, I., Pace, C., Küchenhoff, H., Kretzschmar, H. A., Wolf, E., Weiss, S., Scrapie-infected transgenic mice expressing a laminin receptor decoy mutant reveal a prolonged incubation time associated with low levels of PrPres. *J Mol Biol*, in press

Pflanz, H., Bergmann, A.-K., Dimitriadis, C., Knackmuss, S., Reusch, U., Zuber, C., Haass, C., Little, M., Weiss, S., The LRP-specific antibody IgG1 iS18 reduced Amyloid β secretion in APP-expressing HEK293FT cells. *Nature*. submitted

Im Verlauf wurden folgende Übersichtsartikel publiziert:

Zuber, C., **Ludewigs, H.** and Weiss, S., Therapeutic approaches targeting the prion receptor LRP/LR. *Vet Microbiol* 2007. 4: 387-393.

Ludewigs, H., Zuber, C., Vana, K., Nikles, D., Zerr, I. and Weiss, S., Therapeutic approaches for prion disorders. *Expert Rev Anti Infect Ther* 2007. 4: 613-630.

Vana K., Zuber C., **Pflanz H.,** Kolodziejczak D., Zemora G., Bergmann A. K. and Weiss S., LRP/LR as an alternative promising target in therapy of prion diseases, Alzheimer's disease and cancer. *Infect Disord Drug Targets* 2009. 1: 69-80.

Vorträge auf Konferenzen

- NoE Neuroprion Meeting 14. März 2008, München
- NoE Neuroprion Meeting 10. Oktober 2008, Madrid

Acknowledgements

I would like to thank Prof. Dr. Stefan Weiss for the great opportunity to work in the field of biochemistry and to intensively study neurological disorders, especially prion diseases and Alzheimer´s disease. Such a position is not easy to find for a pharmacist. I appreciate the opportunity to visit international congresses and to give oral presentations. Finally, I am grateful for the creation of the first certificate.

I want to thank Prof. Dr. Dr. Hans A. Kretzschmar for his continuous support, for the attendance in my thesis committee and for the creation of the second certificate.

I would like to thank Prof. Dr. Roland Beckmann, Prof. Dr. Angelika Vollmar, Prof. Dr. Dietmar Martin and Prof. Dr. Klaus Förstemann for attending my thesis committee.

I am grateful to Dr. Gerda Mitteregger for helpful advices in experimental setups and her reliability regarding the animal experiments.

I want to thank all group members of the AG Weiss, particularly Dr. Karen Vana, for developing the projects, I took over, for always being there and caring for a optimistic atmosphere. Especially, I want to thank Dr. Chantal Zuber who was there from the beginning to the last day. I am grateful for her big knowledge, for always being open minded, the long discussions and continuous help. A special thank goes to Dr. Daphne Nikles for having an idea to solve each and every problem, for spreading high spirits even if the experiments didn´t work out.

I am grateful to Ann-Katrin Bergmann, Christoph Dimitriadis and André Heuer for their enthusiastic engagement and pushing forward the Alzheimer project.
I thank Georgeta Zemora, Katharina Häussermann, Cilli Sedlaczek, Dominika (El Dome) Kolodziejczak, Benjamin (the consultant) Hackner, Katja Vlasova and Jennifer Hentrich for the nice atmosphere in the lab, even in the times when only girls were around, for nice cocktail and cinema evenings.

I would like to thank Stephanie Koch and Franziska Siegel for the nice time in Frankfurt, for the best study group ever and for still being my friends today. And I want to thank Stephanie Schmelcher-Mändle for distracting me from work so often and providing kids to distract me from everything else.

I am grateful to Ingo Pflanz for his support and objective opinion, for listening to endless lab-stories, for keeping an eye on my sometimes never-ending working hours and for being my helpdesk in every sense.
Finally, I want to thank my family, especially my parents for giving me the opportunity to study, for their continuous support and their confidence in my decisions.

TABLE OF CONTENTS

Summary

Prion disorders and Alzheimer´s disease (AD) both belong to the group of neurodegenerative diseases and share distinct neuropathological patterns. The key event in prion diseases, such as Creutzfeldt-Jakob disease (CJD) in humans or bovine spongiform encephalopathy (BSE) in cattle, is the conversion of the host encoded cellular prion protein (PrP^{c}) to the disease associated misfolded isoform PrP scrapie (PrP^{Sc}), predominantly accumulating in the brain. In AD the causative agent and the main component of the amyloid plaques is the Amyloid β peptide, emerged from the enzymatic processing of the Amyloid β precursor protein (APP). These protein aggregates lead to neuronal death of the affected individual. So far, there is no therapy available to cure or stall the progression of both diseases, although a lot of efforts have been driven to develop curative or palliative therapies.

Recently, the 37kDa/67kDa non-integrin laminin receptor LRP/LR has been identified as a receptor for the cellular prion protein and the pathogenic isoform PrP^{Sc}. Fluorescent labeled LRP- and PrP-molecules co-localize on the cell surface and in the perinuclear compartment. Furthermore, a fluorescent PrP-mutant lacking the signal sequence partly co-localizes with fluorescence labeled LRP in the nucleus. These results implicate an involvement of LRP/LR in the cellular trafficking of the prion protein.

A series of therapeutic strategies for the treatment of prion diseases targeting LRP/LR have been conducted. Downregulation of LRP/LR by RNAi and the generation of a laminin receptor decoy mutant represent alternative promising approaches. Microinjection of lentiviral vectors encoding for siRNAs directed against the LRP mRNA into the hippocampus resulted in a significant prolongation of the pre-clinical phase in scrapie infected mice. Transgenic mice expressing a laminin receptor decoy mutant show a significantly prolonged incubation time after prion inoculation. Therefore, LRP/LR represents a promising molecular target for the development of prion disease therapeutics.

Very recently, PrP^{c} has been shown to directly regulate β-secretase activity, which participates in the amyloidogenic cleavage process of APP, generating the soluble APPβ (sAPPβ) and the AD-associated Amyloid β peptides (Aβ). Furthermore, PrP^{c} mediates impairment of synaptic plasticity by amyloid-β oligomers. The prion receptor LRP/LR, PrP^{c} and APP all localize at the cell surface. Treatment of HEK293FT cells with the LRP/LR-specific antibody IgG1-iS18 resulted in a significant reduction of the Aβ release and the sAPPβ shedding by 99%, respectively, suggesting that LRP/LR contributes to both processes by promoting the β-secretase. LRP/LR specific pharmaceuticals such as antibodies may have therapeutic potential for the treatment of Alzheimer's Disease.

Chapter I

Therapeutic approaches for prion disorders

published as:

Ludewigs, H., Zuber, C., Vana, K., Nikles, D., Zerr, I. and Weiss, S., Therapeutic approaches for prion disorders. *Expert Rev Anti Infect Ther* 2007. 4: 613-630.

Therapeutic approaches for prion disorders

Heike Ludewigs, Chantal Zuber, Karen Vana, Daphne Nikles, Inga Zerr and Stefan Weiss[†]

Prion diseases are lethal for both humans and animals, and affected individuals die after several months following a rapid disease progression. Although researchers have attempted for decades to develop effective therapeutics for the therapy of human prion disorders, until now no efficient drug has been available on the market for transmissible spongiform encephalopathy (TSE) treatment or cure. Approximately 200 patients worldwide have died or suffer from variant Creutzfeldt–Jakob disease (CJD). Incidences for sporadic and familial CJD are approximately 1.5–2 per million per year and one per 10 million per year, respectively, in Europe. This review summarizes classical and modern trials for the development of effective anti-TSE drugs, introduces potential effective delivery systems, such as lentiviral and adeno-associated virus systems for antiprion components, including antibodies and siRNAs, and presents vaccination trials. Most of the antiprion drugs target prion protein PrP^{C} and/or PrP^{Sc}. Alternative targets are receptors and coreceptors for PrP, that is, the 37/67-kDa laminin receptor and heparan sulfate proteoglycanes. We review clinical trials for the treatment of TSEs and describe hindrances and chances for a breakthrough in therapy of prion disorders.

Expert Rev. Anti Infect. Ther. 5(4), 613–630 (2007)

CONTENTS

[†]*Author for correspondence*
Laboratorium für Molekulare Biologie – Genzentrum – Institut für Biochemie der LMU München, Feodor-Lynen-Str. 25, D-81377 München, Germany
Tel.: +49 892 180 76887
Fax: +49 892 180 76999
weiss@lmb.uni-muenchen.de

KEYWORDS:
37/67-kDa laminin receptor, adeno-associated virus, amyloid, bovine spongiform encephalopathy, Creutzfeldt–Jakob disease, laminin receptor, lentivirus, LRP/LR, pentosan polysulfate, prion, PrP, RNA interference, therapy

Prion diseases & therapy

Prion diseases are a group of neurodegenerative diseases affecting animals and humans [1]. Animal transmissible spongiform encephalopathies (TSEs) occur as scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, transmissible mink encephalopathy in mink (TME), exotic ungulate encephalopathy (EUE) in zoo animals and chronic wasting disease (CWD) in elk and deer [1]. Human prion diseases include kuru, Gerstmann–Sträussler–Scheinker syndrome (GSS), fatal and sporadic familial insomnia (FFI/sFI) and Creutzfeldt–Jakob disease (CJD) [1]. CJD can occur in sporadic (s), familial (f), iatrogenic (i) and variant (v) forms (TABLE 1). vCJD is thought to be caused by BSE and represents a zoonotic disease [1]. The incidence for sCJD is 1.5–2 per million per year [2]. Until April 2007, approximately 200 suspected and confirmed cases of vCJD occurred worldwide, most of them in the UK (164) and France (22). Countries showing between one and four vCJD cases include the Republic of Ireland (4), the USA (3), Canada (1), Japan (1), Portugal (1), Spain (1) and The Netherlands (2) [201]. A clear correlation between the numbers of BSE cases and the numbers of vCJD cases in individual countries is not visible, since Germany with no reported case of vCJD has 405 BSE cases (June 2007) [202]. Recent findings on iatrogenic vCJD transmission via blood and blood products caused major concern in various countries and effective therapeutic or postexposure measurements are urgently needed.

Human TSEs, such as CJD, are lethal, with long incubation times (i.e., the time from the day of infection to the first day TSE-relevant symptoms occur). Survival (i.e., the time from the day the first symptoms occur to the day of death) varies between approximately 6 months for sCJD and 15 months for vCJD. The average age for vCJD patients is approximately 27 years [201]. The youngest patient to date died at the age of 14 years, the eldest at the age of 74 years [3,4].

Until now, no therapeutic is on the market for the treatment or cure of prion diseases. In addition, no vaccination against prion diseases

10.1586/14787210.5.4.613

ISSN 1478-7210

is available. This article reviews therapeutic and vaccination approaches for the treatment and prevention of prion diseases. The effectiveness of a potential drug is, in principal, tested by three systems: *in vitro* systems representing mainly scrapie propagating cells, *in vivo* systems representing scrapie-infected rodents or macaques, and finally clinical studies. Although a series of drugs demonstrated convincing effects *in vitro* and *in vivo*, those tested in clinical trials failed to show significant effects on the prolongation of the incubation time or survival in patients suffering from a TSE. The development of an effective antiprion compound is challenging because the drug has to penetrate the blood–brain barrier. Although many of the compounds are effective in a cell culture system, they lack any effect *in vivo* because of their low penetration into the CNS. Moreover, a prerequisite for an effective treatment, however, represents the development of a reliable preclinical screening test to diagnose the disease at the preclinical stage and initiate the treatment at an early stage of the disease. Some progress has been made toward this, as shown in the work by Malucci *et al.*, suggesting that disease pathology and symptoms might be reversible [5,6].

Polysulfonated, polyanionic substances

Dextran sulfate

The sulfated polyanion dextran sulfate 500 (DS500) is proven to be an effective substance against TSEs *in vivo* (TABLE 2). A single intraperitoneal injection leads to a dose-dependent reduction of susceptibility to scrapie in mice and to a lengthening of incubation time. The effective scrapie titer has even been reduced by 90% [7]. However, DS500 is toxic in mice, which leads to the question of whether this substance should be tested in human prion diseases.

Suramin

Suramin is a polysulfonated aromatic urea derivative (TABLE 2). It inhibits the scrapie prion protein (PrP^{Sc}) formation *in vitro* and *in vivo* by inducing misfolding of cellular PrP (PrP^{c}) in a post-endoplasmic reticulum (ER)/Golgi compartment (FIGURE 1). The protein is retargeted to acidic compartments, thereby preventing PrP^{c} from reaching the cell surface and the cellular compartment(s) of conversion [8]. The derivatives of suramin are also able to inhibit the PrP^{Sc} *de novo* synthesis and to induce aggregation and reduction of the half-life of PrP^{c} without downregulating the PrP^{c} cell surface expression. Therefore, a symmetrical bipolar structure might be necessary [9].

Table 1. Human prion disorders.

Disorder	Cause
Sporadic CJD	Spontaneous?
Familial CJD	*PRNP* mutation
Gerstmann-Sträussler-Scheinker syndrome	*PRNP* mutation
Fatal familial insomnia	*PRNP* mutation
Variant CJD	Acquired
Iatrogenic CJD	Acquired
Kuru	Acquired

CJD: Creutzfeldt–Jakob disease; *PRNP*: Prion protein gene.

Heparan mimetics

Heparan sulfate mimetics (HMs) belong to the group of polysulfated glycans (TABLE 2). The HMs 2602 and 5004 block the PrP^{Sc}-37/67-kDa laminin receptor (LRP/LR)-HSPG binding and internalization into target cells (FIGURE 1) [10]. HM2602 was able to prolong the survival time in scrapie-infected hamsters, whereas HM5004 does not have any effects *in vivo* [11]. CR36 was efficient in reducing proteinase K (PK)-resistant PrP (PrPres) in scrapie-infected cells but had only a marginal effect on the survival time of mice infected with BSE [12].

Pentosan polysulfate (SP54)

Pentosan polysulfate is a large polyglycoside molecule with weak heparin-like activity (TABLE 2). It is able to prevent PrP^{Sc} propagation in cell culture models (FIGURE 1) [13]. It may inhibit the binding of PrP^{Sc} to LRP/LR by competing with the coreceptor heparan sulfate [10]. *In vivo*, SP54 is able to prolong the survival of scrapie-infected mice and even cures two mouse strains (VM and CBA) infected with two defined prion strains (22A and ME7) [14]. Orally or intraperitoneally administered SP54 may not be very effective since the drug may not cross the blood–brain barrier. Case studies for treatment of vCJD in humans were published in which SP54 was delivered by chronic intraventricular infusion. However, no definite clinical benefit was observed [15,16]. Further clinical studies are required to assess the efficacy of SP54 administration in vCJD and other prion diseases (see 'Antiprion drugs in clinical trials' section).

Phosphorothioate oligonucleotides

Nucleic acids are known to interact with PK-sensitive PrP (PrPsen) molecules. In cell-free assays, DNA binds to recombinant PrP and, depending on their concentrations, PrPsen aggregation is promoted or inhibited [17–19]. Natural nucleic acids do not affect PrPres formation in scrapie-infected neuroblastoma cells (ScN2a), but their degenerate single-stranded phosphorothioated analogs (TABLE 2) (FIGURE 1) are found to bind PrP^{c} and inhibit PrPres accumulation [20]. Phosphorothioate oligonucleotides (PS-ONs) administered prophylactically significantly prolong survival of scrapie-infected rodents [20]. Hydrophobicity and molecular size, but not the base composition of PS-ONs, are important to efficiently interact with PrP^{c}, implying an interaction with an amphipathic site that influences conversion. Thus, PS-ONs are not only an attractive new group of agents in TSE therapy but may also help to identify the mechanism for PrPres formation.

Table 2. Summary of anti-infective drugs against prion disorders, their effects *in vitro* and *in vivo* and their suggested mode of action.

Class of compounds	Example	Effect *in vitro*/*in vivo*	Suggested mode of action	Ref.
Polysulfonated, polyanionic and polycationic substances	Dextran sulfate	+/+	Not known	[7]
	Suramin	+/+	Induces aggregation and retargeting of PrP^{c} to endocytic compartment	[8,9]
	Pentosan polysulfate	+/+	May inhibit internalization of PrP^{Sc} by competing with heparan sulfate and blocking the binding to LRP/LR	[10,13–16]
	Heparan sulfate mimetics e.g., HM2602 HM5004 CR36	+/+ +/- +/-	Inhibits PrP^{Sc} internalization by competing with heparan sulfate and blocking the binding to LRP/LR	[10–12]
	Phosphorothioate oligonucleotides	+/+	Binds to PrP and inhibit PrPres formation	[20]
Amyloidotrophic intercalators	Congo red	+/+	May inhibit PrP^{Sc} propagation by overstabilization of the PrP^{Sc} molecule	[23–25, 27,28]
Polyene antibiotics	Amphotericin B	+/+	May inhibit endocytosis of PrP^{Sc}	[29–35]
	MS 8209	+/+	May inhibit endocytosis of PrP^{Sc}	[29–35]
	Filipin	+/n.d.	Reduces endocytosis of PrPsen	[36]
Tetracyclines	Tetracycline, Doxycycline*	+/+	Direct interaction with PrP^{Sc} leads to reduction of infectivity	[37,38]
Cyclic tetrapyrrols	Porphyrines, Phtalocyanines	+/+	May inhibit PrPres formation	[12,39–42]
Polyamines	DOSPA	+/n.d.	Interferes with the accumulation of PrP^{Sc}	[44]
	SuperFect, Polyethyleneimine	+/n.d.	Interferes with PrP^{Sc} propagation	[43,142]
	Spermine, Spermidine	+/n.d.	Prevents polymerization of recombinant human PrP to PrP^{Sc}	[45]
Anthracyclines	IDX	+/+	May prevent polymerization by binding to amyloid PrP	[48,49]
Phenothiazines	Chlorpromazine	+/n.d.	Prevents PrP^{Sc} formation	[50]
Acridines/bisacridines	Quinacrine†	+/-	May prevent PrP^{Sc} formation	[50–52]
Designer peptides	β-sheet breaker	+/+	Reverses conformational changes of pathogen PrP	[54]
RNA aptamers	RNA aptamer Ap1/2 (antiprion effect not proven)	+/-	Specifically binds PrP^{c}, but not PrP^{Sc}	[55]
	RNA aptamer DP7	+/n.d.	Interferes with the *de novo* synthesis of PrP^{Sc}	[56]
Tyrosine kinase inhibitors	STI571	+/n.d.	Induces of the lysosomal degradation of PrP^{Sc}	[57]

*Currently recruiting for a placebo-controlled trial in Italy (Milan) and Germany (Göttingen) (A Randomized, Double-Blinded, Placebo-Controlled Study of the Efficacy of Doxycyclin in the Treatment of Sporadic Creutzfeldt-Jakob Disease).
†Currently recruiting for a Phase II clinical trial (Novel Therapeutics For Prion Diseases: A Randomized, Double-Blinded, Placebo-Controlled Study of the Efficacy of Quinacrine in the Treatment of Sporadic Creutzfeldt-Jakob Disease).
IDX: Iododeoxydoxorubicin; LRP/LR: Laminin receptor; n.d.: Not determined; PrP: Prion protein.

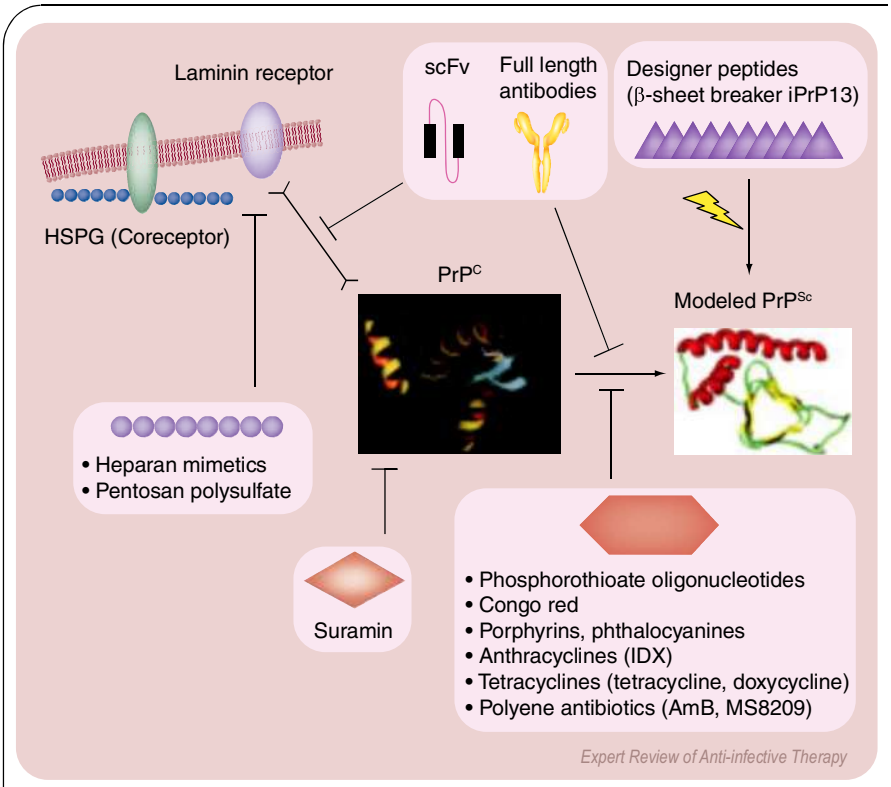


Figure 1. Schematic view of the proposed mode of actions of antiprion drugs, which are effective *in vivo* and *in vitro*. Heparan mimetics and pentosan polysulfate interfere with the PrP^c and PrP^{Sc} binding to the 37/67-kDa laminin receptor (LRP/LR). Antibodies directed against PrP^c or LRP/LR interfere with the PrP^c binding to LRP/LR or PrP^{Sc}. Many anti-transmissible spongiform encephalopathy (TSE) drugs target the PrP^c–PrP^{Sc} conversion, such as phosphorothioate oligonucleotides, Congo red, porphyrins, phtalocyanines (cyclic tetrapyrrols), anthracyclines (IDX), tetracyclines and polyene antibiotics. Some of them (e.g., polyene antibiotics) are thought to interfere with the PrP internalization process. The PrP^c–PrP^{Sc} conversion process might take place either on the cell surface or within compartments of the endocytic pathway, such as endosomes, lysosomes and endolysosomes. Designer peptides might reverse conformational changes of PrP^{Sc} by breaking β-sheets. Suramin aggregates PrP^c to a β-sheet PrP^C structure, which is no longer available as a template for conversion to PrP^{Sc}.
HSPG: Heparan sulfate proteoglycane; PrP: Prion protein; scFv: Single chain antibody.
The image of PrP^c (NMR structure) was modified from [141]. The model of PrP^{Sc} appears courtesy of Fred E Cohen, Department of Cellular & Molecular Pharmacology, University of California San Francisco (UCSF), USA [203].

Congo red as an amyloidotrophic intercalator

Congo red (CR) (TABLE 2), a sulfonated azo dye that was originally used as a histologic stain for amyloids [21,22], was identified to potently inhibit the PrPres accumulation in scrapie-infected cells (FIGURE 1) without affecting the metabolism of PrP^c [23]. Thus, the exact molecular mechanism of CR is not clearly established. However, since CR binds to protein aggregates, it has been proposed that CR inhibits prion propagation by overstabilization of the PrP^{Sc} molecule [24]. Ingrosso and colleagues first reported that *in vivo* administration of CR prolongs the incubation period of hamsters experimentally infected with scrapie. In these studies, the maximal effect was observed when the scrapie agent and CR were coinjected, suggesting that the timing of drug administration is a key determinant [25]. Nevertheless, CR has some properties that make it inappropriate as a therapeutic candidate; for example, CR is not able to cross the blood–brain barrier and it may be toxic after oral administration [26]. Therefore, a number of CR derivatives were synthesized to overcome these unfavorable properties and were tested for their antiprion activity *in vitro* [27] and *in vivo* [28]. In scrapie-infected hamsters, subcutaneous injection of the CR analog CR-A was shown to prolong the survival time comparable to CR [28]. Nevertheless, the adverse effects of these substances make them currently unprofitable for the treatment of human TSEs.

Polyene antibiotics

Polyene antibiotics, such as amphotericin B (AmB) and its synthetic, less-toxic derivative MS-8209 (TABLE 2), are antifungal agents and have been evaluated as anti-TSE agents in scrapie-infected hamsters, delaying disease and PrP^{Sc} accumulation [29,30]. In a murine model of BSE and scrapie, both AmB and MS-8209 were shown to prolong survival times and delay the PrPres and glial fibrillary acidic protein accumulation [31,32]. These substances are thought to interact with cholesterol present in mammalian cell membranes disturbing the PrP^{Sc} endocytosis [33], prevent the propagation of the scrapie agent to the peripheral nervous system [34] or directly affect the conversion of PrP^c to PrP^{Sc} (FIGURE 1) [35]. Another polyene antibiotic, filipin (TABLE 2), has also been shown to inhibit PrPres formation *in vitro*. Filipin reduced the endocytosis of PrPsen and led to a massive secretion of fully matured PrPsen and, thus, decreased the amount of PrP^c for the conversion process [36].

Tetracyclines

This antibiotic, which is produced in streptomyces, and its derivative doxycycline (TABLE 2) have been shown to revert abnormal physicochemical properties and to abolish neurotoxicity of PrP peptides *in vitro* [37]. Based on these findings, it was tested whether tetracyclines interact with PrP^{Sc} derived from vCJD patients and from cattle infected with BSE. Incubation of the infectious material with tetracycline and doxycycline leads to a dose-dependent decrease in PK-resistant PrP^{Sc} (FIGURE 1) [38]. Syrian hamsters injected with scrapie-infected brain homogenate coincubated with tetracycline or doxycycline showed a delayed onset of disease and a prolonged survival [38]. These data suggest a reduction of prion infectivity through a direct interaction of the antibiotics with PrP^{Sc}. Thus, they are potentially useful for the development of inactivation strategies for BSE- or vCJD-contaminated material.

Cyclic tetrapyrroles

Tetrapyrrole compounds, which include porphyrins and phtalocyanines, are known to bind selectively to proteins and induce conformational changes (TABLE 2). Certain molecules have been identified to inhibit the formation of PrPres *in vitro* [39] and increased the survival of TSE-infected animals [40]. Among different tested compounds, phtalocyanine tetrasulfonate [40,41], *meso*-tetra(4-*N*-methylpyridyl)porphine iron (III) [40], deuteroporphyrin IX 2,4-bis-(ethylene glycol) iron (III) [40] and Fe(III)*meso*-tetra(4-sulfonatophenyl)porphine [42] were the most potent inhibitors. Tetrapyrroles (FIGURE 1) have also been shown to inhibit PrPres formation in a cell-free conversion reaction, suggesting a direct interaction of the tetrapyrrole with PrP molecules [39].

Polyamines

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} and led to the testing of several other polyamines (TABLE 2) for their ability to interfere with PrP^{Sc} propagation in chronically infected ScN2a cells. The branched polymers investigated include polyethyleneimine, polypropyleneimine and polyamidoamide dentrimers [43]. 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 [44]. Recently, it has been reported that physiological concentrations of the aliphatic polyamines spermine and spermidine (TABLE 2), which are involved in the cellular metabolism and the stabilization of nucleic acids, prevent the polymerization of human recombinant PrP to PrP^{Sc} in solution [45]. Since nucleic acids have been found to catalyze the conversion of PrP^{c} to the β-sheet conformation [17,46] and, most recently, to infectious PrP^{Sc} employing protein misfolding cyclic amplification and polyA RNA [47], the diminishment of biological amines might lead to oligomerization and polymerization of the PrP^{c} and thus, application of polyamines *in vivo* might be a promising therapeutical approach in TSEs.

Anthracyclines

Anthracyclins are a class of chemotherapeutic agents normally used to treat a wide range of cancers (TABLE 2). Administration of a derivative of doxorubicin, 4′-iodo-4′-deoxy-doxorubicin (IDX), in a murine model of reactive amyloidosis reduced amyloid deposits [48]. In prion diseases, IDX was shown to bind to PrP amyloid and prolonged the survival of scrapie-infected hamsters, when administered intracerebrally at the same time as the scrapie agent [49]. It has been proposed that IDX binds to the abnormal form of PrP, decreasing the availability of template molecules for the conversion process of the normal PrP (FIGURE 1) [49]. Owing to its cytotoxcity and limited ability to cross the blood–brain barrier, IDX is inappropriate for treatment of TSEs. However, owing to its antiprion activity, this substance may act as a template for further compound development.

Phenothiazines

Phenothiazines represent compounds consisting of a tricyclic structure and an aliphatic side chain at the central ring (TABLE 2). Chlorpromazine was shown to inhibit PrP^{Sc} formation in ScN2a cells at micromolar concentrations. After a 6-day treatment, PrP^{Sc} was not detectable in ScN2a cells [50]. Since phenothiazines have been used as antipsychotic drugs in humans for approximately 50 years, their pharmacological profile is well documented. Their ability to cross the blood–brain barrier designates these drugs as good candidates for clinical trials for the treatment of CJD.

Acridines/bisacridines

Quinacrine is one of the most prominent drugs tested as a therapeutic tool against prion diseases (TABLE 2). It is a tricyclic antimalaria drug that has been used in humans for more than 60 years. Therefore, long-term experience in treatment is available and its pharmacological properties are well documented. It is able to cross the blood–brain barrier and can be administered orally. Quinacrine has an approximately tenfold higher antiprion potency than chlorpromazine in a ScN2a cell assay [50] and the S-quinacrine-enantiomer was found to have a higher antiprion activity in preventing PrP^{Sc} formation than the S-enantiomer [51]. However, *in vivo* studies of intraventricular-administered quinacrine into Tg7 mice did not show any prolongation effects [52] and even a paradoxical increase of PrPres was observed when mice were treated with quinacrine by the intraperitoneal route [53].

Nevertheless, it was under investigation in a Safety and Efficacy Study (PRION-1: Quinacrine for Human Prion Disease), which ended in March 2007 and, currently, patients are recruited for a Phase II clinical trial (Novel Therapeutics For Prion Diseases: a Randomized, Double-Blinded, Placebo-Controlled Study of the Efficacy of Quinacrine in the Treatment of Sporadic Creutzfeldt–Jakob Disease).

Designer peptides

β-sheet breaker peptides are a prominent group of designer peptides that are known to interfere with the dimensional structure of PrP (FIGURE 1 & TABLE 2). iPrP13, a 13-residue β-sheet breaker peptide, is able to reverse the structural state of PrP^{Sc} to a similar structure of PrP^{c} in a chinese hamster ovary cell culture assay [54]. *In vivo*, the infectivity of PrP^{Sc}-containing material was decreased by 90–95% and the appearance of clinical symptoms in scrapie-infected mice was delayed when the infectious agent was incubated with iPrP13 prior to injection [54]. Thus, these peptides might provide a useful tool to study the PrP conformational changes during pathogenesis.

RNA aptamers

RNA aptamers were selected from large combinatorial libraries in order to recognize target molecules with high affinity (TABLE 2). The two aptamers AP1 and AP2 were identified to bind specifically to murine, hamster and cattle PrP^{c} but not to native PrP27–30 from mice, suggesting that they recognize the

PK-sensitive N-terminus of PrP^c [55]. This finding might contribute to the development of diagnostic tools to distinguish between cellular and pathogenic PrP. Another promising aptamer DP7 significantly reduced *de novo*-synthesized PK-resistant PrP^{Sc} in ScN2a cells that express a chimeric mouse–hamster–mouse PrP [56] and could, therefore, provide a new class of therapeutic agents against TSEs.

Tyrosine kinase inhibitors

Interference with intracellular signaling has been assumed to play a role in the conversion process of PrP^c to its pathogenic isoform. A screening of inhibitors of specific signaling pathways in prion-infected cells revealed that tyrosine kinase inhibitor STI574 (Gleevec®, imatinib mesylate) (TABLE 2) was effective against PrP^{Sc} propagation [57]. It was shown that STI574 decreases the half-life of PrP^{Sc} in ScN2a cells by inducing its lysosomal degradation and, furthermore, the tyrosine kinase c-Abl was proposed to be probably responsible for the anti-prion effect of STI574 [57]. This novel class of compounds might be a promising tool to investigate the role of cellular signaling pathways in PrP^{Sc} propagation, as well as to develop new therapeutic approaches with substances that are already used for treatment of other human diseases. In this regard, Gleevec is successfully established in the treatment of chronic myeloid leukemia.

Antibodies as immunotherapeutics

Since antibodies are the most rapidly growing class of human therapeutics, the development and design of these tools for antagonizing prion infection have been investigated (TABLE 3). There are only a few antibodies specifically targeting PrP^{Sc}; for example, the 15B3 monoclonal antibody (mAb), which is able to discriminate between the normal PrP^c and the disease-associated form PrP^{BSE} [58]; and 8G8, which also shows a species-specific recognition for bovine PrP^{BSE} [59,60]. Antibodies directed against the repeat motif tyrosine–tyrosine–arginine were able to recognize the pathological isoform of PrP [61]. However, none of the mentioned antibodies were effective in interfering with prion propagation, although, antibodies directed against PrP^c aiming to interfere with PrP^{Sc} propagation are permanently under investigation. Primarily, the use of prion-knockout mice enabled the generation of anti-PrP antibodies that were formerly restricted to immunotolerance. A difficulty for the design of antibodies constitutes the PrP structure. While the C-terminus is well defined (structured), the structure of the N-terminus part remains poorly defined (nonstructured). Furthermore, it is still unclear which PrP epitope exhibits the appropriate target interfering with prion propagation.

Antibodies directed against the C-terminal domain of PrP, such as 6H4 (recognizing PrP epitope amino acids [aa]144–152) [62], or the antigen binding fragment F_{ab} D18/D13 (recognizing aa132–156 and aa95–103, respectively) [63] have been reported to inhibit PrP^{Sc} accumulation in neuroblastoma cells, suggesting that the binding of mAb to the corresponding epitope on PrP^c inhibits the PrP^c–PrP^{Sc} interaction occupying their binding domains. In addition to a complete cure, 6H4 protected N2a cells from a scrapie infection. By contrast, antibodies recognizing the octapeptide repeat region that is suggested to be nonessential for prion propagation [64] are able to antagonize PrP^{Sc} formation [65,66]. It can be concluded that the binding to mature PrP^c on the cell surface is necessary for inhibition of prion propagation. Studies with polyclonal antibodies generated against dimeric recombinant murine PrP in mice confirmed that crosslinking of PrP^c on the cell surface is important for PrP^{Sc} formation [67]. Adjacent to therapeutic approaches, antibodies recognizing different epitopes have been generated to clarify the mechanism of PrP^{Sc} conversion and prion propagation, respectively. One study dealing with hybrid-PrP antibody reagents suggests that the PrP regions aa89–112 and aa136–158 are critical for PrP^c–PrP^{Sc} interaction [68].

Based on cell culture models, several *in vivo* studies have been investigated. Prion pathogenesis has been prevented in transgenic mice expressing anti-PrP 6H4 µ-chain [69]. Treatment of scrapie-infected mice with mAbs termed ICSMs by intraperitoneal injection resulted in a markedly reduced peripheral PrP^{Sc} level and prion activity [70]. ICSM35 was raised against the region aa91–110, whereas ICSM18 was directed against the PrP epitope stretching from aa146 to aa159. Treated mice remained healthy for more than 300 days after continued administration starting from the point of maximal PrP^{Sc} accumulation. However, passive immunotransfer of anti-PrP antibodies revealed no effect in the late incubation period when clinical signs already have developed. In addition, this therapy is efficient only if prions are inoculated into mice by the intraperitoneal route. A postexposure prophylaxis using mAbs 8B4 (residues aa34–52) and 8H4 (aa175–185) resulted in a prolongation of the incubation period in scrapie-infected mice [71].

Several groups reported the use of combinations of different antibodies in cell culture. For instance, SAF34 directed against the octapeptide repeat region and SAF61 recognizing residues aa144–152 resulted in an inhibited PrP^{Sc} formation [66]. The observed acceleration of PrP^c degradation suggests that the disappearance of PrP^{Sc} in cells is directly coupled to PrP^c degradation by reducing its half-life [66]. Since the epitopes for SAF34 and SAF61 correspond to the two different binding sites on the PrP for the 37/67-kDa LRP/LR [72], one possible mechanism might be a disruption of the PrP–LRP/LR interaction resulting in an impaired life cycle and degradation of PrP^c. In addition, the 37/67-kDa LRP/LR was confirmed as a receptor for normal PrP^c [73] and infectious PrP^{Sc} [10], suggesting that antibodies blocking the LRP–PrP interaction might be able to interfere with prion propagation. Indeed, the polyclonal anti-LRP/LR antibody W3 abrogates PrP^{Sc} propagation in scrapie-infected N2a cells [74] and prolongs the survival time in rodents infected intraperitoneally with scrapie [ZUBER C ET AL, UNPUBLISHED DATA]. A single chain anti-LRP/LR antibody (scFv), termed S18, selected by phage display reduced peripheral PrP^{Sc} propagation

Table 3. Antiprion antibodies, passive and active immunotherapeutic strategies.

Antibody	*In vitro*/*in vivo* studies	Suggested mode of action	Ref.
Monoclonal anti-PrP 6H4	+/+ (passive immunotherapy)	Inhibition of the PrP^{c}–PrP^{Sc} interaction by occupying their binding domains	[62,69]
scFv antibody 6H4	+/n.d.	Inhibition of the PrP^{c}–PrP^{Sc} interaction by occupying their binding domains	[81]
Anti-PrP mAb 8B4/8H4	n.d./+ (passive immunotherapy)	Interaction with PrP^{c} and/or PrP^{Sc}	[71]
Antigen binding fragment Fab D18/D13	+/n.d.	Inhibition of the PrP^{c}–PrP^{Sc} interaction	[63]
Antiprion mAb ICSM 18	+/+ (passive immunotherapy)	Almost exclusive binding to PrP^{c}	[70]
Antiprion mAb ICSM 35	+/+ (passive immunotherapy)	Recognize PrP^{Sc} in addition to PrP^{c}	[70]
SAF 34/61	+/n.d.	Inhibition of PrP^{Sc} formation	[66]
Antiprion intrabodies derived from 8H4	+/+ (cell therapy)	Inhibition of the transport of PrP^{c} to the cell surface thus blocking of PrP^{Sc} accumulation	[81]
Anti-LRP/LR polyclonal antibody W3	+/+ (passive immunotherapy)	Interference with PrP-LRP/LR interaction	[74]
scFv S18/N3	+/+ (passive immunotherapy)	Interference with PrP-LRP/LR interaction	[75]

LRP/LR: Laminin receptor; mAb: Monoclonal antibody; n.d.: Not determined; PrP: Prion protein; scFv: Single chain antibody.

in a murine scrapie model [75]. Considering essential functions of PrP, for example, within neuroprotection or within signal transduction pathways that might be impaired by anti-PrP antibodies, inhibition of LRP/LR and not PrP by antibodies might be an alternative strategy for therapeutic intervention in prion diseases.

The exact mechanisms by which anti-PrP antibodies interfere with PrP^{Sc} replication are still unclear. A recent screening of mAbs for their capacity to antagonize prion infection in cell culture suggests an epitope-independent mechanism concerning more global effects on the PrP trafficking and/or transconformation process [59]. Antibodies raised against recombinant PrP^{c} were able to persistently clear neuroblastoma cells (N2a) from PrP^{Sc} and to prevent *de novo* infection, due to the interference in PrP^{Sc} formation both on the cell surface and after internalization in the cytosol [76].

One has to consider that the induction of signal transduction pathways by PrP may be disrupted by the application of anti-PrP antibodies. Therefore, the generation of antibodies recognizing a PrP region that is not implicated in cell signaling might be more favorable. Generally, no toxicity was observed in cell culture studies involving anti-PrP antibodies. However, it was reported recently that crosslinking of PrP^{c} by mAbs directed against the central amino sequence induced neuronal apoptosis, suggesting that PrP^{c} might act as an essential protein in the cell [77]. Furthermore, PrP^{c} itself might play a critical role in prion neuropathology and neuronal cell death.

In addition to polyclonal and monoclonal anti-PrP antibody strategies, alternative approaches are undertaken for novel antibody designs. Phage display-based scFvs directed against PrP were effective in cell culture models [78,79]. Moreover, scFvs derived from 6H4 expressed in a eukaryotic system exert paracrine antiprion activity when cocultured with ScN2a cells [80]. Antiprion intrabodies originated from 8H4 targeted to the ER lumen inhibited the transport of PrP^{c} to the cell surface, which resulted in blockage of PrP^{Sc} accumulation [81]. Mice injected intracerebrally with PC12 cells expressing this intrabody developed no clinical scrapie signs [81].

Active immunization/vaccination trials

Active immunization against prion disease has been at the center of a variety of studies since it became evident that humoral immune responses to the PrP can antagonize prion disease, as described in detail in the previous section. The studies using active immunization approaches, few of which were able to prolong incubation times in animals, have been summarized in detail previously [82]. However, the major aspect of prion immunization has recently turned out to be the induction of a response against the native form of PrP as it is expressed, for example, on the surface of murine T cells. Such responses have been predicted to be the key necessity in successful vaccination against prion disease [83]. By contrast, responses to recombinant PrP often failed to prolong incubation times in animals. The need to break tolerance thus prompted researchers to develop elegant strategies starting from antigen presentation via foreign immunogens, such as retrovirus-derived particles [84] or an attenuated *Salmonella* vaccine strain [85]. Recently, a DNA vaccine that combines antigen expression with lysosomal targeting has been developed [86]. Handisurya and colleagues have recently published a study in which papillomavirus-like particles were generated that display a nine amino acid B-cell epitope of PrP, DWEDRYYRE, resulting in potent conversion inhibitors *in vitro* [87]. Taken together, the new generation of antiprion

vaccines raise the promise to be of complete effectiveness, compared with first-level vaccines developed on the basis of recombinant PrP, by inducing a, so far barely understood, antibody response against native PrP.

An alternative strategy for the development of a vaccine to reduce or prevent spread of CWD in domestic and wild populations of cervids was presented by Miller [88]. Five prion peptides were tested in mice for their suitability to act as possible CWD vaccines. Two of the peptides revealed a significant prolongation of survival in Rocky Mountain Laboratories-infected mice. Since recently infectious prions have been detected in body fluids of deer suffering from CWD [89], a vaccination approach seems to be desirable.

Transdominant negative mutants

Several reports have discussed strategies for inhibiting PrP^{Sc} accumulation. One major problem with drugs shown to exert antiscrapie effects is their intrinsic property to induce a wide variety of side effects [49,90]. Several PrP mutants have been investigated for their therapeutic antiprion potential *in vitro* [91]. Kaneko and collegues first showed that substitution of a basic residue at position 167, 171 or 218 in the PrP prevents PrP^{Sc} formation [91] and claimed that these PrP mutants appear to act as dominant negative mutants by binding to protein X more avidly than the wild-type PrP and thus rendering it unavailable for the prion replication process. Mechanistically, Kaneko *et al.* argued that PrP^{c} initially forms a complex with protein X that might act as a molecular chaperone following PrP^{Sc} binding [91]. Alternatively, deleted PrP molecules inhibiting the accumulation of PrP^{Sc} in a transdominant fashion were designed [92]. Employing scrapie-infected mouse N2a cells as a model system, it has been shown that a deletion of eight amino acids in mouse PrP^{c} (PrP^{c}del114–121) abrogates the conversion of the mutant protein into PrP^{Sc}. In addition, PrP^{c}del114–121 overexpression resulted in inhibition of PrP^{Sc} accumulation [92]. Assuming that any side effects of PrP^{c}del114–121 should be minimal compared with those of the chemical compounds, an alternative therapeutic approach can be envisaged using transdominant PrP mutants.

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* [93]. Here, Perrier *et al.* generated transgenic mice expressing PrP with either the Q167R or Q218K mutation in wild-type mice or on a $Prnp^{0/0}$ background. Following intracerebral prion inoculation into these mice, $Tg(Q167R)Prnp^{+/+}$ and $Tg(Q218K)Prnp^{+/+}$ mice exhibit only low PrP^{Sc} levels or even no PrP^{Sc} accumulation in the brain [93], supporting the possibility of producing prion-resistant livestock.

Recently, in an animal model for iCJD, it has been shown that after a 7-day-lasting intracerebroventricular administration of the dominant negative PrP mutant rPrP-Q218K, the incubation period was prolonged from 117 to 131 days [94].

Delivery of PrP containing dominant negative mutations has been achieved using lentiviral gene transfer [95]. By taking advantage of 'prion-resistant' polymorphisms that naturally exist in sheep and humans (Q171R and E219K), corresponding residues were mutated in the murine *Prnp* gene (Q167R and Q218K) and subcloned into lentiviral vectors. Transduction of prion-infected N2a 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 [95].

Since PrP^{c} is a major cellular requirement for the propagation of infectivity [96], it represents an attractive therapeutic target. However, identification of the 37/67-kDa LRP/LR as the receptor for prions [10,60,73] suggested 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 transdominant negative manner as a decoy by trapping PrP molecules [97]. *In vitro* studies revealed that the LRP mutant is able to reduce the PrP^{Sc} accumulation in scrapie-infected neuronal cells [97] and, thus, might have potential for the development of a TSE therapy.

RNA interference approaches

Reduction of the 37/67-kDa LRP/LR level by RNA interference (RNAi) inhibits PrP^{Sc} accumulation in ScN2a and ScGT1 cells [74], suggesting that LRP/LR represents a promising target for a gene delivery approach using siRNAs directed against LRP mRNA.

Another target for siRNAs is the *Prnp* gene. Transfecting scrapie infected N2a cells with siRNA duplexes directed against the *Prnp* mRNA leads to downregulation of both PrPsen and PrPres levels [98]. Recently, lentiviral gene transfer was used to downregulate PrP^{c} expression by delivering shRNAs into goat fibroblasts, which were used to produce a cloned goat fetus by nuclear transplantation. Analysis of the fetal brain tissue revealed a significant downregulation of PrP^{c} (>90%) [99]. Delivery of the shRNAs was even successful when the recombinant lentiviral vectors were injected into the bovine ova prior to *in vitro* fertilization [99].

The group headed by Kretzschmar reported recently that chronic ScN2a cells revealed an efficient reduction of PrP^{Sc} levels after transduction with lentiviral shRNA512 [100]. Most notably, the group mimicked the clinical situation by generating chimeric mice derived from lentivector-transduced embryonic stem cells. These animals carried the lentiviral shRNAs in a defined percentage of brain cells and expressed reduced PrP^{c} levels. Most importantly, in highly chimeric mice, survival was significantly extended after scrapie infection, strongly suggesting that lentivector-mediated RNAi represents an important approach for the treatment of prion disorders [100].

Disablement of both *Prnp* alleles does not impair normal development and behavior in mice [101]. Recently, PrP^{c}-deficient cattle were generated that appeared to be physiologically,

histopathologically, immunologically and reproductively normal [102]. In addition, transgenic mice [tgN(NSEasLRP)2] were constructed that showed a reduced LRP/LR level in hippocampal and cerebellar brain regions and no abnormal behavior compared with control mice [103]. In addition, mice treated with scFv S18 [75] or polyclonal antibody W3 (ZUBER C *ET AL*, UNPUBLISHED DATA), both directed against LRP/LR by immunotransfer, revealed no side effects, suggesting that ectopic downregulation of LRP/LR in the brain or blocking the prion receptor by antibodies has no phenotype. These findings lead to the conclusion that gene silencing by RNAi targeting PrP and LRP is a promising approach to retard disease progression, especially in familial forms of prion diseases where an early treatment is reasonable owing to a possible diagnosis at an early stage of disease.

The 37/67-kDa LRP/LR as a target in therapy of prion diseases

37/67-kDa LRP/LR – originally identified as a PrP^c interacting protein [104] – acts as a receptor for the PrP^c [73] and the infectious PrP^{Sc} [10]. LRP/LR is required for the internalization of BSE prions by human enterocytes [60]. We are developing a series of therapeutics for the treatment of TSEs targeting LRP/LR (reviewed in [1,105]): the polysulfated glycanes HMs and pentosan polysulfate (SP54) (FIGURE 1) both interfere with the binding of PrP27–30 to LRP/LR at the cell surface [10]. Antisense LRP RNA expressed in scrapie-infected neuronal cells reduced PrP^{Sc} propagation [74]. siRNAs directed against LRP mRNA also hamper PrP^{Sc} propagation in scrapie-infected neuronal cells [74]. The lentiviral gene delivery system will be employed to transfer siRNAs into animals. A transdominant negative LRP decoy mutant termed LRP102–295::FLAG impaired PrP^{Sc} propagation in scrapie-infected neuronal cells [97]. Finally, antibodies directed against LRP/LR seem to be powerful tools for the treatment of TSE. The polyclonal antibody W3 cured PrP^{Sc}-propagating cells from scrapie [74] and prolonged the survival time in scrapie infected mice (ZUBER C ET AL, UNPUBLISHED DATA). scFv antibodies directed against LRP/LR (FIGURE 1) reduced peripheral PrP^{Sc} propagation by passive immunization of mice [75] and through delivery of transgenes encoding for scFvs into mice by recombinant adeno-associated viruses (AAVs) (ZUBER *ET AL*. UNPUBLISHED DATA).

Antiprion drugs in clinical trials

Symptomatic treatment

Given the wide range of symptoms and signs in CJD and current limitations of causal therapy, symptomatic treatment becomes extremely important. Antidementive drugs, such as acetylcholinesterase inhibitors, were not tested systematically in CJD patients, probably because, in most cases, the diagnosis is made in advanced disease stages when dementia is severe. Many CJD patients suffer from psychiatric symptoms, such as depression, anxiety, psychosis and hallucinations. Symptomatic treatment spans a wide range of anxiolytic and antipsychotic drugs, such as benzodiazepines or neuroleptics. Since muscle rigidity and akinesia are frequent in the middle and advanced disease stages, atypical neuroleptics should be used to minimize the adverse effects. A symptomatic therapy exists for CJD-typical myoclonus that responds well to clonazepam or valproate at a standard dose.

Observational trials

So far, only a few case reports are available on therapeutic measures that have an effect on the prognosis and course of the disease [106]. Several compounds have been tested for their potential as an antiprion drug. They belonged to distinct classes, such as analgesic, antidepressant, antipsychotic, antimicrobial and anticoagulant drugs. Most of them were tested in observational trials only on a small number of individuals and mainly case reports for these patients are available. These studies are listed in TABLE 4 [107,109].

In vitro experimental results indicate that pentosan polysulphate (PPS) has an effect on PrP production, replication and associated cell toxicity [110]. In animal experiments, PPS has a prophylactic effect [52]. A major problem is that PPS is believed not to cross the blood–brain barrier; therefore, the compound has to be administered intraventriculary. This has been performed in a number of individuals with vCJD and sCJD [15,16,111]. There are controversial results of PPS in clinical studies. Although prolongation of survival was not always reported, this finding is hard to judge because this compound has not been tested in a case–control study. PPS might still be a candidate for a clinical trial since a significant effect was observed in a murine model of scrapie, BSE and vCJD after oral, intraventricular, intraperitoneal and intracerebral administration of PPS [112].

Case–control trials

Controlled clinical trials using a prospective double-blinded approach were conducted for flupirtine only [113]. Other studies reported to date in the literature compared clinical variables and survival times in treated patients and cohorts with untreated CJD patients. Such trials are available for sporadic and vCJD patients using quinacrine and sCJD patients using doxycycline.

Table 4. Summary of case reports with anti-infective drugs used in prion diseases.

Drug	Ref.
Acyclovir	[144,145]
Amantadine	[109,146–151]
Amphotericin B	[152]
Interferon	[107,153]
Pentosan polysulfate	[15]
Quinacrine	[154–157]
Vidarabin	[158]

The flupirtine trial was conducted in 26 sCJD patients and two iCJD patients [113]. A total of 13 patients were treated with 100 mg (300–400 mg) flupirtine daily; 15 controls received placebo. There was no difference regarding survival, that is, 107 days for the treatment group and 106 for the controls. One of the parameters used to monitor the disease progression was the change in the ADAS-Cog score. Patients treated with flupirtine showed significantly less deterioration in the dementia tests than patients treated with placebo. The mean change in ADAS-Cog (baseline to best) was +8.4 (±15.3) in the flupirtine group and +20.6 (±15.1) in the placebo group ($p = 0.02$, one-sided t-test) [113].

Disease progression was evaluated in 30 sCJD patients and two vCJD patients using 100 mg quinacrine three-times daily. The data were compared with untreated 125 sCJD patients. There was no significant difference on the mean survival time between the treated and untreated groups [114].

According to cell culture and animal experiments, doxycyclin may bind PrP^{Sc} directly, thus preventing further conformational changes [38,115]. A limited number of patients with sCJD were treated with doxycycline and an increased survival in these patients compared with historical sCJD cases was reported [116]. These preobservations have to be confirmed in a prospective multinational study in Italy and Germany, which is currently underway (TAGLIAVINI F, ZERR I, PERS. COMM.). No systematic studies are reported to date for establishment of this treatment in routine clinical practice.

Problems of clinical trials in CJD

Clinical diagnosis

There is a wide scope of clinical phenomenology in human prion disease, regarding the age of onset, presenting features, rate of progression and appearance of other clinical manifestations [117–119]. Owing to clinical heterogeneity, the diagnosis at early stages might be difficult. However, recent advances in clinical diagnostic techniques, such as cerebrospinal fluid (CSF) tests and MRI, enable the recognition of the disease at earlier stages, but better tests are needed to identify patients as early as possible, at best at the preclinical stage [120,121]. On the other hand, clinical studies in patients with prion diseases are hampered by various clinical presentations and variability in the disease course, which are influenced by several factors, such as age at onset, gender, molecular disease subtype and *Prnp* codon 129 genotype [117,122].

At disease onset, the symptoms and signs are not specific; however, during the disease course, almost all patients develop rapid progressive dementia, ataxia, myoclonus and muscle tone abnormalities. The prodromal phase is mainly characterized by unspecific behavioral changes, fear, adynamia and dizziness, which are frequently regarded as unspecific symptoms and no suspicion of a prion disease is raised at that time. Many patients complain of sleep disturbances or relatives might notice eating abnormalities and weight loss. These signs, together with altered social behavior and depressive mood changes, might point toward an organic depression at this phase. Depression, fear and aggression were reported as early psychiatric symptoms [123–127].

With the progression of the disease, patients develop cognitive deficits and focal neurological signs, such as hemianopsia, cerebellar ataxia, pyramidal and extrapyramidal signs, myoclonus and akinetic mutism. In the advanced stage of the disease, the patients have lost contact with the environment, are usually bedridden, akinetic and mute [128]. Muscle tone abnormalities (rigor and/or spasticity) and myoclonus are mostly present. Patients of the classical sCJD variant usually die within months or weeks (median 6 months), whereas others (atypical cases) may survive in this state for 1–2 years [122,129–131].

Differential diagnosis

Owing to the nature of the clinical symptomatology, the differential diagnosis of sCJD includes a large number of neurological and psychiatric diseases. The most frequent differential diagnosis is Alzheimer's disease [132–136]. The rapid disease course, in particular, can rarely be discriminated from CJD when myoclonus is present. Dementia with Lewy bodies and vascular dementias are further diagnoses that are frequently found as a differential diagnosis of CJD in elder patients [118]. Chronic encephalitis is often the differential diagnosis among patients of younger age in both sCJD and vCJD. CSF tests and MRI help to differentiate CJD from other neurodegenerative and inflammatory conditions.

Phenotypic heterogeneity

Phenotypic variability in the clinical syndrome and neuropathological changes in sCJD were recognized a long time ago and some attempts were made to define disease subtypes (such as Heidenhain variant or Brownell–Oppenheim variant). Based on new molecular diagnostic criteria, distinct clinicopathological phenotypes are defined by the codon 129 genotype of *Prnp* together with the type of PK-resistant core of the PrP (either as type 1 or 2). Some of clinicopathological subtypes, formerly known as Heidenhain variant, could be classified as homozygous for methionine (MM)-1/heterozygous (MV)-1 subtype and Brownell–Oppenheim variant as homozygous for valine (VV)-2 subtype [117].

The most frequent subtypes of sCJD are designated as MM-1/MV-1 (the latter are often summarized as one subtype because of clinicopathological similiarities), VV-2 and MV-2 (TABLE 5). Patients with MM1 (median age at onset: 65 years [range 31–81 years]) subtype usually display a short clinical disease course of several months and present with rapid progressive myoclonic dementia. Patients with VV2 subtype usually develop cerebellar ataxia for several months and dementia is usually mild and becomes apparent as the disease progresses, often at late disease stages. The disease course is prolonged (median 7 months). In MV2 subtype, the clinical presentation might vary between extrapyramidal syndrome, ataxia and dementia. The slowly progressive disease, which might span a period of up to 3 years, often hampers the diagnosis, since many other neurodegenerative conditions might be taken into account before CJD is suspected [117,126].

Table 5. Molecular subtypes of sporadic Creutzfeldt–Jakob disease (clinical and pathological characteristics).

Occurence	Molecular disease subtype	Median age at onset, years (range)	Median duration, months (range)	Most prominent clinical signs/symptoms	Neuropathological features
Frequent	MM1/MV1	65 (31–82)	4 (1–18)	Dementia, cortical anopsia and myoclonus	Prominent involvement of occipital cortex, 'synaptic type' PrP staining
	MV2	64 (53–76)	12 (4–27)	Ataxia, dementia and extrapyramidal	Similiar to VV2, focal involvement of the cortex, amyloid-kuru plaques in the cerebellum, plaque-like focal PrP deposits
	VV2	61 (40–76)	7 (3–18)	Ataxia at onset and late dementia	Prominent involvement of subcortical structures, including brain stem nuclei, spongiosis often limited to deep cortical layers, plaque-like PrP staining, prominent perineuronal staining
Rare	MM2-thalamic MM2-cortical	52 (36–71) 64 (49–77)	16 (8–24) 16 (9–36)	Insomnia, dysautonomia at onset, later ataxia and cognitive impairment. Progressive dementia for several months	Atrophy of the thalamus and inferior olive, spongiosis may be absent or focal. Large confluent vacuoles with perivacuolar PrP staining
	VV1	44 (19–55)	21 (17–42)	Dementia at onset, later ataxia and extrapyramidal	Severe pathology in the cerebral cortex and striatum with sparing of brain stem nuclei and cerebellum

CJD: Creutzfeldt-Jakob disease; MM: Homozygous for methionine; MV: Heterozygous for methionine or valine; PrP: Prion protein; VV: Homozygous for valine.
Data from German CJD Surveillance and according to [117,119,129–131].

The median disease duration reported for all forms of sCJD is approximately 6 months [118,122,127]. However, the disease duration appears to vary with respect to the subtypes. The influence of the genotype at codon 129 on disease duration is shown in FIGURE 2. Patients who are homozygous for methionine have the most progressive disease course, followed by those who are homozygous for valine and heterozygous. The latter individuals have the longest survival. In addition, patients who are younger at disease onset might display a disease duration three-times longer than elder individuals with the same genotype (FIGURE 3) [122,137]. Thus, age and codon 129 genotype have to be taken into account when controlled clinical trials are undertaken in CJD patients.

Case identification

Although single prospective, double-blinded trials in CJD were performed [113,114], clinicians face several difficulties with the design. First, CJD is still a rare condition and the incidence rate is 1.5–2 per million per year [138]. Therefore, a monocentric approach has to be chosen, ideally by centralizing the data in one center experienced with case identification and diagnosis. Another major problem is the rapid disease progression. Given a short survival in most patients, an early case identification is crucial to start treatment. A specific problem has to be addressed when a clinical trial has to be carried out in patients with dementia, which might be severe at the time when the CJD diagnosis is made. It is important that ethical issues concerning the trials, which will be carried out on patients unable to give an informed consent, are addressed.

Disease progression

Dementia syndrome in CJD differs from other more common neurodegenerative dementia, such as Alzheimer's disease, but has been poorly characterized and neuropsychological assessment data are limited [124]. Commonly used dementia scales were designed to monitor disease progression in Alzheimer's disease and scales for vascular dementia and frontotemporal dementia are also available [139]. However, these scales and scores might not be suitable to monitor progression of the cognitive decline in CJD patients; therefore, disease-specific scores have to be developed. In addition, scores for monitoring other neurological abnormalities, such as ataxia, rigidity or myoclonus, also have to be applied in clinical trials. Owing to the variability in clinical syndromes and survival across molecular CJD subtypes, scales might be weighted for single subtypes: ataxia is more prominent in VV2 subtype and an ataxia scale might be more useful to monitor the disease progression in this subtype, whereas a score that involves neuropsychiatric assessment might reflect disease progression for the MM1 subtype.

Conclusions

A series of anti-TSE drugs were effective *in vitro* (scrapie-infected neuronal cells) and *in vivo* (mainly scrapie-infected rodent models). However, when administered in clinical studies, none of them so far have revealed significant effects regarding a prolongation of survival times in CJD patients or an improvement of the state of health. A symptomatic therapy for CJD-typical myoclonus exists

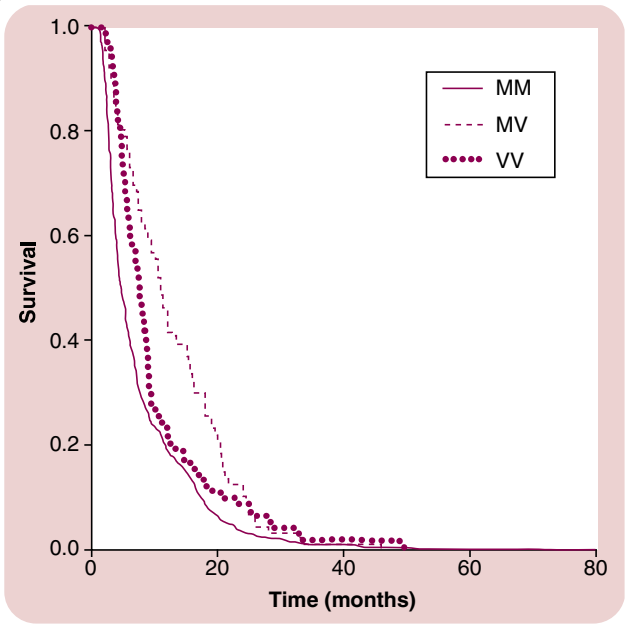


Figure 2. Kaplan–Meyer survival time in Creutzfeldt–Jakob disease patients stratified by codon 129 genotype.
MM: Homozygous for methionine; MV: Heterozygous for methionine and valine; VV: Homozygous for valine.
Reproduced from National TSE reference center, Göttingen, Germany.

that responds well to clonazepam or valproate at a standard dose. A safety and efficacy study with quinacrine ended in March 2007 and patients are currently being recruited for a Phase II clinical trial. Pentosan polysulfate (SP54) was able to cure two mouse strains from two scrapie strains. Clinical trials so far have led to controversial results. HMs might represent alternatives to SP54. Recently, Doh-ura reported prolonged survival times in CJD patients after long-term cerebroventricular administration of the drug [140]. Antibodies directed against PrP and LRP/LR revealed significant effects *in vivo* and might be suitable for passive immunization and vaccination strategies. Promising vaccination trials were reported, including papilloma-like particles that display a nine amino acid B-cell epitope of PrP resulting in potent conversion inhibitors *in vitro*. Prion peptides may act as possible CWD vaccines, which might protect exposed individuals from a CWD infection in the USA. Each attempt to develop a therapeutic strategy considers the development of an early (preclinical screening) test. Until such a test is available, clinical studies in humans will be hampered by the late diagnosis, when extensive neuronal damage has already taken place. On the other hand, many factors have to be taken into account for the analysis of the treatment outcome, such as genetic background and heterogeneous disease course with variable survival times. Disease-specific scores have to be developed to monitor disease progression in single molecular disease subtypes.

Expert commentary

vCJD patients are young and die at a mean age of approximately 27 years. All human TSEs, including the sCJD forms with an incident rate of 1.5–2 cases per million per year, iCJD and the familial forms, fCJD, fatal familial insomnia and GSS syndrome, are lethal and survival times range between approximately 6 months in sporadic and a few years in genetic cases. No therapeutic for the causal treatment of human prion disorders is available on the market worldwide, and TSE patients are treated only symptomatically. Even if the number of vCJD cases declines further, the numbers of sporadic and familial human TSE cases will stay constant or will even increase. Therefore, the development of effective antiprion drugs is essential.

It is hard to predict which of the drugs will show a significant effect on the prolongation of survival in patients. Most of the drugs target PrP. Since PrP does not seem to have essential functions for the organism (PrP-knockout cows reveal no phenotype [102]), depletion of PrP might also be tolerable for the human body. An alternative target is LRP/LR, identified as a receptor for PrP^{c} and PrP^{Sc}. This receptor appears to be necessary for organisms owing to its multifunctional role, such as cell adhesion, growth and development (reviewed in [1,105]). However, ectopic downregulation of LRP in the brain by using an antisense RNA strategy revealed no phenotype [103], suggesting that depletion of LRP in the brain is tolerable for the organism. In addition, intraperitoneal treatment of mice with antibodies directed against LRP/LR, such as W3 and scFv S18 [75], did not reveal any side effects. Therefore, tools blocking LRP/LR might be promising for the treatment of TSEs, especially when delivered by passive immunotransfer, *ex vivo* approaches (grafting of antibody secreting myotubes) and gene delivery systems, including recombinant lentiviral vectors. Here, antibodies directed against LRP/LR, siRNAs directed against LRP mRNA, transdominant negative LRP mutants and polysulfated glycanes, such as SP54 or heparan mimetics, might be interesting tools. In addition, siRNAs directed against PrP mRNA

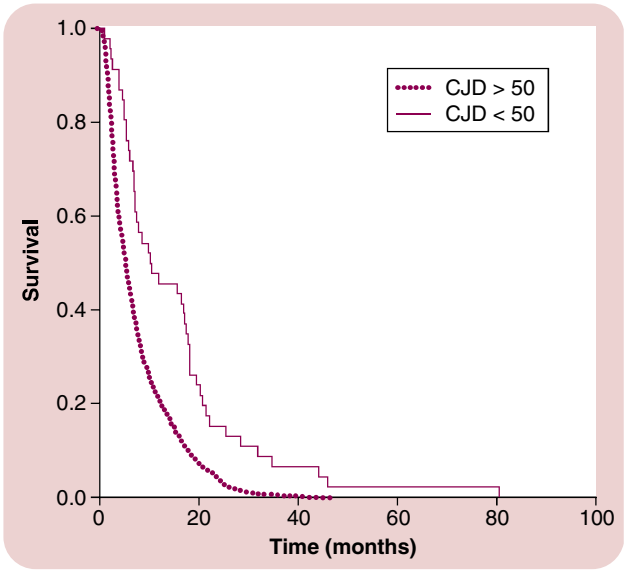


Figure 3. Kaplan–Meyer survival time in CJD patients stratified by age at onset.
CJD: Creutzfeldt–Jakob disease.
Reproduced from National TSE reference center, Göttingen, Germany.

delivered by lentiviral vectors [100] might be promising. The development of preclinical screening tests is a prerequisite for successful treatment of TSE patients. New approaches for the identification of novel drugs for the treatment of TSEs might be required, which target either novel components of the life cycle of prions or well-known interaction partners of the PrP [1]. As long as it is unclear whether CWD and sheep scrapie might cause a zoonotic disease in humans, vaccination trials might be of interest for those people coming in contact with cervids suffering from CWD and/or sheep suffering from scrapie.

Five-year view

Within the next 5 years, we will know whether classical antiprion drugs, such as SP54, HMs, quinacrine, doxycycline, or others, will have the potential to prolong the survival time in TSE patients. We consider the development of novel antiscrapie drugs focusing on further essential components of the life cycle of prions as important. One of the targets is the 37/67-kDa LRP/LR identified as a receptor for PrP^{c} and PrP^{Sc}. Here, antibodies, siRNAs and transdominant negative mutants are currently tested for their antiprion activity in animal models and, in case they are active, also in clinical studies.

A bottleneck in TSE therapy is the delivery system. Antibodies, for instance, can be delivered by passive immunization, *ex vivo* approaches (e.g., grafting of antibody-secreting muscle cells) and gene delivery systems, including AAV and lentiviral vectors. Vaccination approaches include active immunization with PrP and prion peptides, as well as DNA vaccination. Vaccination trials are important, especially for those individuals in contact with animals suffering from a TSE to avoid the development of a zoonotic disease.

siRNAs might represent further powerful therapeutic tools, which can be directed against *Prnp* mRNA, LRP mRNA and mRNAs encoding for further important proteins of the life cycle of prions. Cutting-edge gene delivery systems might include lentiviral vectors, the AAV system or papilloma-like virus particles. A better understanding of the life cycle of prions via identification of all essential components for prion replication will reveal potential alternative targets for development of a powerful antiprion drug. The development of preclinical screening systems seems to be realistic within the next 5 years to enable an early preclinical treatment of TSE patients with powerful antiprion drugs.

Acknowledgements

This work was supported by the European Commission (grant NoE-NeuroPrion FOOD-CT-2004–506579, the Bundeministerium für Bildung und Forschung (grant 01-KO-0514) and the Deutsche Forschungsgemeinschaft (grant WE 2664/2–1).

Financial disclosure

The authors have no relevant financial interests related to this manuscript, including employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Key issues

- Approximately 200 people worldwide have died or suffer from variant Creutzfeldt–Jakob disease (CJD) most likely caused by bovine spongiform encephalopathy.
- The incidences of sporadic and familial CJD are 1.5–2 per million/year and one per 10 million/year, respectively, in Europe.
- No therapeutic is on the market for the treatment of human transmissible spongiform encephalopathies.
- Most of the therapeutics target prion protein $(PrP)^{c}$ and/or PrP^{Sc}.
- Pentosan polysulfate (SP54) is the only drug that cured the mouse strains VM and CBA from scrapie strains 22A and ME7. There are controversial results with SP54 in clinical studies.
- The prion receptor 37/64-kDa laminin receptor (LRP/LR) is targeted by antibodies, polysulfated glycanes, siRNAs directed against LRP mRNA and transdominant negative mutants.
- No vaccination against prion disorders is currently on the market. Most antiprion vaccinations induce a response to PrP.
- A symptomatic therapy exists for CJD-typical myoclonus that responds well to clonazepam or valproate.
- Case reports were conducted with acyclovir, amantadine, amphotericin B, interferon, pentosane, quinacrine and vidarabin.
- Controlled clinical trials were performed with flupiritine only.
- Clinical diagnosis at early stages of CJD is possible with cerebrospinal fluid tests and MRI. Both techniques help to differentiate CJD from other neurodegenerative and inflammatory diseases.
- Preclinical screening tests are required to develop an effective therapeutic strategy, which can be initiated at the preclinical phase.

References

Papers of special note have been highlighted as:
- • of interest
- •• of considerable interest

▶1 Vana K, Zuber C, Nikles D, Weiss S. Novel aspects of prions, their receptor molecules, and innovative approaches for TSE therapy. *Cell. Mol. Neurobiol.* 27(1), 107–128 (2007).

▶2 Ladogana A, Puopolo M, Croes EA *et al.* Mortality from Creutzfeldt–Jakob disease and related disorders in Europe, Australia, and Canada. *Neurology* 64(9), 1586–1591 (2005).

3 Will RG, Ironside JW, Zeidler M *et al.* A new variant of Creutzfeldt–Jakob disease in the UK. *Lancet* 347(9006), 921–925 (1996).
- **First description of variant Creutzfeldt–Jakob disease (vCJD) in the UK.**

4 Will RG, Cousens SN, Farrington CP, Smith PG, Knight RS, Ironside JW. Deaths from variant Creutzfeldt–Jakob disease. *Lancet* 353(9157), 979 (1999).

5 Mallucci G, Dickinson A, Linehan J, Klohn PC, Brandner S, Collinge J. Depleting neuronal PrP in prion infection prevents disease and reverses spongiosis. *Science* 302(5646), 871–874 (2003).
- **Reversion of spongiosis in prion diseases.**

6 Mallucci GR, White MD, Farmer M *et al.* Targeting cellular prion protein reverses early cognitive deficits and neurophysiological dysfunction in prion-infected mice. *Neuron* 53(3), 325–335 (2007).

7 Farquhar CF, Dickinson AG. 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 (1986).

8 Gilch S, Winklhofer KF, Groschup MH *et al.* Intracellular re-routing of prion protein prevents propagation of PrP(Sc) and delays onset of prion disease. *EMBO J.* 20(15), 3957–3966 (2001).
- **Intracellular rerouting prevents prion protein (PrP)^{Sc} propagation.**

9 Nunziante M, Kehler C, Maas E, Kassack MU, Groschup M, Schatzl HM. Charged bipolar suramin derivatives induce aggregation of the prion protein at the cell surface and inhibit PrPSc replication. *J. Cell. Sci.* 118(Pt 21), 4959–4973 (2005).

10 Gauczynski S, Nikles D, El-Gogo S *et al.* The 37-kDa/67-kDa laminin receptor acts as a receptor for infectious prions and is inhibited by polysulfated glycanes. *J. Infect. Dis.* 194(5), 702–709 (2006).
- **37/67-kDa laminin receptor (LRP/LR) acts as a prion receptor.**

11 Adjou KT, Simoneau S, Sales N *et al.* A novel generation of heparan sulfate mimetics for the treatment of prion diseases. *J. Gen. Virol.* 84(Pt 9), 2595–2603 (2003).

12 Larramendy-Gozalo C, Barret A, Daudigeos E *et al.* Comparison of CR36, a new heparan mimetic, and pentosan polysulfate in the treatment of prion diseases. *J. Gen. Virol.* 88(Pt 3), 1062–1067 (2007).

13 Caughey B, Raymond GJ. Sulfated polyanion inhibition of scrapie-associated PrP accumulation in cultured cells. *J. Virol.* 67(2), 643–650 (1993).

14 Farquhar C, Dickinson A, Bruce M. Prophylactic potential of pentosan polysulphate in transmissible spongiform encephalopathies. *Lancet* 353(9147), 117 (1999).
- **Pentosan polysulfate cures two distinct mouse strains from two scrapie strains.**

15 Whittle IR, Knight RS, Will RG. Unsuccessful intraventricular pentosan polysulphate treatment of variant Creutzfeldt–Jakob disease. *Acta Neurochir. (Wien)* 148(6), 677–679 (2006).

16 Todd NV, Morrow J, Doh-ura K *et al.* Cerebroventricular infusion of pentosan polysulphate in human variant Creutzfeldt–Jakob disease. *J. Infect.* 50(5), 394–396 (2005).

17 Cordeiro Y, Machado F, Juliano L *et al.* DNA converts cellular prion protein into the β-sheet conformation and inhibits prion peptide aggregation. *J. Biol. Chem.* 276(52), 49400–49409 (2001).

18 Deleault NR, Geoghegan JC, Nishina K, Kascsak R, Williamson RA, Supattapone S. Protease-resistant prion protein amplification reconstituted with partially purified substrates and synthetic polyanions. *J. Biol. Chem.* 280(29), 26873–26879 (2005).

19 Nandi PK, Leclerc E. Polymerization of murine recombinant prion protein in nucleic acid solution. *Arch. Virol.* 144(9), 1751–1763 (1999).

20 Kocisko DA, Vaillant A, Lee KS *et al.* Potent antiscrapie activities of degenerate phosphorothioate oligonucleotides. *Antimicrob. Agents Chemother.* 50(3), 1034–1044 (2006).

21 Cohen AS, Calkins E, Levene CI. Studies on experimental amyloidosis. I. Analysis of histology and staining reactions of casein-induced amyloidosis in the rabbit. *Am. J. Pathol.* 35, 971–989 (1959).

22 Glenner GG. Amyloid deposits and amyloidosis: the β-fibrilloses (second of two parts). *N. Engl. J. Med.* 302(24), 1333–1343 (1980).

23 Caughey B, Race RE. Potent inhibition of scrapie-associated PrP accumulation by congo red. *J. Neurochem.* 59(2), 768–771 (1992).

24 Caspi S, Halimi M, Yanai A, Sasson SB, Taraboulos A, Gabizon R. The anti-prion activity of Congo red. Putative mechanism. *J. Biol. Chem.* 273(6), 3484–3489 (1998).

25 Ingrosso L, Ladogana A, Pocchiari M. Congo red prolongs the incubation period in scrapie-infected hamsters. *J. Virol.* 69(1), 506–508 (1995).
- **Congo red prolongs scrapie incubation time in hamsters *in vivo*.**

26 Bos RP, Koopman JP, Theuws JLG, Henderson PT. The essential role of the intestinal flora in the toxification of orally-administered benzidine-dyes: internal exposure of rats to benzidine after intestinal azo reduction. *Mutat. Res.* 181, 327 (1987).

27 Rudyk H, Vasiljevic S, Hennion RM, Birkett CR, Hope J, Gilbert IH. Screening Congo red and its analogues for their ability to prevent the formation of PrP-res in scrapie-infected cells. *J. Gen. Virol.* 81(Pt 4), 1155–1164 (2000).

28 Poli G, Martino PA, Villa S *et al.* Evaluation of anti-prion activity of Congo red and its derivatives in experimentally infected hamsters. *Arzneimittelforschung* 54(7), 406–415 (2004).

29 Adjou KT, Demaimay R, Lasmezas C, Deslys JP, Seman M, Dormont D. MS-8209, a new amphotericin B derivative, provides enhanced efficacy in delaying hamster scrapie. *Antimicrob. Agents Chemother.* 39(12), 2810–2812 (1995).

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

31 Adjou KT, Demaimay R, Lasmezas CI, Seman M, Deslys JP, Dormont D. Differential effects of a new amphotericin B derivative, MS-8209, on mouse BSE and scrapie: implications for the mechanism of action of polyene antibiotics. *Res. Virol.* 147(4), 213–218 (1996).

32 Demaimay R, Adjou KT, Beringue V *et al.* Late treatment with polyene antibiotics can prolong the survival time of scrapie-infected animals. *J. Virol.* 71(12), 9685–9689 (1997).

33 Bolard J. Interaction of polyene antibiotics with membrane lipids: physicochemical studies of the molecular basis of selectivity. *Drugs Exp. Clin. Res.* 12(6–7), 613–618 (1986).

34 Beringue V, Lasmezas CI, Adjou KT *et al.* Inhibiting scrapie neuroinvasion by polyene antibiotic treatment of SCID mice. *J. Gen. Virol.* 80(Pt 7), 1873–1877 (1999).
- **The polyene antibiotic MS 8209 inhibits scrapie neuroinvasion in SCID mice.**

35 Hartsel SC, Weiland TR. Amphotericin B binds to amyloid fibrils and delays their formation: a therapeutic mechanism? *Biochemistry* 42(20), 6228–6233 (2003).

36 Marella M, Lehmann S, Grassi J, Chabry J. Filipin prevents pathological prion protein accumulation by reducing endocytosis and inducing cellular PrP release. *J. Biol. Chem.* 277(28), 25457–25464 (2002).

37 Tagliavini F, Forloni G, Colombo L *et al.* Tetracycline affects abnormal properties of synthetic PrP peptides and PrP(Sc) *in vitro*. *J. Mol. Biol.* 300(5), 1309–1322 (2000).

38 Forloni G, Iussich S, Awan T *et al.* Tetracyclines affect prion infectivity. *Proc. Natl Acad. Sci. USA* 99(16), 10849–10854 (2002).
- **Tetracycline prolongs incubation and survival times in scrapie-infected hamsters.**

39 Caughey WS, Raymond LD, Horiuchi M, Caughey B. Inhibition of protease-resistant prion protein formation by porphyrins and phthalocyanines. *Proc. Natl Acad. Sci. USA* 95(21), 12117–12122 (1998).

40 Priola SA, Raines A, Caughey WS. Porphyrin and phthalocyanine antiscrapie compounds. *Science* 287(5457), 1503–1506 (2000).
- **Cyclic tetrapyrroles prolong survival in scrapie-infected mice by up to 300%.**

41 Priola SA, Raines A, Caughey W. Prophylactic and therapeutic effects of phthalocyanine tetrasulfonate in scrapie-infected mice. *J. Infect. Dis.* 188(5), 699–705 (2003).

42 Kocisko DA, Caughey WS, Race RE, Roper G, Caughey B, Morrey JD. A porphyrin increases survival time of mice after intracerebral prion infection. *Antimicrob. Agents Chemother.* 50(2), 759–761 (2006).

43 Supattapone S, Nguyen HO, Cohen FE, Prusiner SB, Scott MR. Elimination of prions by branched polyamines and implications for therapeutics. *Proc. Natl Acad. Sci. USA* 96(25), 14529–14534 (1999).

44 Winklhofer KF, Tatzelt J. Cationic lipopolyamines induce degradation of PrPSc in scrapie-infected mouse neuroblastoma cells. *Biol. Chem.* 381(5–6), 463–469 (2000).

45 Bera A, Nandi PK. Biological polyamines inhibit nucleic-acid-induced polymerisation of prion protein. *Arch. Virol.* 152(4), 655–668 (2007).

46 Deleault NR, Lucassen RW, Supattapone S. RNA molecules stimulate prion protein conversion. *Nature* 425(6959), 717–720 (2003).

47 Deleault NR, Harris BT, Rees JR, Supattapone S. Formation of native prions from minimal components *in vitro*. *Proc. Natl Acad. Sci. USA* 104(23), 9741–9746 (2007).
- •• **Generation of infectious prions from PrP^c by protein misfolding cyclic amplification in the presence of polyA RNA.**

48 Merlini G, Ascari E, Amboldi N *et al.* Interaction of the anthracycline 4'-iodo-4'-deoxydoxorubicin with amyloid fibrils: inhibition of amyloidogenesis. *Proc. Natl Acad. Sci. USA* 92(7), 2959–2963 (1995).

49 Tagliavini F, McArthur RA, Canciani B *et al.* Effectiveness of anthracycline against experimental prion disease in Syrian hamsters. *Science* 276(5315), 1119–1122 (1997).
- **The anthracycline iododeoxydoxorubcin prolongs survival time in hamsters.**

50 Korth C, May BC, Cohen FE, Prusiner SB. Acridine and phenothiazine derivatives as pharmacotherapeutics for prion disease. *Proc. Natl Acad. Sci. USA* 98(17), 9836–9841 (2001).
- **The acridine derivative quinacrine and the phenothiazine derivative chlorpromazine block PrP^{Sc} propagation *in vitro*.**

51 Ryou C, Legname G, Peretz D, Craig JC, Baldwin MA, Prusiner SB. Differential inhibition of prion propagation by enantiomers of quinacrine. *Lab. Invest.* 83(6), 837–843 (2003).

52 Doh-ura K, Ishikawa K, Murakami-Kubo I *et al.* Treatment of transmissible spongiform encephalopathy by intraventricular drug infusion in animal models. *J. Virol.* 78(10), 4999–5006 (2004).

53 Barret A, Tagliavini F, Forloni G *et al.* Evaluation of quinacrine treatment for prion diseases. *J. Virol.* 77(15), 8462–9 (2003).

54 Soto C, Kascsak RJ, Saborio GP *et al.* Reversion of prion protein conformational changes by synthetic β-sheet breaker peptides. *Lancet* 355(9199), 192–197 (2000).
- **Synthetic β-sheet breakers reverse conformational changes in prion proteins.**

55 Weiss S, Proske D, Neumann M *et al.* RNA aptamers specifically interact with the prion protein PrP. *J. Virol.* 71(11), 8790–8797 (1997).

56 Proske D, Gilch S, Wopfner F, Schatzl HM, Winnacker EL, Famulok M. Prion-protein-specific aptamer reduces PrP^{Sc} formation. *Chembiochem* 3(8), 717–725 (2002).

57 Ertmer A, Gilch S, Yun SW *et al.* The tyrosine kinase inhibitor STI571 induces cellular clearance of PrP^{Sc} in prion-infected cells. *J. Biol. Chem.* 279(40), 41918–41927 (2004).

58 Korth C, Stierli B, Streit P *et al.* Prion (PrPSc)-specific epitope defined by a monoclonal antibody. *Nature* 390(6655), 74–77 (1997).
- **15B3 is the first antibody specifically recognizing PrPBSE.**

59 Feraudet C, Morel N, Simon S *et al.* Screening of 145 anti-PrP monoclonal antibodies for their capacity to inhibit PrP^{Sc} replication in infected cells. *J. Biol. Chem.* 280(12), 11247–11258 (2005).

60 Morel E, Andrieu T, Casagrande F *et al.* Bovine prion is endocytosed by human enterocytes via the 37 kDa/67 kDa laminin receptor. *Am. J. Pathol.* 167(4), 1033–1042 (2005).
- **LRP/LR-dependent internalization of bovine prions by human enterocytes.**

61 Paramithiotis E, Pinard M, Lawton T *et al.* A prion protein epitope selective for the pathologically misfolded conformation. *Nat. Med.* 9(7), 893–899 (2003).
- **An antibody directed against the YYR-motif of PrP specifically recognizes PrP^{Sc}.**

62 Enari M, Flechsig E, Weissmann C. Scrapie prion protein accumulation by scrapie-infected neuroblastoma cells abrogated by exposure to a prion protein antibody. *Proc. Natl Acad. Sci. USA* 98(16), 9295–9299 (2001).
- **Monoclonal anti-PrP antibody 6H4 cures scrapie-propagating cells.**

63 Peretz D, Williamson RA, Kaneko K *et al.* Antibodies inhibit prion propagation and clear cell cultures of prion infectivity. *Nature* 412(6848), 739–743 (2001).
- **Anti-PrP antibody F_{ab} D18 cures scrapie-infected cells.**

64 Flechsig E, Shmerling D, Hegyi I *et al.* Prion protein devoid of the octapeptide repeat region restores susceptibility to scrapie in PrP knockout mice. *Neuron* 27(2), 399–408 (2000).

65 Kim CL, Karino A, Ishiguro N, Shinagawa M, Sato M, Horiuchi M. Cell-surface retention of PrP^c by anti-PrP antibody prevents protease-resistant PrP formation. *J. Gen. Virol.* 85(Pt 11), 3473–3482 (2004).

66 Perrier V, Solassol J, Crozet C *et al.* Anti-PrP antibodies block PrP^{Sc} replication in prion-infected cell cultures by accelerating PrP^c degradation. *J. Neurochem.* 89(2), 454–463 (2004).

67 Gilch S, Wopfner F, Renner-Muller I *et al.* Polyclonal anti-PrP auto-antibodies induced with dimeric PrP interfere efficiently with PrP^{Sc} propagation in prion-infected cells. *J. Biol. Chem.* 278(20), 18524–18531 (2003).
- **An anti-PrP autoantibody blocks PrP^{Sc} propagation in cultured cells.**

68 Moroncini G, Kanu N, Solforosi L *et al.* Motif-grafted antibodies containing the replicative interface of cellular PrP are specific for PrP^{Sc}. *Proc. Natl Acad. Sci. USA* 101(28), 10404–10409 (2004).

▶69 Heppner FL, Musahl C, Arrighi I *et al.* Prevention of scrapie pathogenesis by transgenic expression of anti-prion protein antibodies. *Science* 294(5540), 178–182 (2001).

• **Transgenic expression of a prion antibody prevents scrapie pathogenesis.**

▶70 White AR, Enever P, Tayebi M *et al.* Monoclonal antibodies inhibit prion replication and delay the development of prion disease. *Nature* 422(6927), 80–83 (2003).

• **Monoclonal anti-PrP antibody delays development of prion diseases.**

▶71 Sigurdsson EM, Sy MS, Li R *et al.* Anti-prion antibodies for prophylaxis following prion exposure in mice. *Neurosci. Lett.* 336(3), 185–187 (2003).

▶72 Hundt C, Peyrin JM, Haik S *et al.* Identification of interaction domains of the prion protein with its 37-kDa/67-kDa laminin receptor. *EMBO J.* 20(21), 5876–5886 (2001).

• **HSPGs act as cofactors/coreceptors for PrP^c.**

▶73 Gauczynski S, Peyrin JM, Haik S *et al.* The 37-kDa/67-kDa laminin receptor acts as the cell-surface receptor for the cellular prion protein. *EMBO J.* 20(21), 5863–5875 (2001).

• **LRP/LR acts as the receptor for cellular PrP^c.**

▶74 Leucht C, Simoneau S, Rey C *et al.* The 37 kDa/67 kDa laminin receptor is required for PrP(Sc) propagation in scrapie-infected neuronal cells. *EMBO Rep.* 4(3), 290–295 (2003).

• **Anti-LRP antibody W3 cures N2a cells from scrapie and siRNAs directed against LRP mRNA block PrP^{Sc} propagation *in vitro*.**

75 Zuber C, Knackmuss S, Rey C *et al.* Single chain Fv antibodies directed against the 37kDa/ 67kDa laminin receptor as therapeutic tools in prion diseases. *Mol. Immunol.* (2007) (Epub ahead of print).

▶76 Pankiewicz J, Prelli F, Sy MS *et al.* Clearance and prevention of prion infection in cell culture by anti-PrP antibodies. *Eur. J. Neurosci.* 23(10), 2635–2647 (2006).

▶77 Solforosi L, Criado JR, McGavern DB *et al.* Cross-linking cellular prion protein triggers neuronal apoptosis *in vivo*. *Science* 303(5663), 1514–1516 (2004).

• **Crosslinking of PrP by anti-PrP antibodies induces apotosis**

▶78 Adamson CS, Yao Y, Vasiljevic S, Sy MS, Ren J, Jones IM. Novel single chain antibodies to the prion protein identified by phage display. *Virology* 358(1), 166–177 (2007).

▶79 Leclerc E, Liemann S, Wildegger G, Vetter SW, Nilsson F. Selection and characterization of single chain Fv fragments against murine recombinant prion protein from a synthetic human antibody phage display library. *Hum. Antibodies* 9(4), 207–214 (2000).

▶80 Donofrio G, Heppner FL, Polymenidou M, Musahl C, Aguzzi A. Paracrine inhibition of prion propagation by anti-PrP single-chain Fv miniantibodies. *J. Virol.* 79(13), 8330–8338 (2005).

▶81 Vetrugno V, Cardinale A, Filesi I *et al.* KDEL-tagged anti-prion intrabodies impair PrP lysosomal degradation and inhibit scrapie infectivity. *Biochem. Biophys. Res. Commun.* 338(4), 1791–1797 (2005).

• **Anti-PrP intrabodies inhibit scrapie infectivity (cell therapeutic approach)**

▶82 Buchholz CJ, Bach P, Nikles D, Kalinke U. Prion protein-specific antibodies for therapeutic intervention of transmissible spongiform encephalopathies. *Expert Opin. Biol. Ther.* 6(3), 293–300 (2006).

▶83 Polymenidou M, Heppner FL, Pellicioli EC *et al.* Humoral immune response to native eukaryotic prion protein correlates with anti-prion protection. *Proc. Natl Acad. Sci. USA* 101(Suppl. 2), 14670–14676 (2004).

▶84 Nikles D, Bach P, Boller K *et al.* 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(7), 4033–4042 (2005).

▶85 Goni F, Knudsen E, Schreiber F *et al.* Mucosal vaccination delays or prevents prion infection via an oral route. *Neuroscience* 133(2), 413–421 (2005).

▶86 Fernandez-Borges N, Brun A, Whitton JL *et al.* DNA vaccination can break immunological tolerance to PrP in wild-type mice and attenuates prion disease after intracerebral challenge. *J. Virol.* 80(20), 9970–9976 (2006).

▶87 Handisurya A, Gilch S, Winter D *et al.* Vaccination with prion peptide-displaying papillomavirus-like particles induces autoantibodies to normal prion protein that interfere with pathologic prion protein production in infected cells. *FEBS J.* 274(7), 1747–1758 (2007).

• **Prion peptide-displaying papillomavirus-like particles induce autoantibodies against PrP.**

88 Pillon JL, Loiacono CM, Okeson D *et al.* Early Success in the development of a PrP^{Sc} vaccine using a mouse model system. Presented at: *Prion 2006 Conference (Strategies, Advances and Trends Towards Protection of Society)*. Torino, Italy, 3–6 October 2006.

•• **Proof of infectious prions in saliva and blood of deer suffering from chronic wasting disease.**

▶89 Mathiason CK, Powers JG, Dahmes SJ *et al.* Infectious prions in the saliva and blood of deer with chronic wasting disease. *Science* 314(5796), 133–136 (2006).

•• **Proof of infectious prions in saliva and blood of deer suffering from chronic wasting disease.**

▶90 Priola SA, Caughey B. Inhibition of scrapie-associated PrP accumulation. Probing the role of glycosaminoglycans in amyloidogenesis. *Mol. Neurobiol.* 8(2–3), 113–120 (1994).

▶91 Kaneko K, Zulianello L, Scott M *et al.* Evidence for protein X binding to a discontinuous epitope on the cellular prion protein during scrapie prion propagation. *Proc. Natl Acad. Sci. USA* 94(19), 10069–10074 (1997).

▶92 Hölscher C, Delius H, Bürkle A. Overexpression of nonconvertible PrPc δ114–121 in scrapie-infected mouse neuroblastoma cells leads to trans-dominant inhibition of wild-type PrP(Sc) accumulation. *J. Virol.* 72(2), 1153–1159 (1998).

▶93 Perrier V, Kaneko K, Safar J *et al.* Dominant-negative inhibition of prion replication in transgenic mice. *Proc. Natl Acad. Sci. USA* 99(20), 13079–13084 (2002).

▶94 Furuya K, Kawahara N, Yamakawa Y *et al.* Intracerebroventricular delivery of dominant negative prion protein in a mouse model of iatrogenic Creutzfeldt–Jakob disease after dura graft transplantation. *Neurosci. Lett.* 402(3), 222–226 (2006).

▶95 Crozet C, Lin YL, Mettling C *et al.* Inhibition of PrP^{Sc} formation by lentiviral gene transfer of PrP containing dominant negative mutations. *J. Cell. Sci.* 117(Pt 23), 5591–5597 (2004).

▶96 Bueler H, Aguzzi A, Sailer A *et al.* Mice devoid of PrP are resistant to scrapie. *Cell* 73(7), 1339–1347 (1993).

•• **PrP^c depletion in mice leads to resistance to a scrapie infection.**

▶97 Vana K, Weiss S. A trans-dominant negative 37kDa/67kDa laminin receptor mutant impairs PrP(Sc) propagation in scrapie-infected neuronal cells. *J. Mol. Biol.* 358(1), 57–66 (2006).

▶98 Daude N, Marella M, Chabry J. Specific inhibition of pathological prion protein accumulation by small interfering RNAs. *J. Cell. Sci.* 116(Pt 13), 2775–2779 (2003).

99 Golding MC, Long CR, Carmell MA, Hannon GJ, Westhusin ME. Suppression of prion protein in livestock by RNA interference. *Proc. Natl Acad. Sci. USA* 103(14), 5285–5290 (2006).

100 Pfeifer A, Eigenbrod S, Al-Khadra S *et al.* Lentivector-mediated RNAi efficiently suppresses prion protein and prolongs survival of scrapie-infected mice. *J. Clin. Invest.* 116(12), 3204–3210 (2006).

•• **RNA interference directed against PrP mRNA delivered by lentiviral vectors prolongs survival in scrapie-infected mice.**

101 Bueler H, Fischer M, Lang Y *et al.* Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. *Nature* 356(6370), 577–582 (1992).

•• **PrP^c-deficient mice behave normal.**

102 Richt JA, Kasinathan P, Hamir AN *et al.* Production of cattle lacking prion protein. *Nat. Biotechnol.* 25(1), 132–8 (2007).

•• **PrP^c-deficient cattle show a normal phenotype.**

103 Leucht C, Vana K, Renner-Muller I *et al.* Knock-down of the 37-kDa/67-kDa laminin receptor in mouse brain by transgenic expression of specific antisense LRP RNA. *Transgenic Res.* 13(1), 81–85 (2004).

104 Rieger R, Edenhofer F, Lasmezas CI, Weiss S. The human 37-kDa laminin receptor precursor interacts with the prion protein in eukaryotic cells. *Nat. Med.* 3(12), 1383–1388 (1997).

• **Identification of LRP/LR as a PrP^c accomplice.**

105 Zuber C, Ludewigs H, Weiss S. Therapeutic approaches targeting the prion receptor LRP/LR. *Vet. Microbiol.* 123, 387–393 (2007).

106 Trevitt CR, Collinge J. A systematic review of prion therapeutics in experimental models. *Brain* 129, 2241–2265 (2006).

107 Gresser I, Maury C, Chandler RL. Failure to modify scrapie in mice by administration of interferon or anti-interferon globulin. *J. Gen. Virol.* 64, 1387–1389 (1983).

108 Grigoriev V, Adjou KT, Sales N *et al.* Effects of the polyene antibiotic derivative MS-8209 on the astrocyte lysosomal system of scrapie-infected hamsters. *J. Mol. Neurosci.* 18, 271–281 (2002).

109 Terzano MG, Montanari E, Calzetti S, Mancia D, Lechi A. The effect of amantadine on arousal and EEG patterns in Creutzfeldt–Jakob disease. *Arch. Neurol.* 40(9), 555–559 (1983).

110 Dealler S, Rainov N. Pentosan polysulfate as a prophylactic and therapeutic agent against prion disease. I. *Drugs* 6, 470–478 (2003).

111 Parry A, Baker I, Stacey R, Wimalaratna S. Long term survival in a patient with variant Creutzfeldt–Jakob disease treated with intraventricular pentosan polysulphate. *J. Neurol. Neurosurg. Psychiatry* 78(7), 733–734 (2007).

112 Farquard C. Sodium pentosan polysulfate use in rodent models of Scrapie and BSE. Oral presentation at: *Prion 2006 Conference (Strategies, Advances and Trends Towards Protection of Society).* Torino, Italy, 3–6 October 2006.

113 Otto M, Cepek L, Ratzka P *et al.* Efficacy of flupirtine on cognitive function in patients with CJD: a double-blind study. *Neurology* 62, 714–718 (2004).

114 Haik S, Brandel JP, Salomon D *et al.* Compassionate use of quinacrine in Creutzfeldt–Jakob disease fails to show significant effects. *Neurology* 63(12), 2413–2415 (2004).

115 Forloni G, Colombo L, Girola L, Tagliavini F, Salamona M. Anti-amyloidogenic activity of tetracyclines: studies *in vitro*. *FEBS Lett.* 487(3), 404–407 (2001).

116 Tagliavini F. Prion disease therapeutics: Tetracyclic compounds in *in vitro* models, animal models and patients with Creutzfeldt–Jakob Disease. Oral presentation at: *6th National TSE-Forschungsplattform Congress*. Greifswald, Germany, 26–28 June 2007.

117 Parchi P, Giese A, Capellari S *et al.* Classification of sporadic Creutzfeldt–Jakob disease based on molecular and phenotypic analysis of 300 subjects. *Ann. Neurol.* 46, 224–233 (1999).

118 Heinemann U, Krasnianski A, Meissner B *et al.* Creutzfeldt–Jakob disease in Germany: a prospective 12-years surveillance. *Brain* 130, 1350–1359 (2007).

• **Prospective CJD surveillance study in Germany.**

119 Zerr I, Schulz-Schaeffer WJ, Giese A *et al.* Current clinical diagnosis in CJD: identification of uncommon variants. *Ann. Neurol.* 48, 323–329 (2000).

120 Sanchez-Juan P, Green A, Ladogana A *et al.* Cerebrospinal fluid tests in the differential diagnosis of CJD. *Neurology* 67(4), 637–643 (2006).

• **Cerebrospinal fluid tests in CJD diagnosis.**

121 Tschampa HJ, Kallenberg K, Urbach H *et al.* MRI in the diagnosis of sporadic Creutzfeldt–Jakob disease: a study on inter-observer agreement. *Brain* 128(Pt 9), 2026–2033 (2005).

122 Pocchiari M, Poupolo M, Croes EA *et al.* Predictors of survival in sporadic Creutzfeldt–Jakob disease and other human transmissible spongiform encephalopathies. *Brain* 10, 2348–2359 (2004).

123 Cordery RJ, Hall M, Cipolotti L *et al.* Early cognitive decline in Creutzfeldt–Jakob disease associated with human growth hormone treatment. *J. Neurol. Neurosurg. Psychiatry* 74, 1412–1416 (2003).

124 Cordery RJ, Alner K, Cipolotti L *et al.* The neuropsychology of variant CJD: a comparative study with inherited and sporadic forms of prion disease. *J. Neurol. Neurosurg. Psychiatry* 76, 330–336 (2005).

125 Hamaguchi T, Kitamoto T, Sato T *et al.* Clinical diagnosis of MM2-type sporadic Creutzfeldt–Jakob disease. *Neurology* 64, 643–8 (2005).

126 Zerr I, Poser S. Clinical diagnosis and differential diagnosis of CJD and vCJD. With special emphasis on laboratory tests. *APMIS* 110, 88–98 (2002).

127 Brown P, Gibbs CJ Jr, Rodgers-Johnson P *et al.* Human spongiform encephalopathy: the National Institutes of Health series of 300 cases of experimentally transmitted disease. *Ann. Neurol.* 35(5), 513–529 (1994).

128 Otto A, Zerr I, Lantsch M, Weidehaas K, Riedemann C, Poser S. Akinetic mutism as a classification criterion for the diagnosis of Creutzfeldt–Jakob disease. *J. Neurol. Neurosurg. Psychiatry* 64(4), 524–528 (1998).

129 Krasnianski A, Meissner B, Schulz-Schaeffer W *et al.* Clinical features and diagnosis of the MM2 cortical subtype of sporadic Creutzfeldt–Jakob disease. *Arch. Neurol.* 63(6), 876–880 (2006).

130 Krasnianski A, Schulz-Schaeffer WJ, Kallenberg K *et al.* Clinical findings and diagnostic tests in the MV-2 subtype of sporadic CJD. *Brain* 129, 2288–2296 (2006).

131 Meissner B, Westner I, Kallenberg K *et al.* Sporadic Creutzfeldt–Jakob disease: clinical and diagnostic characteristics of the rare VV1 type. *Neurology* 65(10), 1544–1550 (2005).

132 de Silva R. Human spongiform encephalopathy. In: *Prion Diseases.* Baker HF, Ridley RM (Eds). Humana Press, NJ, USA 15–33 (1996).

133 Haïk S, Brandel JP, Sazdovitch V *et al.* Dementia with Lewy bodies in a neuropathologic series of suspected Creutzfeldt–Jakob disease. *Neurology* 55, 1401–1404 (2000).

134 Poser S, Mollenhauer B, Krauss A *et al.* How to improve the clinical diagnosis of Creutzfeldt–Jakob disease. *Brain* 122, 2345–2351 (1999).

135 Tschampa HJ, Neumann M, Zerr I *et al.* Patients with Alzheimer's disease and dementia with Lewy bodies mistaken for Creutzfeldt–Jakob disease. *J. Neurol. Neurosurg. Psychiatry* 71, 33–39 (2001).

136 Van Everbroeck B, Dobbeleir I, De Waele M, De Deyn P, Martin J-J, Cras P. Differential diagnosis of 201 possible Creutzfeldt–Jakob disease patients. *J. Neurol.* 251, 298–304 (2004).

137 Boesenberg C, Schulz-Schaeffer WJ, Meissner B *et al.* Clinical course in young patients with sporadic Creutzfeldt–Jakob disease. *Ann. Neurol.* 58, 533–543 (2005).

138 Ladogana A, Puopolo M, Croes EA *et al.* Mortality from Creutzfeldt–Jakob disease and related disorders in Europe, Australia, and Canada. *Neurology* 64, 1586–1591 (2005).

139 Braaten AJ, Parsons TD, McCue R, Sellers A, Burns WJ. Neurocognitive differential diagnosis of dementing diseases: Alzheimer's dementia, vascular dementia, frontotemporal dementia, and major depressive disorder. *Int. J. Neurosci.* 116(11), 1271–1293 (2006).

140 Doh-ura K. Pentosan polysulfate and amyloidophilic chemicals for prion diseases. Presented at: *Prion 2006 Conference (Strategies, Advances and Trends Towards Protection of Society).* Torino, Italy, 3–6 October 2006.

141 Riek R, Hornemann S, Wider G, Billeter M, Glockshuber R, Wuthrich K. NMR structure of the mouse prion protein domain PrP(121–321). *Nature* 382(6587), 180–182 (1996).

- **First nuclear magnetic resonance structure of PrP (121–231).**

142 Supattapone S, Wille H, Uyechi L *et al.* Branched polyamines cure prion-infected neuroblastoma cells. *J. Virol.* 75(7), 3453–3461 (2001).

143 Beringue V, Vilette D, Mallinson G *et al.* PrP^{Sc} binding antibodies are potent inhibitors of prion replication in cell lines. *J. Biol. Chem.* 279(38), 39671–39676 (2004).

144 David AS, Grant R, Ballantyne JP. Unsuccessful treatment of Creutzfeldt–Jakob disease with acyclovir [letter]. *Lancet* 1(8375), 512–513 (1984).

145 Newman PK. Acyclovir in Creutzfeldt–Jakob disease [letter]. *Lancet* 1(8380), 793 (1984).

146 Braham J. Amantadine in the treatment of Creutzfeldt–Jakob disease [letter]. *Arch. Neurol.* 41(6), 585–586 (1984).

147 Ratcliffe J, Rittman A, Wolf S, Verity MA. Creutzfeldt–Jakob disease with focal onset unsuccessfully treated with amantadine. *Bull. Los Angeles Neurol. Soc.* 40(1), 18–20 (1975).

148 Terzano MG, Mancia D, Manzoni GC. Periodic activities in Creutzfeldt–Jakob disease and epilepsy. *Prog. Clin. Biol. Res.* 124(199), 199–226 (1983).

149 Braham J. Jakob-Creutzfeldt disease: treatment by amantadine. *Br. Med. J.* 4, 212–213 (1971).

150 Sanders WL. Creutzfeldt–Jakob disease treated with amantadine. *J. Neurol. Neurosurg. Psychiatry* 42(10), 960–961 (1979).

151 Sanders WL, Dunn TL. Creutzfeldt–Jakob disease treated with amantidine. A report of two cases. *J. Neurol. Neurosurg. Psychiatry* 36(4), 581–584 (1973).

152 Masullo C, Macchi G, Xi YG, Pocchiari M. Failure to ameliorate Creutzfeldt–Jakob disease with amphotericin B therapy [letter]. *J. Infect. Dis.* 165(4), 784–785 (1992).

153 Kovanen J, Haltia M, Cantell K. Failure of interferon to modify Creutzfeldt–Jakob disease. *Br. Med. J.* 280, 902 (1980).

154 Nakajima M, Yamada T, Kusuhara T *et al.* Results of quinacrine administration to patients with Creutzfeldt–Jakob disease. *Dement. Geriatr. Cogn. Disord.* 17, 158–163 (2004).

155 Benito-Leon J. Compassionate use of quinacrine in Creutzfeldt–Jakob disease fails to show significant effects. *Neurology* 64(10), 1824 (2005).

156 Satoh K, Shirabe S, Eguchi K *et al.* Toxicity of quinacrine can be reduced by co-administration of P-glycoprotein inhibitor in sporadic Creutzfeldt–Jakob disease. *Cell. Mol. Neurobiol.* 24(6), 873–875 (2004).

157 Scoaazec JY, Krolak-Salmon P, Casez O *et al.* Quinacrine-induced cytolytic hepatitis in sporadic Creutzfeldt–Jakob disease. *Ann. Neurol.* 53(4), 546–547 (2003).

158 Furlow TJ, Whitley RJ, Wilmes FJ. Repeated suppression of Creutzfeldt–Jakob disease with vidarabine [letter]. *Lancet* 2(8297), 564–565 (1982).

Websites

201 The National Creutzfeldt-Jakob Disease Surveillance Unit www.cjd.ed.ac.uk

202 The Federal Ministry of Food, Agriculture and Consumer Protection www.verbraucherministerium.de

203 Department of Cellular and Molecular Pharmacology, UCSF, USA http://cmp.ucsf.edu/faculty/pdb_show.html?id=cohen

Affiliations

- *Heike Ludewigs*
  Pharmacist, PhD student, Laboratorium für Molekulare Biologie – Genzentrum – Institut für Biochemie der LMU München, Feodor-Lynen-Str. 25, D-81377 München, Germany
  Tel.: +49 892 180 76885
  Fax: +49 892 180 76999
  ludewigs@lmb.uni-muenchen.de
- *Chantal Zuber*
  Biologist, PhD student, Laboratorium für Molekulare Biologie – Genzentrum – Institut für Biochemie der LMU München, Feodor-Lynen-Str. 25, D-81377 München, Germany
  Tel.: +49 892 180 76885
  Fax: +49 892 180 76999
  zuber@lmb.uni-muenchen.de
- *Karen Vana, PhD*
  Postdoctoral fellow, Laboratorium für Molekulare Biologie – Genzentrum – Institut für Biochemie der LMU München, Feodor-Lynen-Str. 25, D-81377 München, Germany
  Tel.: +49 892 180 76886
  Fax: +49 892 180 76999
  vana@lmb.uni-muenchen.de
- *Daphne Nikles, PhD*
  Postdoctoral fellow, Laboratorium für Molekulare Biologie – Genzentrum – Institut für Biochemie der LMU München, Feodor-Lynen-Str. 25, D-81377 München, Germany
  Tel.: +49 892 180 76885
  Fax: +49 892 180 76999
  nikles@lmb.uni-muenchen.de
- *Inga Zerr, MD*
  Professor, National Reference Center for TSE Surveillance, Dept. of Neurology, Georg-August-University Göttingen, Robert-Koch-Str, 40, D-37075 Göttingen, Germany
  Tel.: +49 551 396 636
  Fax: +49 551 397 020
  ingazerr@med.uni-goettingen.de
- *Stefan Weiss, PhD*
  Assistant Professor, Laboratorium für Molekulare Biologie – Genzentrum – Institut für Biochemie der LMU München, Feodor-Lynen-Str. 25, D-81377 München, Germany
  Tel.: +49 892 180 76887
  Fax: +49 892 180 76999
  weiss@lmb.uni-muenchen.de

Chapter II

Therapeutic approaches targeting the prion receptor LRP/LR

published as:

Zuber, C., Ludewigs, H. and Weiss, S., Therapeutic approaches targeting the prion receptor LRP/LR. *Vet Microbiol* 2007. 4: 387-393.

Available online at www.sciencedirect.com
ScienceDirect
Veterinary Microbiology 123 (2007) 387–393
veterinary microbiology
www.elsevier.com/locate/vetmic

Therapeutic approaches targeting the prion receptor LRP/LR

Chantal Zuber, Heike Ludewigs, Stefan Weiss *

Laboratorium für Molekulare Biologie-Genzentrum, Institut für Biochemie der Ludwig-Maximilians-Universität München, Munich, Germany

Abstract

Transmissible spongiform encephalopathies known as prion diseases are a group of fatal neurodegenerative disorders that affect both humans and animals. The generally accepted principle of the disease is that the conversion of the cellular prion protein (PrP^c) into the disease associated isoform PrP^{Sc} leads to spongiform degeneration of the brain and amyloid plaque formation. Until now no therapy leading to potential alleviation or even cure of the disease exists. It is therefore important to develop therapeutic approaches for the treatment of TSEs since these infections are inevitably fatal and, especially in the case of vCJD, they affect youngsters. Besides current conventional therapeutic strategies, this review summarizes new therapeutic tools targeting the prion receptor LRP/LR.

Keywords: Bovine spongiform encephalopathy; 37 kDa/67 kDa laminin receptor; LRP/LR; Prion; PrP; Transmissible spongiform encephalopathy; Therapy

1. Introduction

Prion diseases or TSEs are neurological disorders associated with the aggregation of a pathologic isoform of a host-encoded prion protein (PrP). Conversion of the cellular prion protein (PrP^c) into the disease-associated form PrP^{Sc} leads to conformational changes resulting in aggregation and accumulation. Deposition of this abnormal protein takes place mainly in the brain and the lymphoreticular system, accompanied with neuronal vacuolation (spongiosis) and neuronal death. After extremely long incubation times, affected individuals show progressive neurological symptoms terminating in death. Conventional therapeutic approaches use anti-prion compounds which can prolong incubation times but do not lead to a cure. It has been demonstrated that prion propagation *in vitro* requires the laminin receptor (Leucht et al., 2003) implicating that approaches downregulating LRP/LR are a promising alternative strategy for the treatment of prion diseases.

2. Prion diseases in humans and animals

Prion diseases involve rapid neurological decline, accompanied by neuronal loss and spongiform changes caused by accumulation of the aggregated and misfolded prion protein. The most common type

* Corresponding author. Tel.: +49 89 2180 76887; fax: +49 89 2180 76999.
E-mail address: weiss@lmb.uni-muenchen.de (S. Weiss).

0378-1135/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetmic.2007.04.005

of human prion diseases, termed Creutzfeldt-Jakob disease (CJD), can be classified into four categories: sporadic (sCJD), inherited/familial (fCJD), iatrogenic (iCJD) and variant (vCJD). Whereas it has been suggested that the latter results from ingestion/consumption of BSE-contaminated food (Bruce et al., 1997), familial disorders (fCJD) are the inheritance of autosomal-dominant mutations within the *Prn*-p locus. Transplantation of tissues or injection of hormones originating from individuals suffering from CJD or the use of contaminated surgical instruments resulted in the iatrogenic form of CJD. Gerstmann–Sträussler–Scheinker syndrome (GSS), fatal familial insomnia (FFI), its sporadic form (sFI) and kuru are other human prion diseases.

Animal TSEs have been observed in different species: Bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goat, chronic wasting disease (CWD) in cervids such as deer, elk or captive mule and feline spongiform encephalopathy (FSE) in cats or transmissible mink encephalopathy (TME). In addition, some exotic diseases were observed including exotic ungulate encephalopathy (EUE) and primate spongiform encephalopathy (PSE). Transmission of BSE to pigs has been experimentally proven (Wells et al., 2003). Recently, new forms of TSEs with unusual characteristics e.g. an atypical scrapie case (Nor98) (Benestad et al., 2003) have been discovered. Apart from the existing species barrier, the different modes of transmission are not yet understood.

3. Prions and different forms of prion proteins

The term prion was defined by Stanley Prusiner as a ‘‘small proteinaceous particle that resists inactivation by procedures which modify nucleic acids’’, suggesting that a new agent exists beside the commonly known pathogenic organisms such as bacteria, virus or fungi (Prusiner, 1982). The infectious ‘‘agent’’, the prion, and the exact infectious mechanism for prion disorders is just as little understood as the mechanism by which they kill neurons.

PrP^{c} is an ubiquitous membrane-bound glycoprotein attached to the cell surface by a glycosylphosphatidylinositol (GPI) anchor, expressed in many tissues and cell types. Its conversion leads to the disease-associated form PrP^{Sc}, which exhibits a higher β-sheet content correlating with a high tendency to form aggregates. PrP^{Sc} is characterized as insoluble and partially resistant to proteases (Cohen and Prusiner, 1998). Digestion of PrP^{c} with protease K results in the truncated form PrPres (a 27–30 kDa fragment) demonstrating insolubility in aqueous and organic solvents as well as in non-ionic detergents. Additionally, it is completely resistant to proteases. PrP^{Sc} and PrP27-30 both have the tendency to form amyloid fibrils.

4. The 37 kDa/67 kDa LRP/LR as the receptor for PrP^{c}

In a yeast two-hybrid screen we identified the 37 kDa laminin receptor precursor (LRP) as an interaction partner for the prion protein (Rieger et al., 1997). Further *in vitro* studies on neuronal and non-neuronal cells proved that both the 37 kDa LRP and the 67 kDa high affinity laminin receptor function as the receptor for the cellular prion protein (Gauczynski et al., 2001). Direct and indirect heparan sulphate proteoglycane (HSPG)-dependent binding domains on LRP/LR and on PrP have been identified suggesting that HSPGs act as co-factors or co-receptors for PrP^{c} (Hundt et al., 2001). It has been suggested that the 37 kDa LRP is the precursor of the 67 kDa form which was first isolated 1983 from melanoma cells due to its high binding capacity to laminin (Rao et al., 1983). The relationship between the 37 kDa precursor form and the mature 67 kDa isoform is still unknown. Regarding the function of LRP/LR, the 37 kDa LRP appears to be a multifunctional protein involved in the translational machinery (Auth and Brawerman, 1992) and has also been identified as a ribosome-associated protein termed p40 (Makrides et al., 1988). LRP has also been localized in the nucleus, where it is closely associated with nuclear structures (Sato et al., 1996) and binds to DNA through connections with histones H2A, H2B and H4 (Kinoshita et al., 1998). The 37 kDa/67 kDa LRP/LR has been described 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), Dengue virus (Tio et al., 2005) and Adeno-Associated Viruses (Akache et al., 2006). In addition, studies have been carried out

in order to detect the isoforms that are present in the central nervous system and that bind PrP. Several maturation states of the receptor were identified, including a 44 kDa, 60 kDa, 67 kDa and a 220 kDa form. All of these isoforms were able to bind PrP, suggesting a physiological role for the laminin receptor/PrP interaction in the brain (Simoneau et al., 2003). Although LRP consists of a transmembrane domain (amino acid residue 86–101 (Castronovo et al., 1991)) it is abundant in the cytoplasm (Romanov et al., 1994). In mammalian cells both the 37 kDa LRP and the 67 kDa LR are present in plasma membrane fractions (Gauczynski et al., 2001).

5. The role of LRP/LR in PrP^{Sc} propagation

LRP/LR not only acts as a receptor for the cellular prion protein but also for the infectious PrP27-30, an N-terminal truncated version of PrP^{Sc} (Gauczynski et al., 2006). The importance of LRP/LR in PrP^{Sc} propagation was verified using a polyclonal anti-LRP/LR antibody termed W3 which was able to block and prevent the binding of PrP^{Sc} and to cure scrapie-infected neuroblastoma cells (ScN2a) from PrP^{Sc} (Leucht et al., 2003).

LRP/LR-dependent binding of PrP^{c} and PrP^{Sc} to the cell surface (either alone or together with other co-factors) is accompanied by internalisation which is thought to occur in clathrin-coated pits. After this receptor-mediated endocytosis the conversion of PrP^{c} molecules into the disease-associated form probably takes place in endosomes, lysosomes or endolysosomes. Heparan sulphates also play an essential role in prion uptake and cell infection (Horonchik et al., 2005) suggesting that both the LRP/LR and heparan sulphates act presumably in synergy for PrP^{Sc} binding and internalisation.

The fact that LRP/LR is present in higher amounts in several organs and tissues of scrapie-infected mice and hamsters suggests a correlation between LRP/LR levels and PrP^{Sc} propagation (Rieger et al., 1997). Furthermore, expression studies revealed distribution of the laminin receptor in the intestinal epithelial/brush border confirming that the prion protein uptake and therefore the infection is mediated and supported by this receptor (Shmakov et al., 2000). After oral exposure, TSE agents accumulate in lymphoid tissue, spleen, lymph nodes, tonsils, appendices and Peyer's patches. For this reason prion particles have to cross the intestinal epithelial barrier. Besides the proposition that M-cells are responsible for the uptake of prions (Heppner et al., 2001) has also been suggested that enterocytes are involved in this process, due to the fact that bovine prions are rapidly endocytosed in the presence of LRP/LR (Morel et al., 2005). By preincubating the human enterocytes with the polyclonal anti-LRP/LR antibody, endocytosis of PrP^{BSE} was reduced.

Distribution studies in adult rats revealed that the 67 kDa LR form is highly present in brain regions, classically associated with prion-related neurodegeneration, whereas the 37 kDa form was detected in a subclass of interneurons known to be particularly sensitive to abnormal prion accumulation and cell death during the early stages of CJD (Baloui et al., 2004).

6. Conventional therapeutic strategies for the treatment of TSEs

In recent years, various studies gave evidence that substantial neuropathological changes (e.g. nerve cell degeneration) already occur prior to the onset of symptoms and might be related to PrP^{Sc} accumulation. Accordingly, any effective intervention must aim to start directly after inoculation. Unfortunately, no diagnostic tests are available to detect the disease prior to the onset of symptoms, except for individuals carrying pathogenic mutations within the *Prn*-p gene. Inhibition of PrP^{Sc} formation is the most studied target and can be achieved through (i) inhibition of PrP^{c} synthesis or prevention of its transport to the cell surface, (ii) stabilization of the PrP^{c} structure to make its conformational change unfavourable, (iii) destruction of PrP^{Sc} aggregates, (iv) reversion of PrP^{Sc} to a protease-sensitive form and (v) inhibition of the prion protein receptor(s).

A series of compounds efficiently interfere with PrP^{Sc} accumulation, such as Congo red (Ingrosso et al., 1995) and analogs (Demaimay et al., 1997), certain cyclic tetrapyrrols (Priola et al., 2000) and sulphated polyanions. Although many other compounds have been identified, only flupirtine, an analgetic, had beneficial effects on cognitive function

for human CJD patients (Otto et al., 2004). The anthracycline 4′-iodo-4′-deoxy-doxorubicin (IDX) was able to delay clinical signs of the disease and prolong the survival time in scrapie-infected hamsters (Tagliavini et al., 1997). It was also shown that quinacrine reduces the protease resistance of PrP peptide aggregates and is able to inhibit the *in vitro* conversion of the normal prion protein (PrP^c) to the abnormal form (PrPres) (Barret et al., 2003). Chlorpromazine was reported to increase incubation time in mice after intracerebral, but not intraperitoneal, injection (Roikhel et al., 1984), but was less effective in cell culture than quinacrine (Korth et al., 2001). Unfortunately, most substances that inhibit PrP^{Sc} formation show only significant effects when administered long before the clinical onset of the disease. Since no effective therapy for clinically affected TSE patients is available these diseases are inevitably fatal.

PrP-specific antibodies, a promising alternative tool in TSE treatment, counteract prion propagation both *in vitro* (Enari et al., 2001; Peretz et al., 2001; Perrier et al., 2004) and *in vivo* (Buchholz et al., 2006). In a murine model, treatment with a monoclonal anti-PrP antibody delayed the development of prion disease (White et al., 2003). Application of monoclonal antibodies raised against recombinant PrP also resulted in a reduction of PrP^{Sc} levels in infected mouse neuroblastoma cells (Pankiewicz et al., 2006). Paracrine secretion of single chain antibodies (scFv) directed against PrP^c revealed an anti-prion effect in neuroblastoma cells (Donofrio et al., 2005).

7. Therapeutic approaches targeting LRP/LR

7.1. Trans-dominant negative LRP mutants

Recently, it has been shown that an LRP mutant encompassing the extracellular domain of LRP/LR (LRP102-295::FLAG) might act in a trans-dominant negative way as a decoy by trapping PrP molecules (Fig. 1, I) (Vana and Weiss, 2006). *In vitro* studies revealed that the LRP mutant is able to reduce the

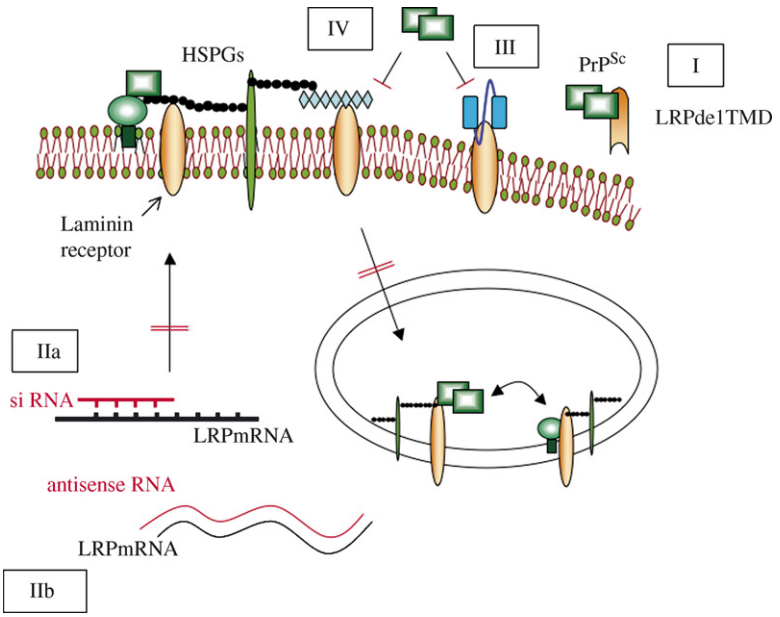


Fig. 1. Molecules inhibiting the PrP-LRP interaction and reducing the LRP/LR levels. LRP/LR (orange) is able to bind both the cellular prion protein (green circle) and the infectious PrP^{Sc} (green rectangle). After binding to the LRP/LR–HSPG complex the prion protein becomes internalized into endo-/lysosomal compartments where the conversion of PrP^c to PrP^{Sc} might take place. Prevention of the binding to LRP/LR is achieved by (I) trapping PrP^{Sc} by a LRP mutant (delTMD) encompassing the extracellular domain (LRP102-295), downregulation of LRP/LR by (IIa) siRNAs and (IIb) antisense RNA directed against LRP mRNA, (III) anti-LRP/LR antibodies (blue rectangles) competing with PrP for LRP binding sites and (IV) heparan mimetics interfering with the binding of PrP^{Sc} to the LRP/LR/HSPG complex.

PrP^{Sc} formation in scrapie-infected neuronal cells (Vana and Weiss, 2006) and might therefore represent a promising novel tool in TSE therapy.

7.2. RNA interference and antisense RNA

A further strategy used to influence the PrP^{Sc} propagation level is the knockdown of LRP/LR by siRNA and antisense RNA technology. This has already successfully been shown for PrP using *Prn*-p-specific sequences. Thus, the transfection of siRNAs corresponding to the murine *Prn*-p triggered specific *Prn*-p-gene silencing in scrapie-infected neuroblastoma cells. This caused a rapid loss of their PrPres content (Daude et al., 2003). Accordingly, it was shown that transfection of either LRP antisense RNA or LRP-specific siRNAs in scrapie-infected neuronal cells results in downregulation of LRP/LR expression and prevention of PrP^{Sc} propagation (Fig. 1, II) (Leucht et al., 2003).

Furthermore, a permanent effect of knockdown of disease-relevant genes using RNAi has been achieved using a lentivirus-mediated gene transfer (Ralph et al., 2005; Raoul et al., 2005). Recently it was shown that lentivector-mediated RNAi efficiently suppressed the prion protein and prolonged survival of scrapie infected mice (Pfeifer et al., 2006). This suggests that an alternative lentivirus-based RNAi gene therapy approach using HIV-derived vectors expressing LRP-specific siRNAs might represent another promising approach in TSE treatment.

7.3. Antibodies directed against the LRP/LR

The PrP binding capacity of LRP offers strategies in therapeutic approaches against prion diseases. The curative effect of the polyclonal anti-LRP/LR antibody (W3) on scrapie infected N2a cells recommends anti-LRP antibodies as therapeutic tools for the treatment of prion diseases (Leucht et al., 2003). On the molecular level this antibody (i) prevents the binding of infectious prions to mammalian cells (Fig. 1, III) (Gauczynski et al., 2006) and (ii) blocks endocytosis of PrP^{BSE} by enterocytes mediated by the LRP/LR is inhibited after treatment with W3 (Morel et al., 2005). Moreover W3 was able to prolong the incubation/survival time in scrapie mice (Zuber et al., submitted).

Since a polyclonal antibody format is not suitable for a therapy in animals or humans the development of single-chain antibodies directed against LRP/LR provides a promising alternative therapeutic strategy. Smaller (30 kDa) and with better tissue penetration, they can be delivered via passive immunotransfer for example intracerebrally into the brain region where massive prion propagation takes place. So far, no immune response or side reactions have been observed after application of scFvs. In a murine scrapie model passively delivered anti-LRP/LR single chain antibodies reduced peripheral prion propagation (Zuber et al., in press). To circumvent the problem of the short half-life in organisms a permanent delivery of single chain antibodies directed against LRP/LR may be achieved by gene therapeutic strategies employing AAV-based or lentiviral vector systems.

7.4. Polysulphated glycans

Polysulphated glycans such as heparan mimetics (HM) or pentosan polysulphate interfere with the binding of the infectious PrP27-30 to the LRP/LR–HSPG complex and are therefore alternative promising therapeutic tools (Fig. 1, IV) (Gauczynski et al., 2006). Treatment of scrapie-infected mice with pentosan polysulphate resulted in a prolonged incubation time and even in the cure of two mouse strains infected with two different scrapie strains (Farquhar and Dickinson, 1986). Moreover, it has been shown that GAGs (Hijazi et al., 2005), especially heparan sulphate, (Horonchik et al., 2005) also act as receptors for the infectious PrP^{Sc}. Polysulphated glycans such as SP54 and PS3 (phycarin sulphate) also show an inhibitory effect on the binding of PrP27-30 to LRP hyperexpressing BHK cells (Gauczynski et al., 2006). Both pentosan polysulphates and heparan sulphate mimetics are able to prolong the incubation time in rodent models and interfere with PrP^{Sc} propagation in neuronal cells due to the inhibition of the LRP/LR dependent binding of prions to target cells.

8. Conclusions

So far, there is no TSE treatment available that is able to cure affected individuals. Alternative therapeutic strategies targeting LRP/LR might be promising since it

acts as the receptor for PrP^{c} and PrP^{Sc}. Molecules targeting the LRP–PrP interaction such as LRP mutants, LRP/LR-specific antibodies and polysulphated glycans or tools downregulating the LRP/LR levels such as siRNAs and antisense RNAs might be effective in the treatment of prion disorders.

Acknowledgments

We thank the Federal Ministry of Research and Education (grants 01-KO-0106 and KO-01-0514), the Bavarian Prion Research Foundation (grant LMU 4) and the European Commission (grants QLRT-2000-02085 and NoE NeuroPrion FOOD-CT-2004-506579) for financial support.

References

Akache, B., Grimm, D., Pandey, K., Stephen, R.Y., Xu, H., Kay, M.A., 2006. The 37/67-Kilodalton Laminin Receptor is a receptor for Adeno-Associated Virus Serotypes 8,2,3, and 9. J. Virol. 80, 9831–9836.

Ardini, E., Pesole, G., Tagliabue, E., Magnifico, A., Castronovo, V., Sobel, M.E., Colnaghi, M.I., 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.

Auth, D., 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.

Baloui, H., von Boxberg, Y., Vinh, J., Weiss, S., Rossier, J., Nothias, F., 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.

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., Deslys, J.P., 2003. Evaluation of quinacrine treatment for prion diseases. J. Virol. 77, 8462–8469.

Benestad, S.L., Sarradin, P., Thu, B., Schonheit, J., Tranulis, M.A., Bratberg, B., 2003. Cases of scrapie with unusual features in Norway and designation of a new type, Nor98. Vet. Rec. 153, 202–208.

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

Buchholz, C.J., Bach, P., Nikles, D., Kalinke, U., 2006. Prion protein-specific antibodies for therapeutic intervention of transmissible spongiform encephalopathies. Expert. Opin. Biol. Ther. 6, 293–300.

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

Cohen, F.E., Prusiner, S.B., 1998. Pathologic conformations of prion proteins. Annu. Rev. Biochem. 67, 793–819.

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

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

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

Enari, M., Flechsig, E., 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, 9259–9299.

Farquhar, C.F., 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.

Gauczynski, S., Nikles, D., El-Gogo, S., Papy-Garcia, D., Rey, C., Alban, S., Barritault, D., Lasmezas, C.I., Weiss, S., 2006. The 37-kDa/67-kDa laminin receptor acts as a receptor for infectious prions and is inhibited by polysulfated glycanes. J. Infect. Dis. 194, 702–709.

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

Heppner, F.L., Christ, A.D., Klein, M.A., Prinz, M., Fried, M., Kraehenbuhl, J.P., Aguzzi, A., 2001. Transepithelial prion transport by M cells. Nat. Med. 7, 976–977.

Hijazi, N., Kariv-Inbal, Z., Gasset, M., Gabizon, R., 2005. PrPSc incorporation to cells requires endogenous glycosaminoglycan expression. J. Biol. Chem. 280, 17057–17061.

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

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

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

Kinoshita, K., Kaneda, Y., Sato, M., Saeki, Y., Wataya-Kaneda, M., Hoffmann, A., 1998. LBP-p40 binds DNA tightly through associations with histones H2A, H2B, and H4. Biochem. Biophys. Res. Commun. 253, 277–282.

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

Leucht, C., Simoneau, S., Rey, C., Vana, K., Rieger, R., Lasmezas, C.I., 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.

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

Makrides, S., Chitpatima, S.T., Bandyopadhyay, R., 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.

Morel, E., Andrieu, T., Casagrande, F., Gauczynski, S., Weiss, S., Grassi, J., Rousset, M., Dormont, D., Chambaz, J., 2005. Bovine prion is endocytosed by human enterocytes via the 37 kDa/67 kDa laminin receptor. Am. J. Pathol. 167, 1033–1042.

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., Prange, H., 2004. Efficacy of flupirtine on cognitive function in patients with CJD: a double-blind study. Neurology 62, 714–718.

Pankiewicz, J., Prelli, F., Sy, M.S., Kascsak, R.J., Kascsak, R.B., Spinner, D.S., Carp, R.I., Meeker, H.C., Sadowski, M., Wisniewski, T., 2006. Clearance and prevention of prion infection in cell culture by anti-PrP antibodies. Eur. J. Neurosci. 23, 2635–2647.

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., Prusiner, S.B., 2001. Antibodies inhibit prion propagation and clear cell cultures of prion infectivity. Nature 412, 739–743.

Perrier, V., Solassol, J., Crozet, C., Frobert, Y., Mourton-Gilles, C., Grassi, J., Lehmann, S., 2004. Anti-PrP antibodies block PrPSc replication in prion-infected cell cultures by accelerating PrPc degradation. J. Neurochem. 89, 454–463.

Pfeifer, A., Eigenbrod, S., Al-Khadra, S., Hofmann, A., Mitteregger, G., Moser, M., Bertsch, U., Kretzschma, H., 2006. Lentivector-mediated RNAi efficiently suppresses prion protein and prolongs survival of scrapie-infected mice. J. Clin. Invest. 116, 3204–3210.

Priola, S.A., Raines, A., 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.

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., 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., 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., 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.

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

Roikhel, V.M., Fokina, G.I., Pogodina, V.V., 1984. Influence of aminasine on experimental scrapie in mice. Acta Virol. 28, 321–324.

Romanov, V., Sobel, M.E., pinto da Silva, P., Menard, S., 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.

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

Shmakov, A.N., Bode, J., Kilshaw, P.J., 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.

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

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., Post, C., 1997. Effectiveness of anthracycline against experimental prion disease in Syrian hamsters. Science 276, 1119–1122.

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

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

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

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

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

Zuber, C., Knackmuss, S., Rey, C., Reusch, U., Röttgen, P., Fröhlich, T., Arnold, G., Mitteregger, G., Pace, C., Kretzschmar, H.A., Little, M., Weiss, S., 2007. Single chain Fv antibodies directed against the 37kDa/67kDa laminin receptor as therapeutic tools in prion diseases. Mol. Imm., in press.

Chapter III

LRP/LR as an alternative promising target in therapy of prion diseases, Alzheimer´s disease and cancer

published as:

Vana K., Zuber C., Pflanz H., Kolodziejczak D., Zemora G., Bergmann A. K. and Weiss S., LRP/LR as an alternative promising target in therapy of prion diseases, Alzheimer's disease and cancer. *Infect Disord Drug Targets* 2009. 1: 69-80.

LRP/LR as an Alternative Promising Target in Therapy of Prion Diseases, Alzheimer's Disease and Cancer

Karen Vana, Chantal Zuber, Heike Pflanz, Dominika Kolodziejczak, Georgeta Zemora, Ann-Katrin Bergmann and Stefan Weiss*

Laboratorium für Molekulare Biologie - Genzentrum-Institut für Biochemie der LMU München, Feodor-Lynen-Str. 25, 81377 München, Germany

Abstract: The 37 kDa/67 kDa laminin receptor (LRP/LR) represents a key player for cell adhesion, is associated with the metastatic potential of solid tumors and is required for maintenance of cell viability by preventing apoptosis. LRP/LR acts as a receptor for viruses such as Sindbis virus, Venezuelean Equine Encephalitis (VEE) virus, Adeno-associated-viruses (AAV) and Dengue Virus, the latter causing 50 to 100 million infections in humans per year. LRP/LR acts further as a receptor for prions and represents a multifunctional protein subcellularly located to the nucleus, the cytoplasm and the cell surface. The receptor represents an alternative target for therapy of viral infections, cancer and prion disorders and might play additional roles in further neurodegenerative diseases such as Alzheimer's disease. The species barrier in prion disorders might be at least in part determined by the presence of LRP/LR in enterocytes of the intestinal epithelium. Anti-LRP/LR antibodies, siRNAs directed against LRP mRNA, polysulfated glycanes such as pentosan polysulfate and heparan mimetics and LRP decoy mutants are promising tools for blocking or downregulating the receptor and may represent alternative therapeutics for the treatment of prion disorders, Alzheimer's Disease and metastatic cancer.

Keywords: 37kDa/67kDa laminin receptor LRP/LR, prion, protein, PrP, neurodegenerative disease, HSPG, pentosan polysulfate, antibody, siRNA, species barrier, zoonotic disease, CWD, scrapie, BSE, therapy, cancer, Alzheimer's Disease, CJD, metastasis, dengue virus.

1. THE 37kDa/ 67kDa LAMININ RECEPTOR LRP/LR

In a yeast two-hybrid screen, the 37 kDa laminin receptor precursor (LRP) was identified as an interaction partner for the prion protein [1]. Coinfection and cotransfection studies in insect and mammalian cells confirmed the interaction, and led to the assumption that LRP might act as the receptor for the cellular PrP [1]. Further *in vitro* studies on neuronal and non-neuronal cells validated the hypothesis and showed that the laminin receptor LRP/LR acts as the receptor for the cellular prion protein [2]. Using the yeast two-hybrid system domains on PrP and LRP have been identified to be involved in the PrP-LRP interaction. Two binding domains for LRP on PrP were discovered: a direct binding domain (PrPLRPbd1, aa144-179) and an indirect one (PrPLRPbd2, aa53-93), which depends on the presence of heparan sulfate proteoglycans (HSPGs) that function as co-factors or co-receptors for the binding of PrP^c to the LRP/LR [3]. Furthermore, the yeast two-hybrid system localized the direct PrP-binding domain on LRP between amino acid residues 161 and 180 [3] (Fig. **1**). A second HSPG-dependent binding site, which has not been identified so far, might be located between amino acid 180 and 285. HSPGs are multifunctional macromolecules characterized by a core polypeptide to which glycosaminoglycans (GAGs) are covalently attached and have also been shown to act as initial attachment receptors for several viruses (Table **1**) and are associated with Aβ deposits in Alzheimer's disease (AD) [4].

The 37 kDa LRP is thought to be the precursor of the 67 kDa high-affinity laminin receptor (LR), which was first isolated from melanoma cells due to its high binding capacity to laminin [5]. Although the LRP has a transmembrane domain (amino acid residue 86-101, [3, 6]) (Fig. **1**), it is abundantly localized in the cytoplasm [7]. In mammalian cells, it has been demonstrated that both the 37 kDa LRP and the 67 kDa LR are present in plasma membrane fractions [2]. The exact mechanism by which the 37 kDa precursor forms the mature 67 kDa isoform is up to now still unclear. Data from a yeast two-hybrid analysis and a size exclusion chromatography on recombinant LRP showed that LRP failed to interact with itself [3], which is an argument against the hypothesis of a direct homodimerization. Analysis of the membrane-bound 67 kDa LR indicated, that acylation of LRP might be involved in the processing of the receptor [8]. Additional studies suggested that the 67 kDa LR might be a heterodimer stabilized by fatty acid-mediated interactions [9]. Mammalian genomes contain multiple copies of the LRP gene, particularly 6 copies in the mouse and 26 copies in the human genome [10]. Sequencing revealed that over 50% of the 37 kDa LRP gene copies were pseudogenes most probably generated by retrotranspositional events. The finding of multiple pseudogenes for the 37 kDa 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 [11].

Interestingly, the 37kDa LRP appears to be a multifunctional protein involved in the translational machinery [12] and has also been identified as p40 ribosome-associated protein [13]. In addition, LRP has been found in the nucleus, where it is tightly associated with nuclear structures [14]. The 37kDa/67kDa LRP/LR has been described to act as a receptor for laminin, elastin and carbohydrates [15] as well as a receptor for several viruses [16-19, 103] (Table **1**).

*Adress correspondence to this author at the Genzentrum- Institut für Biochemie der LMU München, Feodor-Lynen-Str. 25, D-81377 München; E-mail: weiss@lmb.uni-muenchen.de

1871-5265/09 $55.00+.00

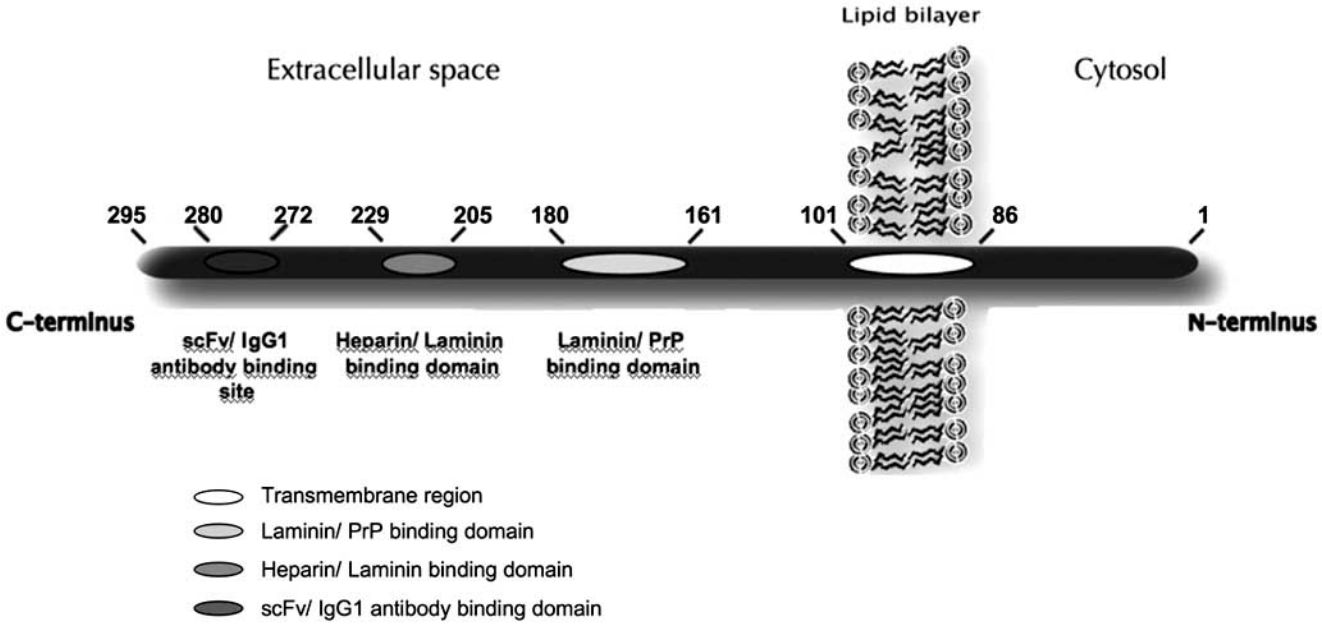


Fig. (1). Functional domains of the 37kDa/ 67kDa laminin receptor LRP/LR.
LRP/LR consists of 295 amino acids (aa) and belongs to the group of Type II membrane proteins, spanning the plasma membrane once (aa 86-101) with its C-terminus exposed to the extracellular space. PrP/Laminin and Heparin/Laminin binding sites have been characterized at aa positions 161-180 and 205-229, respectively. In addition, the scFv/IgG1 antibody binding site has been mapped to aa 272-280 [47,50].

Table 1. General Conspectus of LRP/LR and HSPGs as Initial Attachment Receptors for Viruses

Viruses using LRP/LR as receptor	Reference	Viruses using HSPGs as receptors (selection)	Reference
Dengue Virus (Serotype 1, 2, 3)	[18]	Adeno-Associated Virus (Serotype 1,6)	[102]
Sindbis Virus	[19, 103]	Adenovirus (Serotype 2, 5)	[104]
Venezuelean Equine Encephalitis Virus	[17]	Human Papilloma Virus	[105]
Adeno-Associated Virus (Serotype 2, 3, 8, 9)	[16]	Hepatitis B Virus Hepatitis C Virus	[106] [107]
		Human Immunodeficiency Virus	[108]
		Murine Leukaemia Virus	[109]
		Herpes Simplex Virus Type 1	[110]
		Pseudorabies Virus	[111]

Recent studies investigated the role of LRP/LR in the maintainance of cell viability and it has been shown that "knockdown" of the laminin receptor via RNA interference induced apoptosis [20]. Furthermore, it has been reported that LRP/LR might regulate microgliosis [21] and might serve as a binding factor for inflammatory macrophages to basement membranes [22]. 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 [2]. 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 [2]. 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 [23]. Several LRP/LR isoforms corresponding to different maturation states of the receptor were identified, including a 44 kDa, 60 kDa, 67 kDa and a 220 kDa form. Furthermore, it could be demonstrated, that all of these isoforms were able to bind PrP, supporting a physiological role for the laminin receptor/PrP interaction in the brain [23]. A closer insight into the fine cellular distribution of LRP/LR in the central

nervous system was reached by using immunohistochemistry in adult rat brain [24]. It has been shown, that the 67 kDa 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 [24]. In contrast, the 37 kDa 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 Creutzfeldt-Jakob Disease (CJD) [25]. Recent data indicate that LRP levels are increased in the spinal cord microglia after activation by peripheral injury or a traumatic lesion [26]. In addition, recent studies showed, that LRP/LR is not only involved in the PrP^{c} metabolism, but fullfills also a crucial role in prion propagation. Using antisense LRP RNA or 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 [27]. Very recently it was reported that microinjection of lentiviral vectors expressing siRNAs directed against LRP mRNA into the brain prolongs the pre-clinical phase in scrapie-infected mice [28].

Due to the facts, that a (natural) infection with prions mostly occur via an oral route and that LRP/LR act as receptor for prions [2, 29] potential binding sites for PrP in the intestinal mucosa were examined. Tissue expression studies of the 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. Employing immunohistochemistry LR expression has also been observed in the perinuclear/Golgi apparatus region and in the Paneth cell secretory granules [30]. A colocalization with PrP^{c} in the perinuclear compartment has recently been proven [31]. 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 67 kDa LR may be an increased susceptibility to an oral infection with prions [30]. It has been demonstrated, that the oral transmission of infectious prion particles led to a rapid accumulation of PrP^{Sc} in Peyer's patches [32]. PrP^{Sc} has also been detected in enterocytes of the villous epithelium of the small intestine of primates after oral exposure to prions [33]. Enterocytes represent the major cell population of the intestinal epithelium [34] 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 [35]. These results led to the hypothesis that enterocytes might play an important role for the uptake of infectious prion particles. Previously, it has been demonstrated that bovine PrP^{Sc} is internalized by human enterocytes via an LRP/LR-mediated endocytosis [36]. 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 [37]. In summary, an important role of the 37 kDa/ 67 kDa LRP/LR in mediating binding and internalization of the prion protein and its involvement in pathological mechanisms was demonstrated.

2. ROLE OF LRP/LR AS A TARGET IN PRION DISEASES

2.1. Antibodies as Therapeutic Tools

Antibodies gained increasing attention in the development of therapeutics for human disease. Especially monoclonal antibodies are used in clinical trials and many of them already obtained the approval from the U.S. Food and Drug Administration (FDA) for therapy e.g. in cancer. Several anti-prion antibodies harboring different formats have already been developed antagonizing prion infection [38-42]. However, none of them achieved striking results, which render them suitable for application in human transmissible spongiform encephalopathies (TSEs).

The 37kDa/67kDa laminin receptor represents the receptor for the cellular prion protein PrP^{c} [2] and a receptor for the infectious $PrP^{27\text{-}30}$ [29], implicating that LRP/LR might represent a valuable and alternative target for antibody development in prion disease therapy [39, 44]. Therefore, different antibody formats (Table **2**, Fig. **2**) directed against the LRP/LR have been developed to block or prevent binding and internalization of the prion protein and therefore a possible infection of cells.

A polyclonal anti-LRP antibody termed W3 (Fig. **2**) reduced the PrP^{Sc} propagation in cell culture [27], hampered the binding of BSE prions to human enterocytes [45] and prevented the binding of PrP27-30 to mammalian cells [29]. Passive immunotransfer of W3 into scrapie infected mice significantly reduced peripheral PrP^{Sc} propagation by 66% and prolonged the survival of scrapie infected mice 1.8-fold [46]. Since the amount of the polyclonal antibody W3 is limited, single chain Fv antibodies (scFv) have been selected via phage display on recombinant LRP [47]. Due to a smaller size (approx. 30 kDa) they display a better tissue penetration, might pass the blood brain barrier, and might therefore reach the brain, where prions replicate predominantly. Application of the anti-LRP scFv antibody termed S18 via passive immunotransfer into scrapie infected mice reduced PrP^{Sc} levels in the spleen by approx. 40%, indicating that the peripheral propagation is impaired (Fig. **2**). The fact that incubation times and survival were not prolonged might be explained with the low stability and short half life (approx. 12 hours in blood) of the scFvs. To circumvent these problems, we developed a permanent delivery system based on recombinant Adeno-associated viral vectors (rAAV). rAAV mediated gene delivery is currently investigated in clinical trials for human therapy. A recent study reported the administration of anti-PrP antibodies into scrapie infected mice via rAAV serotype 2, which resulted in a delayed onset of disease [48]. Intracerebral injection of rAAVs encoding for the anti-LRP/LR scFv antibodies S18 and N3 into scrapie infected mice efficiently reduced the peripheral PrP^{Sc} propagation by approx. 60% and 32%, respectively [49]. This remarkable finding can be explained by the fact that trafficking of intracerebrally administered rAAV to the spleen occurred, resulting in a direct scFv expression and secretion [49].

Table 2. Anti-LRP/LR Tools as Therapeutics in Prion Diseases

Anti-LRP/LR tools	Effect *in vitro*	Application *in vivo*	Effect *in vivo*			Reference
			Peripheral PrP^{Sc} propagation	Incubation time	survival	
Antibodies						
Polyclonal antibody W3	blocks PrP^{Sc} propagation and cures scrapie infected cells	passive immunotransfer via i.p. injection	reduced by 66%	no prolongation	1.8 fold prolongation	[46]
Single chain Fv antibody scFv S18	n.d.	passive immunotransfer via i.p. injection	reduced by 40%	no prolongation	no prolongation	[47]
		delivery by i.c. injection of rAAV	reduced by 60%	no prolongation	no prolongation	[49]
Laminin receptor decoy mutant						
LRP102-295::FLAG	Reduces PrP^{Sc} propagation in transfected $N2aSc^{+}$ cells	Generation of transgenic mice	in progress	in progress	in progress	[61]
Strategies downregulating LRP/LR expression						
Antisense LRP RNA	Prevents PrP^{Sc} propagation in transfected ScMNB cells	Generation of transgenic mice	n.d.	n.d.	n.d.	[27, 59]
LRP-specific siRNAs	Prevents PrP^{Sc} propagation in transfected $N2aSc^{+}$ cells	i.c. injection of lentiviral vectors expressing siRNAs (gene therapy)	n.d.	significant prolongation	not prolonged	[27, 28]

Anti-LRP/LR tools blocking or downregulating the 37kDa/67kDa LRP/LR were applied in cell as well as in animal studies to investigate their therapeutic potential in prion diseases. i.p. intraperitoneal; i.c. intracerebral, n.d. not determined; $ScN2a^{+}$ Scrapie infected mouse neuroblastoma cells; ScMNB Scrapie mouse neuroblastoma cells; peripheral PrP^{Sc} propagation was determined analysing the PrP^{Sc} content in the spleen of scrapie infected mice 90 days post infection; incubation time describes the time span from the scrapie infection until the mice display the first TSE relevant symptoms (Ataxia of gait, tremor, rigidity in the tail or difficulty righting from a supine position). Survival represents the time span from the day one of the four TSE symptoms occur until the day two of these symptoms are observed for three days [43].

Although scFvs provide better tissue penetration and probably pass the blood brain barrier, a full length IgG format is much more stable in the organism and displays a longer half life up to 21 days. In contrast to the small scFvs, only the polyclonal anti-LRP/LR antibody W3 prolonged survival in scrapie infected mice. Using an improved version of the scFv S18 (iS18), as a template for the antigen-binding regions, a full length IgG1-iS18 antibody has been engineered [50] and will be applied for prophylaxis and treatment of scrapie infected mice expecting a more pronounced effect regarding survival.

The mode of action of the anti-LRP antibodies as a prion disease therapeutic, remains to be further investigated. Although an epitope mapping of the anti-LRP antibodies scFv S18 and full length IgG1-iS18 exhibits recognition at the very C-terminal part of LRP (Fig. **1**) and not at the direct PrP binding site (aa161-180) [47, 50], the antibodies might act through sterical hindrance preventing the binding of infectious PrP^{Sc} to the receptor. A full length IgG format reveals higher stability *in vivo* and provides a higher affinity to the laminin receptor resulting in a probable more permanent prevention of the LRP/LR-PrP^{Sc} interaction.

2.2. Pentosan Polysulfate

Pentosan polysulfate (PPS) is a large polysulfated glycane with weak heparin-like activity and has been shown to prevent PrP^{Sc} propagation in cell culture models [51]. Recently, it has been proposed that PPS functions via inhibition of the binding of PrP^{Sc} to LRP/LR [29] (Fig. **2**). In mouse models, PPS is able to prolong the survival time of scrapie infected animals or even cures of the mouse strains VM and CBA from 22A and ME7 prions, respectively [52]. Furthermore, survival time of scrapie-infected mice was increased using a combined treatment of PPS and Fe(III) meso-tetra(4-sulfonato-phenyl)porphine [53]. Orally or intraperitoneally administered PPS, however, is thought to be not strinkingly effective for the treatment of TSEs due to the assumed inability of the drug to cross the blood-brain-barrier [54]. Several case studies for the treatment of vCJD in humans were performed showing that delivery of PPS by

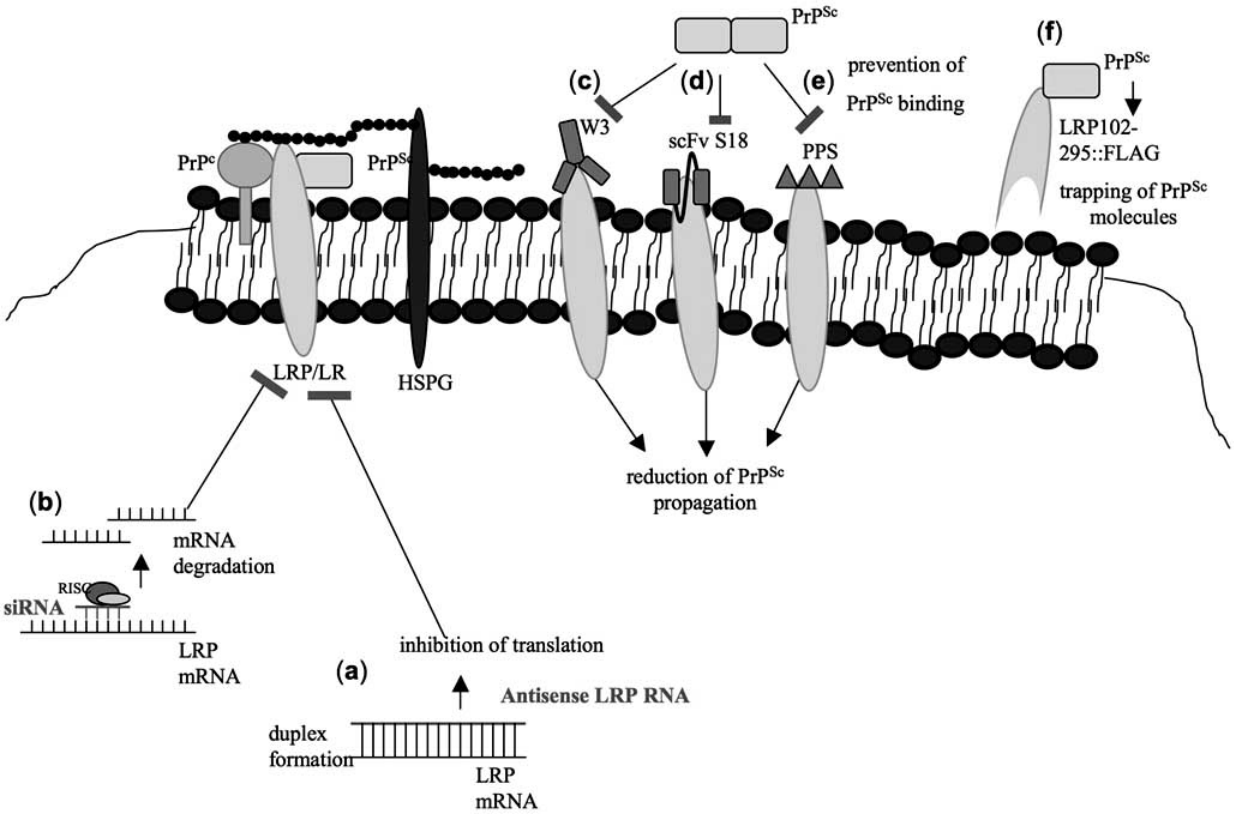


Fig. (2). Therapeutic approaches targeting LRP/LR.

LRP/LR (oval) has been identified as receptor for cellular (circle) and infectious prions (square). Several strategies can be employed to interfere with either the expression of LRP/LR (a, b) or binding of PrP^{Sc} (c-f). To ablate LRP/LR expression an antisense RNA (**a**) and a small interfering RNA (**b**) approach have been used resulting in prevention of PrP^{Sc} propagation in scrapie-infected cells [27]. Microinjection of lentiviral vectors expressing siRNAs directed against LRP mRNA significantly prolonged the pre-clinical phase in scrapie-infected mice [28]. Both the polyclonal anti-LRP/LR antibody W3 (immunoglobuline structure) (**c**) and the single chain (scFv) anti-LRP/LR antibody S18 (single chain antibody structure) (**d**) have been shown to interfere with the PrP^{Sc} propagation [27, 46, 47]. In addition, drugs such as pentosan polysulfate (PPS) (triangles) (**e**) were able to reduce PrP^{Sc} binding *in vitro* [29] possibly due to an inhibition of the LRP/LR-dependent binding of prions to the cell. As an alternative therapeutic system, a LRP/LR decoy mutant (LRP102-295::FLAG) (semi-oval) (**f**) has been used to interfere with the PrP^{Sc} propagation in scrapie-infected neuroblastoma cells [61].

chronic intra-ventricular infusion resulted in no definite clinical benefits [55, 56]. However, continuous intra-ventricular treatment of a 22-year-old vCJD patient with PPS over a period of 31 month resulted in a prolonged survival [57]. In addition, very recently the effect of continuous intraventricular infusion of PPS was investigated in seven UK patients suffering from human prion diseases. In this report, it has been demonstrated that a pentosan polysulfate therapy during 6 months extended the mean survival of all patients [58]. Nevertheless, further *in vivo* animal experiments and clinical studies, respectively, are required to assess the efficacy of PPS administration in variant Creutzfeldt-Jakob Disease (vCJD) and other prion diseases.

2.3. RNA Interference Approaches

Since the 37kDa/67kDa laminin receptor (LRP/LR) has been identified to act as the cell-surface receptor for the cellular prion protein [2] it was further discovered that limited expression of LRP inhibits PrP^{Sc} accumulation in scrapie infected neuronal cells (ScN2a and ScGT1) [27]. Transfection of ScN2a and ScGT1 cells with vectors encoding for antisense LRP mRNA led to elimination of LRP expression concomitant with an absence of PrP^{Sc} propagation [27] (Fig. **2**). In addition, transgenic mice [tgN (NSEasLRP)] were generated showing a reduced LRP/LR level in hippocampal and cerebellar brain regions and no abnormal behavior compared to control mice [59]. Another approach to knock down LRP expression deals with small interfering (si)RNAs directed against the LRP mRNA. Transfection of ScN2a cells resulted in a strong downregulation of LRP correlating with a complete abolishment of PrP^{Sc} propagation 72 hours post transfection [27] (Fig. **2**).

These findings lead to the conclusion that post-transcriptional gene silencing of LRP by RNAi is a promising approach to delay PrP^{Sc} propagation. In deed, microinjection of lentiviral vectors expressing siRNAs directed against (LRP mRNA revealed a prolonged pre-clinical phase (incubation time) in scrapie infected mice [28].

2.4. Transdominant Negative Laminin Receptor Mutant RP102-295::FLAG

The expression of cellular PrP (PrP^{C}) is a major requirement of scorpie infection [60] and therefore

represents a promising therapeutic target. However, identification of the 37kDa/67kDa laminin receptor LRP/LR as the receptor for PrP^{C} [2] and PrP^{Sc} [29, 45] led to an alternative target for the development of TSE therapeutics. Recently, it has been shown that an LRP mutant encompassing only the extra-cellular domain of LRP/LR (LRP102-295::FLAG) is secreted into the extracellular space and therefore might act in a trans-dominant negative manner as a decoy by trapping PrP molecules [61]. Baby hamster kidney (BHK) cells expressing and secreting the laminin receptor mutant show a reduced binding of PrP 27-30, and in scrapie-infected neuronal cells the mutant is able to reduce the PrP^{Sc} accu-mulation [61] (Fig. **2**). Thus, the transdominant negative LRP-mutant might have potential for the development of a TSE therapy. An *in vivo* study with transgenic animals expressing the LRP mutant will reveal if the decoy mutant efficiently influences incubation time and/or survival during scrapie infection.

3. ROLE OF THE 37kDa/67kDa LAMININ RECEPTOR LRP/LR IN ZOONOTIC DISEASES

The phenomenon that the transmission of prion diseases from one species to another results in prolonged incubation times and survival compared to those of intraspecies transmission is called the "species barrier" [62]. This barrier is due to the variety of prion strains encompassing various infectious potential [63]. In natural animal populations the intraspecies transmission of prion diseases is much more efficient than an interspecies transmission [45], although these efficiencies can vary depending on the animal species, the used infectious agent and the route of infection: BSE and sheep Scrapie have a low intraspecies transmission efficiency [64, 65] whereas CWD is rapidly transmitted in captive deer populations [66] (Fig. **3**). Until today, mechanisms underlying an intraspecies transmission remain unclear. But prions from one species are often less infectious to other species. This is thought to depend on differences in host prion protein sequences [67] (Fig. **4**) but also may result from sequence differences of the 37kDa/67kDa LRP/LR (Fig. **5**). The species barrier of an interspecies transmission is sometimes so strong that peripheral injection, oral transmission and sometimes even an intracerebral inoculation with the agent causing a transmissible spongiform encephalopathy (TSE) fails to develop neurodegenerative signs. Nevertheless, susceptible hosts show clinical signs when they are inoculated with brain homogenates of resistant species [67]. Bovine spongiform encephalopathy (BSE) is the only known TSE agent that was transmitted to humans causing the zoonotic disease vCJD [68]. Due to the fact that a natural infection with prions mostly occurs via oral uptake,

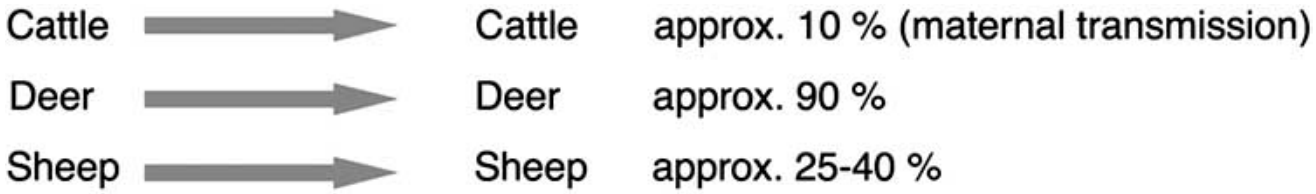


Fig. (3). Intraspecies transmission efficiencies of TSEs.
Experimental transmission of prion diseases have been performed to examine neuropathological profiles, risk factors and incidence rates. A long-term cohort study in cows investigating maternally-associated risk factors for BSE revealed a statistically significant risk of 10% for a transmission to the offspring [64]. For an intraspecies transmission of sheep scrapie, a rate of 25-40% has been calculated [66]. Chronic wasting disease (CWD) is naturally transmitted with remarkably efficacy of approximately 90% that has been studied in a captive mule deer herd [64].

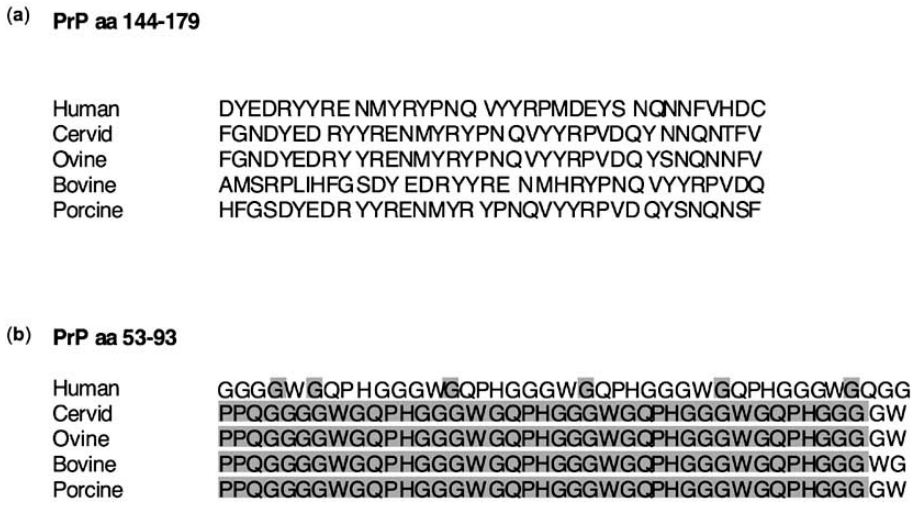


Fig. (4). Comparison of amino acid sequences of the direct and indirect PrP binding sites for LRP/LR in different species.
PrP comprises two binding sites for LRP/LR, a direct (aa 144-179) and an indirect one (aa 53-93), that is mediated by HSPGs [3]. (**a**) Sequence alignment for the direct PrP binding site in human, cervid, ovine, bovine and porcine PrP resulted in no sequence homology, whereas the (**b**) alignment for the indirect PrP binding domain for cervid, ovine, bovine and porcine PrP, respectively, revealed approximately 95% sequence identity (amino acids marked in dark grey).

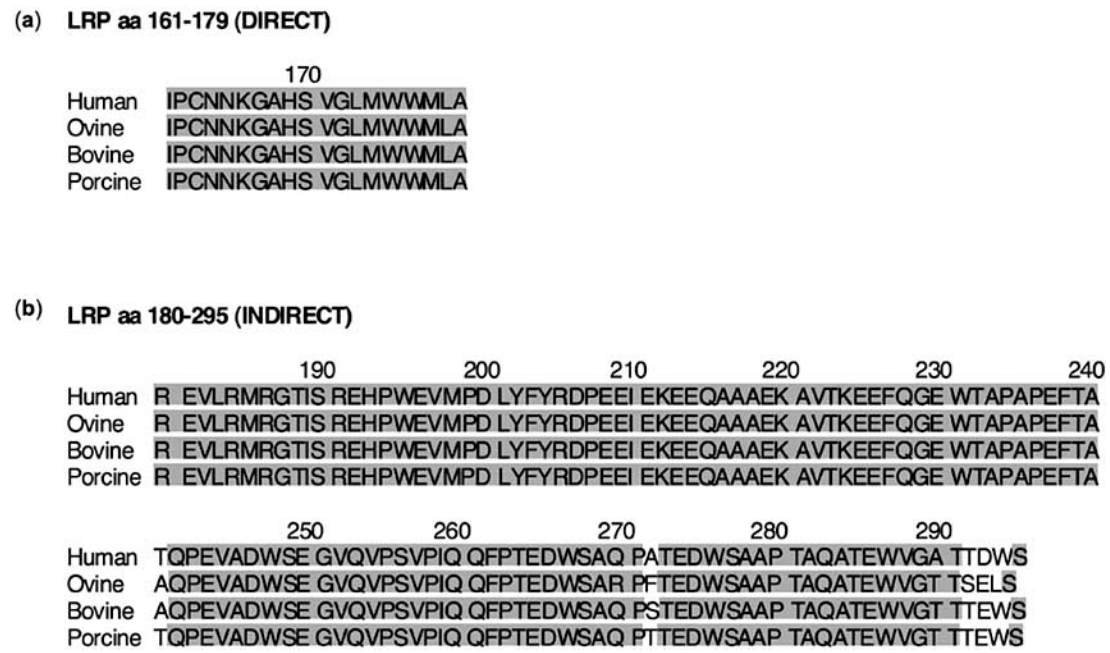


Fig. (5). Comparison of amino acid sequences of the direct and indirect LRP/LR binding sites for PrP in different species.
Binding of LRP/LR to PrP occurs via a direct binding site located between aa 161-179 and an indirect HSPG-dependent domain encompassing aa 180-295 [3]. (**a**) Sequence alignment of the direct LRP/LR binding site for PrP resulted in complete identical sequences for the human, ovine, bovine and porcine proteins. (**b**) Human, ovine, bovine and porcine sequences, respectively, of the indirect LRP/LR binding domain showed approximately 90% homology (amino acids marked in dark grey).

like the transmission of BSE to human, further analyses were performed to study the initial prion uptake. Enterocytes represent the major cell population in the intestinal epithelium and express the 37 kDa/67 kDa LRP/LR on their cell surface. It was hypothesized that both enterocytes and the LRP/LR may play a major role in oral uptake of infectious prions and the species barrier. Shmakov and colleagues demonstrated that the 67 kDa LR is expressed in the apical brush border of the small intestine and in the perinuclear region of Paneth cell secretory granules [30]. These findings suggest that LRP/LR might play a crucial role in secretory and endocytic functions in the human small intestine epithelium and that the consequence of the expression of the 37 kDa/67 kDa LRP/LR may result in an increased susceptibility to oral infection with prions by LRP/LR mediated uptake by human enterocytes [30]. Studies of Morel *et al*. showed that bovine prions were specifically internalized by human enterocytes (Caco-2/TC7 cells) via the LRP/LR, whereas mouse-adapted scrapie prions were not endocytosed [45]. It has been demonstrated that BSE prion uptake was significantly reduced after preincubating the cells with the anti-LRP/LR antibody W3 demonstrating that the 37 kDa/67 kDa laminin receptor is required for BSE prion internalization [45]. Other studies on oral and parenteral interspecies transmission in animals showed that BSE could be transmitted orally to sheep and goat [69], mink [70], mouse [71], but not to poultry [69]. Also pigs failed to be susceptible to oral BSE transmission [69], whereas a parenteral inoculation was successful [72] (Fig. **6**). However, the mode of interspecies transmission of BSE to humans still remains unclear. Nevertheless, some interspecies interaction profiles of prions have already been elaborated employing yeast two-hybrid and conversion assays *in vitro* and transmission studies *in vivo* (Table **4**). Further binding studies with prions of different species on bovine, porcine, cervid, ovine and human enterocytes, respectively, will help to unravel the species barrier in prion disorders and will prove whether different animal prions such as CWD or sheep Scrapie might cause further zoonotic diseases.

4. LRP/LR AS A DRUG TARGET IN CANCER

Both, the 37kDa LRP and the 67kDa LR play an important role in cancer. The 67 kDa isoform was first isolated from tumor cells [5] and is termed the high affinity laminin receptor due to its strong binding capacity to laminin. Interaction of the LRP/LR with laminin, a major cell adhesion molecule, is crucial for the invasion of tumorigenic cells. There are at least two binding sites for laminin on LRP, one stretching from aa 205-229 and a second so called Peptide G sequence encompassing aa 161-180 [6] (Fig. **1**) and comprising a heparin dependent laminin binding site [73-75], which is equivalent to the direct PrP binding site. The non-integrin laminin receptor LRP/LR is involved in the metastatic behavior of neoplastic cells compromising lami-nin mediated basement membrane attachment, accompanied by local degradation and cell movement. Overexpression of the 67kDa LR has been detected in several cancer tissues (Table **3**) including gastric cancer [76], colon carcinoma [77], colorectal carcinoma [78], cervical [79], breast [80], lung [81], ovary [82], pancreatic [83] and prostate cancer [84]. In addition to a proposed prognostic role in the metastatic tumor progression [85, 86], LRP/LR provides a suitable target for therapy in cancer. In a murine model it has been demonstrated that human fibrosarcoma cells pretreated with an anti-LRP antibody display less lung metastases compared to those

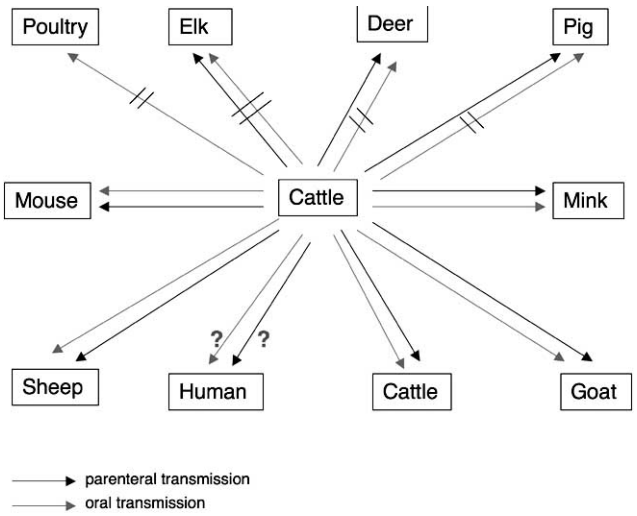


Fig. (6). Oral and parenteral interspecies transmission of BSE. Obviously, a transmission of BSE to humans can not be tested due to ethical reasons. Oral transmission of the BSE agent (light gray) has been performed succesfully to sheep and goats [97], minks [70], mice [71] and cows [98], respectively. However, several experiments demonstrated the existence of a species barrier for the oral transmission of TSEs (crossed light grey arrows), e.g. in elk and deer, pigs [99] and poultry [69], respectively, and parenteral transmission (= circumvention of the intestinal tract, crossed dark grey arrows) to elk. Interestingly, in pigs, a parenteral BSE transmission (dark grey) has been reported to be succesful [100]. Furthermore, the BSE agent has been effectively parenteral transmitted to cattle [98], sheep and goats [97], minks [Bradley, Paris Symposium, 1996] and deer [101] (dark grey arrows).

Table 3. Expression of the 67kDa LR has been Reported in Human Tumors

Human tumors with LR expression	Reference
Colorectal carcinoma	[78]
Colon cancer	[112]
Cervical preneoplastic and neoplastic squamous epithelial lesions	[79]
Gastric adenocarcinoma	[113]
Breast carcinoma	[80, 114]
Acute myeloid leukemia (AML)	[115]
Human laryngeal sqamous cell carcinoma	[116]
Human small cell lung cancer	[117]
Prostate cancer	[84]
Ovarian carcinoma	[82]
Uterine adenocarcinoma	[118]

injected with untreated cells [87]. Furthermore a suppressed LRP expression was followed by a reduced lung cancer cell proliferation and *in vivo* tumor formation [88].

Many tools targeting the LRP/LR for prion disease therapy [89], are also suitable for a tumor intervening therapy. Blocking or downregulating LRP/LR, significantly reduced the invasive potential of tumorigenic human fibrosarcoma cells (HT1080) [50]. Anti-LRP/LR scFv antibodies, the above mentionend full length format IgG, as well as heparan mimetics and pentosan polysulfate efficiently hamper the invasion through the perturbance of the laminin-LRP interaction on the cell surface [50]. These data suggest that tools targeting LRP/LR might represent effective alternative instruments to interfere with metastatic cancer. Very recently, a critical role of LRP/LR for the maintainance of cell viability has been reported [20]. The fact that neoplastic cells such as HT1080 reveal an increased LRP/LR level compared to non-tumorigenic cells [50] together with the finding that LRP/LR prevents apoptotic processes [20] suggests that the receptor has multiple effects in cancer development by supporting metastasis and inhibiting apoptosis.

5. ROLE OF LRP/LR IN OTHER NEURODEGENERATIVE AND INFECTIOUS DISEASES

5.1. Alzheimer's Disease (AD)

Alzheimer´s and prion diseases belong to the group of fatal neurological disorders. Both have in common to form amyloid plaques on neuronal tissues. In the case of Alzheimer´s disease (AD) the cleavage of the amyloid β precursor protein (APP) is thought to be the crucial process causing AD by producing the Aβ-peptide (Aβ) [90]. In the non-amyloidogenic pathway APP is cleaved by the α- and γ-secretase and the emerging soluble peptides are shedded into the extracellular matrix (Fig. **7**). However, if the proteolytic processing of APP is performed by β- and γ-secretase, the emerging β-peptide (Aβ) which is secreted into the extracellular matrix, accumulates in amyloid plaques on neurons, accompanied by neurotoxic effects and neurodegeneration. Tau-fibres may additionally contribute to the development of the Alzheimer disease phenotype [91].

In prion diseases, misfolding of cellular prion protein (PrP^c) to a proteinase K resistant and infectious isoform PrP^{Sc} is thought to be the causative pathogenic mechanism of TSEs. PrP^c passes through the secretory pathway and is then anchored to the cell surface by a glycosyl phosphatidyl inositol (GPI) anchor.

It has been shown that cellular PrP has a regulatory effect on the β-secretase cleavage of APP [92]. Overexpression of PrP^c inhibited β-secretase cleavage and reduced Aβ formation in neuronal cells [92]. Vice versa downregulation of PrP^c by RNAi leads to the secretion of increased amounts of Aβ into the cell culture medium [92]. Additionally, PrP is suggested to inhibit β-secretase (BACE1, β-site APP cleaving enzyme) activity involving interaction with glucosaminoglycanes [92].

The 37kDa/67kDa LRP/LR has been shown to act as a receptor for both, PrP^C [2] and the pathogenic isoform PrP^{Sc} [29, 45]. LRP/LR interacts with PrP via an indirect binding domain depending on the presence of heparan sulfate proteoglycans (HSPGs) [29] which belong to the group of GAGs. LRP/LR and APP share the same subcellular locali-

Table 4. Comparison of Interspecies Interactions in the Yeast Two-Hybrid System with Interconversion Studies by *In Vitro* Conversion and Transmission in Transgenic and Non-Transgenic Animals

PrP species	PrP species	Interspecies interactions by yeast two-hybrid system	Interconversion by *in vitro* conversion assays	Interspecies transmission in transgenic mice	Interspecies transmission in animals
Human	Cattle	+	+ [119]	+ [68, 120]	+ [121-123]
Sheep	Cattle	+	+ [124]	+ [125, 126]	+ [127,128]
Sheep	Human	+	+ [119]	+ [125]	n.d.
Hamster	Human	-	-	n.d.	Via guinea pigs [129]
Hamster	Cattle	-	- [124]	n.d.	- [69]
Cervid	Cattle	n.d.	n.d.	- [130]	n.d.
Cervid	Human	n.d.	n.d.	- [130,131]	n.d.
Cervid	Sheep	n.d.	n.d.	+ [131]	n.d.
Mouse	Human	n.d.	+ [132]	- [133]	n.d.
Mouse	Sheep	n.d.	n.d.	+ [133]	n.d.
Mouse	Hamster	n.d.	n.d.	+ [133]	n.d.

"+" : Interaction in described system
"- " : No interaction in described system
"n.d." : not determined

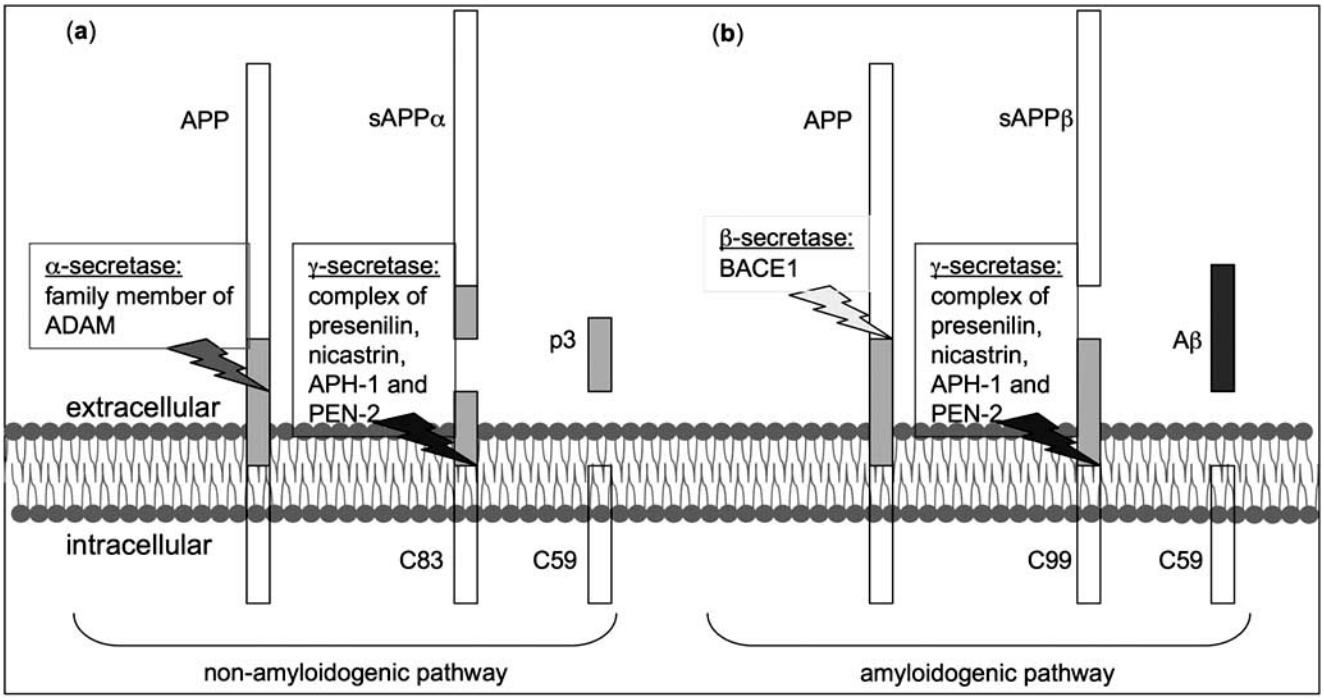


Fig. (7). Overview of the non-amyloidogenic and amyloidogenic APP cleavage pathway.
(**a**) In the non-amyloidogenic pathway, APP (Amyloid β precursor protein) is proteolytically cleaved by the α-secretase, member of the ADAM (a disintegrin and metalloprotease domain) family. A soluble fragment (sAPPα) is secreted into the extracellular space and the C-terminal domain stays membrane-bound. Afterwards the γ-secretase cleaves within membrane-bound C-terminus and releases a soluble peptide (p3) of approximately 3kDa. The γ-secretase minimally consists of 4 individual proteins: the presenilins (aspartyl proteases) form the catalytic subunit, nicastrin, APH-1 (anterior pharynx-defective 1) and PEN-2 (presenilin enhancer). (**b**) In the amyloidogenic pathway, APP is first cleaved by β-secretase (BACE1, β-site APP cleaving enzyme), releasing a soluble fragment (sAPPβ) into the extracellular space, followed by γ-secretase cleavage. Thereby, the amyloid β peptide (Aβ), which is the primary constituent of Alzheimer's Disease (AD), is shed off the cell surface and aggregates in the brain of AD patients.

zation on the cell surface. Therefore, it is conceivable that LRP/LR might be involved in secretase mediated cleavage of App and shedding of sAPPα, sAPPβ and the β-peptide.

5.2. Viral Diseases

As an initial step in viral diseases, most viruses enter the host cell by receptor-mediated endocytosis. Several protein receptors have been identified as initial virus attachment receptors including human immunodeficiency virus (HIV) which use the chemokine co-receptors CCR5 and CXCR4 in addition to the CD4 receptor [93] and the Epstein-Barr virus that is associated with infectious mononucleosis and the development of cancer, respectively, and use the complement receptor CR2 [94]. For alphaviruses, e.g. Sindbis Virus, the high-affinity laminin receptor has been described as major receptor in mammalian cells [19] (Tab. **1**). Hamster cells, that overexpress the laminin receptor at the cell surface were more susceptible to a Sindbis virus infection compared to cells transfected with the antisense gene. Moreover, it has been demonstrated that the 67kDa laminin receptor also functions as the Sindbis virus entry receptor in mosquitos [19]. In addition, the 37kDa/67kDa laminin receptor has been identified as a receptor for (i) dengue virus, which is the causative agent of dengue fever and dengue hemorrhagic fever, in human liver cells [95], porcine kidney cells [18] and mosquito cells [96], respectively, and (ii) for several AAV subtypes [16] (Tab. **1**). Thus, the identification of the 37kDa/67kDa LRP/LR as an initial entry receptor for mosquito-borne viral diseases opened up new vistas to establish a specific receptor-based antiviral prophylaxis and/or therapy.

ACKNOWLEDGEMENT

This work was supported by the Bundesministerium für Bildung und Forschung (BMBF; grant 01-KO-0514), the European Commission (grant NoE-NeuroPrion FOOD-CT-2004-506579) and the Deutsche Forschungsgemeinschaft (DFG; grant: WE 2664/2-1).

ABBREVIATIONS

LRP/LR	=	Laminin receptor precursor/laminin receptor
PrP (PrP^c, PrP^{Sc}, PrP27-30)	=	Prion protein (cellular form, scrapie form, protease-resistant core)
GPI	=	Glycosylphosphatidylinositol
TSE	=	Transmissible Spongiform Encephalopathy
CJD, vCJD	=	Creutzfeldt-Jakob Disease, variant Creutzfeldt-Jakob Disease
BSE	=	Bovine Spongiform Encephalopathy
CWD	=	Chronic Wasting Disease
HSPG	=	Heparan sulfate proteoglycan
GAG	=	Glycosaminoglycan
PPS	=	Pentosan polysulfate
siRNA	=	Small interfering ribonucleic acid
AD	=	Alzheimer's Disease
App	=	Amyloid precursor protein
Aβ	=	Amyloid beta protein (proteolytic fragment of APP)
FDA	=	U.S. Food and Drug Administration
AAV	=	Adeno-Associated Virus

REFERENCES

[1] Rieger, R.; Edenhofer, F.; Lasmezas, C.I.; Weiss, S. *Nat. Med.*, **1997,** *3*, 1383-1388.
[2] Gauczynski, S.; Peyrin, J.M.; Haik, S.; Leucht, C.; Hundt, C.; Rieger, R.; Krasemann, S.; Deslys, J.P.; Dormont, D.; Lasmezas, C.I.; Weiss, S. *EMBO J.*, **2001,** *20*, 5863-5875.
[3] Hundt, C.; Peyrin, J.M.; Haik, S.; Gauczynski, S.; Leucht, C.; Rieger, R.; Riley, M.L.; Deslys, J.P.; Dormont, D.; Lasmezas, C.I.; Weiss, S. *EMBO J.*, **2001,** *20*, 5876-5886.
[4] Snow, A.D.; Sekiguchi, R.T.; Nochlin, D.; Kalaria, R.N.; Kimata, K. *Am. J. Pathol.*, **1994,** *144*, 337-347.
[5] Rao, N.C.; Barsky, S.H.; Terranova, V.P.; Liotta, L.A. *Biochem. Biophys. Res. Commun.*, **1983,** *111*, 804-808.
[6] Castronovo, V.; Taraboletti, G.; Sobel, M.E. *J. Biol. Chem.*, **1991,** *266*, 20440-20446.
[7] Romanov, V.; Sobel, M.E.; pinto da Silva, P.; Menard, S.; Castronovo, V. *Cell Adhes. Commun.*, **1994,** *2*, 201-209.
[8] Landowski, T.H.; Dratz, E.A.; Starkey, J.R. *Biochemistry*, **1995,** *34*, 11276-11287.
[9] Buto, S.; Tagliabue, E.; Ardini, E.; Magnifico, A.; Ghirelli, C.; van den Brule, F.; Castronovo, V.; Colnaghi, M.I.; Sobel, M.E.; Menard, S. *J. Cell Biochem.*, **1998,** *69*, 244-251.
[10] Jackers, P.; Minoletti, F.; Belotti, D.; Clausse, N.; Sozzi, G.; Sobel, M.E.; Castronovo, V. *Oncogene*, **1996,** *13*, 495-503.
[11] Jackers, P.; Clausse, N.; Fernandez, M.; Berti, A.; Princen, F.; Wewer, U.; Sobel, M.E.; Castronovo, V. *Biochim. Biophys. Acta*, **1996,** *1305*, 98-104.
[12] Auth, D.; Brawerman, G. *Proc. Natl. Acad. Sci. USA*, **1992,** *89*, 4368-4372.
[13] Makrides, S.; Chitpatima, S.T.; Bandyopadhyay, R.; Brawerman, G. *Nucleic Acids Res.*, **1988,** *16*, 2349.
[14] Sato, M.; Kinoshita, K.; Kaneda, Y.; Saeki, Y.; Iwamatsu, A.; Tanaka, K. *Biochem. Biophys. Res. Commun.*, **1996,** *229*, 896-901.
[15] Ardini, E.; Pesole, G.; Tagliabue, E.; Magnifico, A.; Castronovo, V.; Sobel, M.E.; Colnaghi, M.I.; Menard, S. *Mol. Biol. Evol.*, **1998,** *15*, 1017-1025.
[16] Akache, B.; Grimm, D.; Pandey, K.; Yant, S.R.; Xu, H.; Kay, M.A. *J. Virol.*, **2006,** *80*, 9831-9836.
[17] Ludwig, G.V.; Kondig, J.P.; Smith, J.F. *J. Virol.*, **1996,** *70*, 5592-5599.
[18] Tio, P.H.; Jong, W.W.; Cardosa, M.J. *Virol. J.*, **2005,** *2*, 25.
[19] Wang, K.S.; Kuhn, R.J.; Strauss, E.G.; Ou, S.; Strauss, J.H. *J. Virol.*, **1992,** *66*, 4992-5001.
[20] Susantad, T.; Smith, D.R. *Cell Mol. Biol. Lett.*, **2008**, *13*, 452-464.
[21] Wang, T.; Zhang, W.; Pei, Z.; Block, M.; Wilson, B.; Reece, J.M.; Miller, D.S.; Hong, J.S. *FASEB J.*, **2006,** *20*, 906-915.
[22] Huard, T.K.; Malinoff, H.L.; Wicha, M.S. *Am. J. Pathol.*, **1986,** *123*, 365-370.
[23] Simoneau, S.; Haik, S.; Leucht, C.; Dormont, D.; Deslys, J.P.; Weiss, S.; Lasmezas, C. *Biol. Chem.*, **2003,** *384*, 243-246.
[24] Baloui, H.; von Boxberg, Y.; Vinh, J.; Weiss, S.; Rossier, J.; Nothias, F.; Stettler, O. *Eur. J. Neurosci.*, **2004,** *20*, 2605-2616.
[25] Belichenko, P.V.; Miklossy, J.; Belser, B.; Budka, H.; Celio, M.R. *Neurobiol. Dis.*, **1999,** *6*, 269-279.
[26] Baloui, H.; Stettler, O.; Weiss, S.; Nothias, F.; Boxberg, Y. *J. Neurotrauma*, in press.
[27] Leucht, C.; Simoneau, S.; Rey, C.; Vana, K.; Rieger, R.; Lasmezas, C.I.; Weiss, S. *EMBO Rep.*, **2003,** *4*, 290-295.
[28] Pflanz, H.; Vana, K.; Mitteregger G.; Pace C.; Messow, D.; Sedlaczek, C.; Nikles, D.; Kretzschmar, H.A.; Weiss, S. *J. Gen. Virol.*, **2009**, *90*, 269-274.
[29] Gauczynski, S.; Nikles, D.; El-Gogo, S.; Papy-Garcia, D.; Rey, C.; Alban, S.; Barritault, D.; Lasmezas, C.I.; Weiss, S. *J. Infect. Dis.*, **2006,** *194*, 702-709.
[30] Shmakov, A.N.; Bode, J.; Kilshaw, P.J.; Ghosh, S. *J. Pathol.*, **2000,** *191*, 318-322.

[31] Nikles, D.; Vana, K.; Gauczynski, S.; Knetsch, H.; Ludewigs, H.; Weiss, S. *Biochim. Biophys. Acta*, **2008,** *1782*, 335-340.
[32] Maignien, T.; Lasmezas, C.I.; Beringue, V.; Dormont, D.; Deslys, J.P. *J. Gen. Virol.*, **1999,** *80* (Pt 11) 3035-3042.
[33] Bons, N.; Mestre-Frances, N.; Belli, P.; Cathala, F.; Gajdusek, D.C.; Brown, P. *Proc. Natl. Acad. Sci. USA*, **1999,** *96*, 4046-4051.
[34] Booth, C.; Potten, C.S. *J. Clin. Invest.*, **2000,** *105*, 1493-1499.
[35] Morel, E.; Fouquet, S.; Chateau, D.; Yvernault, L.; Frobert, Y.; Pincon-Raymond, M.; Chambaz, J.; Pillot, T.; Rousset, M. *J. Biol. Chem.*, **2004,** *279*, 1499-1505.
[36] Morel, E.; Andrieu, T.; Casagrande, F.; Gauczynski, S.; Weiss, S.; Grassi, J.; Rousset, M.; Dormont, D.; Chambaz, J. *Am. J. Pathol.*, **2005,** *167*, 1033-1042.
[37] Hooper, N.M. *Mol. Membr. Biol.*, **1999,** *16*, 145-156.
[38] Enari, M.; Flechsig, E.; Weissmann, C. *Proc. Natl. Acad. Sci. USA*, **2001,** *98*, 9295-9299.
[39] Ludewigs, H.; Zuber, C.; Vana, K.; Nikles, D.; Zerr, I.; Weiss, S. *Expert Rev. Anti. Infect. Ther.*, **2007,** *5*, 613-630.
[40] Miyamoto, K.; Nakamura, N.; Aosasa, M.; Nishida, N.; Yokoyama, T.; Horiuchi, H.; Furusawa, S.; Matsuda, H. *Biochem. Biophys. Res. Commun.*, **2005,** *335*, 197-204.
[41] Perrier, V.; Solassol, J.; Crozet, C.; Frobert, Y.; Mourton-Gilles, C.; Grassi, J.; Lehmann, S. *J. Neurochem.*, **2004,** *89*, 454-463.
[42] White, A.R.; Enever, P.; Tayebi, M.; Mushens, R.; Linehan, J.; Brandner, S.; Anstee, D.; Collinge, J.; Hawke, S. *Nature*, **2003,** *422*, 80-83.
[43] Sethi, S.; Lipford, G.; Wagner, H.; Kretzschmar, H. *Lancet* **2002**, *360*, 229-230.
[44] Zuber, C.; Ludewigs, H.; Weiss, S. *Vet. Microbiol.*, **2007,** *123*, 387-393.
[45] Morel, E.; Andrieu, T.; Casagrande, F.; Gauczynski, S.; Weiss, S.; Grassi, J.; Rousset, M.; Dormont, D.; Chambaz, J. *Am. J. Pathol.*, **2005,** *167*, 1033-1042.
[46] Zuber, C.; Mitteregger, G.; Pace, C.; Zerr, I.; Kretzschmar, H.A.; Weiss, S. *Prion*. **2007,** *1*, 207-212.
[47] Zuber, C.; Knackmuss, S.; Rey, C.; Reusch, U.; Rottgen, P.; Frohlich, T.; Arnold, G.J.; Pace, C.; Mitteregger, G.; Kretzschmar, H.A.; Little, M.; Weiss, S. *Mol. Immunol.*, **2008,** *45*, 144-151.
[48] Wuertzer, C.A.; Sullivan, M.A.; Qiu, X.; Federoff, H.J. *Mol. Ther.*, **2008,** *16*, 481-486.
[49] Zuber, C.; Mitteregger, G.; Schuhmann, N.; Rey, C.; Knackmuss, S.; Rupprecht, W.; Reusch, U.; Pace, C.; Little, M.; Kretzschmar, H.A.; Hallek, M.; Büning, H.; Weiss, S. *J. Gen. Virol.*, **2008,** *89*, 2054-2060.
[50] Zuber, C.; Knackmuss, S.; Zemora, G.; Reusch, U.; Vlasova, E.; Diehl, D.; Mick, V.; Hoffmann, K.; Nikles, D.; Frohlich, T.; Arnold, G.J.; Brenig, B.; Wolf, E.; Lahm, H.; Little, M.; Weiss, S. *J. Mol. Biol*., **2008**, *378*, 530-539.
[51] Caughey, B.; Raymond, G.J. *J. Virol*, **1993,** *67*, 643-650.
[52] Farquhar, C.; Dickinson, A.; Bruce, M. *Lancet*, **1999,** *353*, 117.
[53] Kocisko, D.A.; Caughey, B.; Morrey, J.D.; Race, R.E. *Antimicrob Agents Chemother*, **2006,** *50*, 3447-3449.
[54] Dealler, S.; Rainov, N.G. *IDrugs*, **2003,** *6*, 470-478.
[55] Whittle, I.R.; Knight, R.S.; Will, R.G. *Acta. Neurochir, (Wien)* **2006,** *148*, 677-679.
[56] Todd, N.V.; Morrow, J.; Doh-ura, K.; Dealler, S.; O'Hare, S.; Farling, P.; Duddy, M.; Rainov, N.G. *J. Infect.*, **2005,** *50*, 394-396.
[57] Parry, A.; Baker, I.; Stacey, R.; Wimalaratna, S. *J. Neurol. Neurosurg. Psychiatry*, **2007,** *78*, 733-734.
[58] Bone, I.; Belton, L.; Walker, A.S.; Darbyshire, J. *Eur. J. Neurol.*, **2008,** *15*, 458-464.
[59] Leucht, C.; Vana, K.; Renner-Muller, I.; Dormont, D.; Lasmezas, C.I.; Wolf, E.; Weiss, S. *Transgenic Res.*, **2004,** *13*, 81-85.
[60] Bueler, H.; Aguzzi, A.; Sailer, A.; Greiner, R.A.; Autenried, P.; Aguet, M.; Weissmann, C. *Cell*, **1993,** *73*, 1339-1347.
[61] Vana, K.; Weiss, S. *J. Mol. Biol.*, **2006,** *358*, 57-66.
[62] Pattison, I.H. *Vet. Rec.*, **1965,** *77*, 1388-1390.
[63] Kremer, W.; Kachel, N.; Kuwata, K.; Akasaka, K.; Kalbitzer, H.R. *J. Biol. Chem.*, **2007**, *282*, 22689-22698.
[64] Sigurdson, C.J.; Miller, M.W. *Br. Med. Bull.*, **2003,** *66*, 199-212.
[65] Wilesmith, J.W.; Wells, G.A.; Ryan, J.B.; Gavier-Widen, D.; Simmons, M.M. *Vet. Rec.*, **1997,** *141*, 239-243.
[66] Hourrigan, J.L.; Klingsporn, A.; Clark, W.W., C.M., d. In: Prusiner, S.B.; Hadlow, W.J. Eds., *Slow transmissible diseases of the nervous system: clinical, epidemiological, genetic and pathological aspects of the spongiform encephalopathies*, **1979,** *1*, 331-356.
[67] Aguzzi, A.; Heikenwalder, M.; Polymenidou, M. *Nat. Rev. Mol. Cell Biol.*, **2007,** *8*, 552-561.
[68] Hill, A.F.; Desbruslais, M.; Joiner, S.; Sidle, K.C.; Gowland, I.; Collinge, J.; Doey, L.J.; Lantos, P. *Nature*, **1997,** *389*, 448-450, 526.
[69] Bradley, R.; Wilesmith, J.W. *Br. Med. Bull.*, **1993,** *49*, 932-959.
[70] Robinson, M.M.; Hadlow, W.J.; Huff, T.P.; Wells, G.A.; Dawson, M.; Marsh, R.F.; Gorham, J.R. *J. Gen. Virol.*, **1994,** *75* (Pt 9) 2151-2155.
[71] Barlow, R.M.; Middleton, D.J. *Vet. Rec.*, **1990,** *126*, 111-112.
[72] Scott, A.C.; Wells, G.A.; Stack, M.J.; White, H.; Dawson, M. *Vet. Microbiol.*, **1990,** *23*, 295-304.
[73] Magnifico, A.; Tagliabue, E.; Buto, S.; Ardini, E.; Castronovo, V.; Colnaghi, M.I.; Menard, S. *J. Biol. Chem.*, **1996,** *271*, 31179-31184.
[74] Kazmin, D.A.; Hoyt, T.R.; Taubner, L.; Teintze, M.; Starkey, J.R. *J. Mol. Biol*. **2000,** *298*, 431-445.
[75] Guo, N.H.; Krutzsch, H.C.; Vogel, T.; Roberts, D.D. *J. Biol. Chem.*, **1992,** *267*, 17743-17747.
[76] de Manzoni, G.; Guglielmi, A.; Verlato, G.; Tomezzoli, A.; Pelosi, G.; Schiavon, I.; Cordiano, C. *Oncology*, **1998,** *55*, 456-460.
[77] Cioce, V.; Castronovo, V.; Shmookler, B.M.; Garbisa, S.; Grigioni, W.F.; Liotta, L.A.; Sobel, M.E. *J. Natl. Cancer Inst.*, **1991,** *83*, 29-36.
[78] Sanjuan, X.; Fernandez, P.L.; Miquel, R.; Munoz, J.; Castronovo, V.; Menard, S.; Palacin, A.; Cardesa, A.; Campo, E. *J. Pathol.*, **1996,** *179*, 376-380.
[79] al-Saleh, W.; Delvenne, P.; van den Brule, F.A.; Menard, S.; Boniver, J.; Castronovo, V. *J. Pathol.*, **1997,** *181*, 287-293.
[80] Nadji, M.; Nassiri, M.; Fresno, M.; Terzian, E.; Morales, A.R. *Cancer*, **1999,** *85*, 432-436.
[81] Fontanini, G.; Vignati, S.; Chine, S.; Lucchi, M.; Mussi, A.; Angeletti, C.A.; Menard, S.; Castronovo, V.; Bevilacqua, G. *Clin. Cancer Res.*, **1997,** *3*, 227-231.
[82] van den Brule, F.A.; Berchuck, A.; Bast, R.C.; Liu, F.T.; Gillet, C.; Sobel, M.E.; Castronovo, V. *Eur. J. Cancer*, **1994,** *30A*, 1096-1099.
[83] Pelosi, G.; Pasini, F.; Bresaola, E.; Bogina, G.; Pederzoli, P.; Biolo, S.; Menard, S.; Zamboni, G. *J. Pathol.*, **1997,** *183*, 62-69.
[84] Waltregny, D., de Leval, L., Menard, S., de Leval, J., Castronovo, V. *J. Natl. Cancer Inst.*, **1997,** *89*, 1224-1227.
[85] Gasparini, G., Barbareschi, M., Boracchi, P., Bevilacqua, P., Verderio, P., Dalla Palma, P., Menard, S. *Int. J. Cancer*, **1995,** *60*, 604-610.
[86] Menard, S., Tagliabue, E., Colnaghi, M.I. *Breast Cancer Res. Treat.*, **1998,** *52*, 137-145.
[87] Narumi, K.; Inoue, A.; Tanaka, M.; Isemura, M.; Shimo-Oka, T.; Abe, T.; Nukiwa, T.; Satoh, K. *Jpn. J. Cancer Res.*, **1999,** *90*, 425-431.
[88] Satoh, K.; Narumi, K.; Abe, T.; Sakai, T.; Kikuchi, T.; Tanaka, M.; Shimo-Oka, T.; Uchida, M.; Tezuka, F.; Isemura, M.; Nukiwa, T. *Br. J. Cancer*, **1999,** *80*, 1115-1122.
[89] Vana, K.; Zuber, C.; Nikles, D.; Weiss, S. *Cell. Mol. Neurobiol.*, **2006**, *27*, 107-128.
[90] Sisodia, S.S.; Price, D.L. *FASEB J.*, **1995,** *9*, 366-370.
[91] Ballatore, C.; Lee, V.M.; Trojanowski, J.Q. *Nat. Rev. Neurosci.*, **2007,** *8*, 663-672.
[92] Parkin, E.T.; Watt, N.T.; Hussain, I.; Eckman, E.A.; Eckman, C.B.; Manson, J.C.; Baybutt, H.N.; Turner, A.J.; Hooper, N.M. *Proc. Natl. Acad. Sci. USA*, **2007,** *104*, 11062-11067.
[93] Alkhatib, G.; Berger, E.A. *Eur. J. Med. Res.*, **2007,** *12*, 375-384.
[94] Cooper, N.R.; Bradt, B.M.; Rhim, J.S.; Nemerow, G.R. *J. Invest. Dermatol.*, **1990,** *94*, 112S-117S.
[95] Thepparit, C.; Smith, D.R. *J. Virol.*, **2004,** *78*, 12647-12656.
[96] Sakoonwatanyoo, P.; Boonsanay, V.; Smith, D.R. *Intervirology*, **2006,** *49*, 161-172.
[97] Foster, J.D.; Hope, J.; Fraser, H. *Vet. Rec.*, **1993,** *133*, 339-341.
[98] Bradley, R. *Dev. Biol. Stand.*, **1993,** *80*, 157-170.
[99] Wells, G.A.; Hawkins, S.A.; Austin, A.R.; Ryder, S.J.; Done, S.H.; Green, R.B.; Dexter, I.; Dawson, M.; Kimberlin, R.H. *J. Gen. Virol.*, **2003,** *84*, 1021-1031.
[100] Dawson, M.; Wells, G.A.; Parker, B.N.; Scott, A.C. *Vet. Rec.*, **1990,** *127*, 338.

[101] Dagleish, M.P.; Martin, S.; Steele, P.; Finlayson, J.; Siso, S.; Hamilton, S.; Chianini, F.; Reid, H.W.; Gonzalez, L.; Jeffrey, M. *BMC. Vet. Res*., **2008,** *4*, 17.
[102] Wu, Z.; Miller, E.; Agbandje-McKenna, M.; Samulski, R.J. *J. Virol.,* **2006,** *80*, 9093-9103.
[103] Strauss, J.H.; Wang, K.S.; Schmaljohn, A.L.; Kuhn, R.J.; Strauss, E.G. *Arch. Virol. Suppl.,* **1994,** *9*, 473-484.
[104] Dechecchi, M.C.; Melotti, P.; Bonizzato, A.; Santacatterina, M.; Chilosi, M.; Cabrini, G. *J. Virol.,* **2001,** *75*, 8772-8780.
[105] Shafti-Keramat, S.; Handisurya, A.; Kriehuber, E.; Meneguzzi, G.; Slupetzky, K.; Kirnbauer, R. *J. Virol.,* **2003,** *77*, 13125-13135.
[106] Leistner, C.M.; Gruen-Bernhard, S.; Glebe, D. *Cell Microbiol.,* **2008,** *10*, 122-133.
[107] Barth, H.; Schafer, C.; Adah, M.I.; Zhang, F.; Linhardt, R.J.; Toyoda, H.; Kinoshita-Toyoda, A.; Toida, T.; Van Kuppevelt, T.H.; Depla, E.; Von Weizsacker, F.; Blum, H.E.; Baumert, T.F. *J. Biol. Chem.,* **2003,** *278*, 41003-41012.
[108] de Witte, L.; Bobardt, M.; Chatterji, U.; Degeest, G.; David, G.; Geijtenbeek, T.B.; Gallay, P. *Proc. Natl. Acad. Sci. USA,* **2007,** *104*, 19464-19469.
[109] Kureishy, N.; Faruque, D.; Porter, C.D. *Biochem. J.,* **2006,** *400*, 421-430.
[110] Dechecchi, M.C.; Tamanini, A.; Bonizzato, A.; Cabrini, G. *Virology*, **2000,** *268*, 382-390.
[111] Karger, A.; Mettenleiter, T.C. *J. Virol.,* **1996,** *70*, 2138-2145.
[112] Campo, E.; Monteagudo, C.; Castronovo, V.; Claysmith, A.P.; Fernandez, P.L.; Sobel, M.E. *Am. J. Pathol.,* **1992,** *141*, 1073-1083.
[113] Lee, W.A.; Kim, W.H.; Kim, Y.I.; Yang, H.K.; Kim, J.P.; Kleinman, H.K. *Pathol. Res. Pract.,* **1996,** *192*, 1195-1201.
[114] Martignone, S.; Menard, S.; Bufalino, R.; Cascinelli, N.; Pellegrini, R.; Tagliabue, E.; Andreola, S.; Rilke, F.; Colnaghi, M.I. *J. Natl. Cancer Inst.,* **1993,** *85*, 398-402.
[115] Montuori, N.; Selleri, C.; Risitano, A.M.; Raiola, A.M.; Ragno, P.; Del Vecchio, L.; Rotoli, B.; Rossi, G. *Clin. Cancer Res.,* **1999,** *5*, 1465-1472.
[116] Zhou, L.; Xie, M.; Zhou, J.Q.; Tao, L. *Laryngoscope,* **2006,** *116*, 28-32.
[117] Satoh, K.; Narumi, K.; Isemura, M.; Sakai, T.; Abe, T.; Matsushima, K.; Okuda, K.; Motomiya, M. *Biochem. Biophys. Res. Commun*., **1992,** *182*, 746-752.
[118] van den Brule, F.A.; Buicu, C.; Berchuck, A.; Bast, R.C.; Deprez, M.; Liu, F.T.; Cooper, D.N.; Pieters, C.; Sobel, M.E.; Castronovo, V. *Hum. Pathol.,* **1996,** *27*, 1185-1191.
[119] Raymond, G.J.; Bossers, A.; Raymond, L.D.; O'Rourke, K.I.; McHolland, L.E.; Bryant, P.K.; 3rd, Miller, M.W.; Williams, E.S.; Smits, M.; Caughey, B. *EMBO J.,* **2000,** *19*, 4425-4430.
[120] Scott, M.R.; Will, R.; Ironside, J.; Nguyen, H.O.; Tremblay, P.; DeArmond, S.J.; Prusiner, S.B. *Proc. Natl. Acad. Sci. USA,* **1999,** *96*, 15137-15142.
[121] Bruce, M.E.; Will, R.G.; Ironside, J.W.; McConnell, I.; Drummond, D.; Suttie, A.; McCardle, L.; Chree, A.; Hope, J.; Birkett, C.; Cousens, S.; Fraser, H.; Bostock, C.J. *Nature*, **1997,** *389*, 498-501.
[122] Lasmezas, C.I.; Deslys, J.P.; Demaimay, R.; Adjou, K.T.; Lamoury, F.; Dormont, D.; Robain, O.; Ironside, J.; Hauw, J.J. *Nature*, **1996,** *381*, 743-744.
[123] Lasmezas, C.I.; Fournier, J.G.; Nouvel, V.; Boe, H.; Marce, D.; Lamoury, F.; Kopp, N.; Hauw, J.J.; Ironside, J.; Bruce, M.; Dormont, D.; Deslys, J.P. *Proc. Natl. Acad. Sci. USA,* **2001,** *98*, 4142-4147.
[124] Raymond, G.J.; Hope, J.; Kocisko, D.A.; Priola, S.A.; Raymond, L.D.; Bossers, A.; Ironside, J.; Will, R.G.; Chen, S.G.; Petersen, R.B.; Gambetti, P.; Rubenstein, R.; Smits, M.A.; Lansbury, P.T. Jr.; Caughey, B. *Nature,* **1997,** *388*, 285-288.
[125] Scott, M.R.; Peretz, D.; Nguyen, H.O.; Dearmond, S.J.; Prusiner, S.B. *J. Virol.,* **2005,** *79*, 5259-5271.
[126] Scott, M.R.; Supattapone, S.; Nguyen, H.O.; DeArmond, S.J.; Prusiner, S.B. *Arch. Virol. Suppl.,* **2000**, 113-124.
[127] Cutlip, R.C.; Miller, J.M.; Race, R.E.; Jenny, A.L.; Katz, J.B.; Lehmkuhl, H.D.; DeBey, B.M.; Robinson, M.M. *J. Infect. Dis.,* **1994,** *169*, 814-820.
[128] Clark, W.W.; Hourrigan, J.L.; Hadlow, W.J. *Am. J. Vet. Res.,* **1995,** *56*, 606-612.
[129] Manuelidis, E.E.; Gorgacz, E.J.; Manuelidis, L. *Proc. Natl. Acad. Sci. USA,* **1978,** *75*, 3432-3436.
[130] Kong, Q.; Huang, S.; Zou, W.; Vanegas, D.; Wang, M.; Wu, D.; Yuan, J.; Zheng, M.; Bai, H.; Deng, H.; Chen, K.; Jenny, A.L.; O'Rourke, K.; Belay, E.D.; Schonberger, L.B.; Petersen, R.B.; Sy, M.S.; Chen, S.G.; Gambetti, P. *J. Neurosci.,* **2005,** *25*, 7944-7949.
[131] Tamguney, G.; Giles, K.; Bouzamondo-Bernstein, E.; Bosque, P.J.; Miller, M.W.; Safar, J.; DeArmond, S.J.; Prusiner, S.B. *J. Virol.,* **2006,** *80*, 9104-9114.
[132] Vanik, D.L., Surewicz, K.A., Surewicz, W.K. *Mol. Cell.,* **2004,** *14*, 139-145.
[133] Barron, R.M.; Thomson, V.; Jamieson, E.; Melton, D.W.; Ironside, J.; Will, R.; Manson, J.C. *EMBO J.*, **2001,** *20*, 5070-5078.

Received: June 17, 2008 Revised: July 9, 2008 Accepted: July 10, 2008

Chapter IV

Scrapie-infected transgenic mice expressing a laminin receptor decoy mutant reveal a prolonged incubation time associated with low levels of PrPres

published as:

Pflanz, H., Vana, K., Mitteregger, G., Renner-Müller, I., Pace, C., Küchenhoff, H., Kretzschmar, H. A., Wolf, E., Weiss, S., Scrapie-infected transgenic mice expressing a laminin receptor decoy mutant reveal a prolonged incubation time associated with low levels of PrPres. *J Mol Biol*, in press

Scrapie-Infected Transgenic Mice Expressing a Laminin Receptor Decoy Mutant Reveal a Prolonged Incubation Time Associated with Low Levels of PrPres

Heike Pflanz[1] , Karen Vana[1] , Gerda Mitteregger[2] , Ingrid Renner-Müller[3], Claudia Pace[2], Helmut Küchenhoff[4], Hans A. Kretzschmar[2], Eckhard Wolf[3] and Stefan Weiss[1]*

[1]Laboratorium für Molekulare Biologie – Genzentrum - Institut für Biochemie der LMU München, Germany

[2]Zentrum für Neuropathologie und Prionforschung, LMU München, Germany

[3]Lehrstuhl für Molekulare Tierzucht und Biotechnologie – Genzentrum, LMU München, Germany

[4]Statistical Consulting Unit, Department of Statistics, LMU München, Germany

These authors contributed equally to this work.

*Corresponding author: Stefan Weiss, Genzentrum - Institut für Biochemie der LMU München, Feodor-Lynen-Str. 25, D-81377 München, E-mail: weiss@lmb.uni-muenchen.de

Running title: A LRP mutant prolongs incubation time in scrapie-infected mice

Key words: prion; PrP; laminin receptor; LRP/LR; trans-dominant negative mutant; therapy;

The 37kDa/67kDa laminin receptor (LRP/LR) was identified as a cell surface receptor for prion proteins. The laminin receptor mutant LRP102-295::FLAG interfered with PrP^{Sc} propagation in murine neuronal cells presumably acting as a decoy in a trans-dominant negative fashion by trapping PrP molecules in the ECM. Here, we generated hemizygous transgenic mice expressing LRP102-295::FLAG in the brain. Scrapie-infected transgenic mice exhibit a significantly prolonged incubation time in comparison to scrapie-infected wild-type mice (FVB). At the terminal stage, transgenic mice revealed significantly reduced Proteinase K-resistant PrP levels by 71% compared to wild-type mice. Our results recommend the laminin receptor decoy mutant as an alternative therapeutic tool for treatment of transmissible spongiform encephalopathies.

Introduction

Prion diseases are neurodegenerative disorders occurring in mammals, naturally in many ruminants (scrapie, BSE), deer (CWD) and mink (TME), as well as in humans (CJD, FFI, GSS)[1; 2; 3] and are characterized by the accumulation of an abnormal, partially Proteinase K-resistant isoform (PrPres) of the cellular prion protein (PrP^{c}). The infectious isoform of the prion protein (PrP^{Sc}) tends to aggregate in brains of affected humans or animals[4]. All prion diseases show long incubation periods but are typically rapidly progressive and emerge in the CNS with neuropathological changes including spongiosis, astrogliosis and neuronal loss[5].

The 37kDa/67kDa laminin receptor (LRP/LR) was identified as the cell surface receptor for cellular PrP[6; 7] and for infectious prions[8]. Bovine prions are endocytosed in a LRP/LR dependent manner by human enterocytes[9] confirming the importance of LRP/LR by the oral uptake of prions.

Thereupon, several therapeutic approaches regarding transmissible spongiform encephalopathies (TSEs) targeting LRP/LR have been developed (for review:[10; 11; 12; 13] including (i) anti-LRP/LR antibodies[14; 15; 16], (ii) polysulfated glycans[8], (iii) a transdominant-negative LRP/LR decoy mutant [17] and (iv) siRNAs directed against the LRP mRNA[18; 19]. In addition, LRP/LR acts as a receptor for several viruses and is implicated in a wide variety of biological processes including neuronal cell adhesion, differentiation, migration and neurite outgrowth as well as cell survival[20] and tumor metastasis formation (for review:[12]), respectively. Recently, it has been demonstrated that blocking or downregulating the 37kDa/67kDa LRP/LR hampered the invasion of tumorigenic cells in vitro[21].

LRP/LR is a multifunctional protein and is expressed within the cytoplasm, the nucleus and the plasma membrane, respectively (for review:[11; 12]), and several isoforms of LRP/LR are present in mouse brain[22] with the 67kDa LR as the major receptor form [23]. In contrast, LRP levels are increased in spinal cord microglia after activation by peripheral injury or traumatic lesion[24].

Recently, we demonstrated that LRP/LR is required for the prion propagation in scrapie-infected neuronal cells[18] and showed that secretion of the LRP102-295::FLAG mutant, which represents solely the extracellular domain of LRP/LR, reduced PrP^{Sc} accumulation in scrapie-infected mouse neuroblastoma cells[17]. Moreover, the LRP102-295::FLAG mutant reduced the binding of PrP27-30 to cells, indicating that the laminin receptor mutant may act in a trans-dominant negative manner as a decoy by trapping PrP^{Sc} molecules[17]. Here we report the generation of hemizygous transgenic mice expressing the LRP mutant in the brain under the control of the rat neuron-specific enolase (NSE) promoter to investigate the decoy effect in vivo. Assuming that the NSE promoter-containing construct is more efficient in various neuronal cell types than in non-neuronal tissues[25] we concentrated on the analysis of the central nervous system (CNS). Previously, transgenic mice expressing trans-dominant negative mutants of PrP (Q167R or Q218K) have been investigated for their therapeutic potential in prion diseases and showed a strong effect on the reduction of PrP^{Sc} formation in vivo[26]. Furthermore, lentiviral gene transfer to deliver transdominant negative PrP mutant virions into the brains of scrapie-infected mice by microinjection resulted in a prolongation of survival[27]. These studies encouraged us to use the trans-dominant negative LRP102-295::FLAG

mutant of the prion protein receptor LRP/LR[17] as a therapeutic approach for treatment of prion disorders in vivo.

Results

Generation of transgenic mice

Transgenic mice were generated by microinjection of DNA into the pronuclei of fertilized oocytes (FVB) following transfer of the zygotes into pseudopregnant recipient mice. Subsequently, potential founders were genotyped by PCR to confirm the presence of the transgene. The plasmid, which was microinjected into the pronuclei, served as positive control for the correct fragment size of 711bp. The founder animal showed a specific single band at the correct fragment size in comparison to the wild-type mouse, which matched in age and showed no PCR product (Fig. 1a). A hemizygous mouse line TgN(NSE_LRP102-295::FLAG) was established by mating the founder animal with wild-type (FVB) mice. The offspring were routinely screened for the integration of the pCIneoNSE_LRP102-295FLAG construct by PCR analysis, followed by 1% agarose-gel electrophoresis. The genomic DNAs of all littermates of the F3-generation show a distinct band of the size of 711bp (Fig. 1b), verified by comparison with the PCR product of the plasmid pCIneoNSE_LRP102-295FLAG, which was used for microinjection. The genomic DNAs from mice of the F3-generation from another litter, previously genotyped as transgenic and non-transgenic (non-tg), respectively, served as positive and negative control animals, respectively (Fig. 1b). A control sample without DNA (H_2O) was performed to exclude any contamination. The genotyped littermates (F3) were the offsprings a transgenic mouse of the F2-generation homozygous for NSE_LRP102-

295FLAG (determined by back-crossing with wild-type mice) and a wild-type mouse and are consequently hemizygous (+/-) for the transgene.

Transgenic mice were visually compared with wild-type mice for variations in body size, body weight, movement and coordination, respectively, and showed no visible phenotype.

The laminin receptor mutant LRP102-295::FLAG is expressed in the brains of transgenic mice

Initially, the expression pattern of the LRP102-295::FLAG mutant was analyzed in brain and spleen samples from non-infected transgenic (+/-) mice of the F3-generation and age matched wild-type (FVB) mice (Fig. 2a). Brains and spleens were homogenized and subjected to 12% SDS-PAGE followed by Western blot. The mutant LRP102-295::FLAG was detected with an antibody directed against the FLAG-tag, showing a distinct band at approx. 32 kDa[17]. The analysis revealed that the laminin receptor mutant LRP102-295::FLAG is expressed in the brains of transgenic mice, whereas no expression was detected in neither splenic tissue of transgenic mice nor in brain and spleen of non-transgenic littermates. These results verify that the NSE promotor facilitates the expression of the transgene in neuronal tissues but not in the periphery of transgenic mice.

To assess the ratio of the dominant negative LRP-mutant and endogenously expressed LRP, brain homogenates of transgenic (+/-) mice of the F3-generation and wild-type mice (FVB) were analyzed by Western Blotting employing the IgG1 iS18 antibody directed against the C-terminus of LRP[21]. Endogenous 37kDa LRP and LRP102-

295::FLAG (approx. 32 kDa[17]) was detected in the brain of transgenic mice, whereas only the 37 kDa form was detected in wild-type mice (Fig. 2b). Densitometric analysis of the western blot data revealed no significant differences regarding the endogenous LRP levels in transgenic and wild-type mice (Fig. 2c). In the brain of transgenic mice, the ratio of LRP102-295::FLAG and endogenous LRP was approx. 1 to 1.8.

The cellular PrP level of brain and spleen remains unaltered in transgenic LRP102-295::FLAG expressing mice

The cellular PrP levels in brain and spleen homogenates of uninfected hemizygous (+/-) transgenic mice of the F3-generation and wild-type mice (FVB) were analyzed to investigate whether the laminin receptor mutant might influence PrP^c levels. Densitometric quantification (Fig. 3b) of western blot data (Fig. 3a) revealed no significant alteration of PrP^c expression levels in the brain and spleen of transgenic mice (set to 100%) in comparison to wild-type mice (95% and 82%, respectively, p-value > 0.05, student´s t-test). Thus, hyperexpression of the laminin receptor decoy mutant in the brain of transgenic mice did not influence cellular PrP expression levels, neither in the brain nor in splenic tissue. Statistical evaluations revealed that the levels of neither the diglycosylated nor the non-glycosylated form of PrP^c are significantly altered in the brain of transgenic compared to wild-type mice (Fig. 3a).

Scrapie-infected transgenic mice show a significantly prolonged incubation time and reveal a significantly reduced PrPres level in the brain at the terminal stage

To investigate whether expression of the laminin receptor decoy mutant prolongs the incubation time of scrapie-infected mice, intracerebral (i.c.) prion inoculation studies with transgenic (+/-) mice of the F3-generation expressing LRP102-295::FLAG and wild-type mice were performed. Six weeks old transgenic mice were intracerebrally inoculated with the Rocky Mountain Laboratories (RML) scrapie strain. The animals were sacrificed at the day when two of the TSE-relevant symptoms[28] were present over three consecutive days. This time point is defined as the terminal stage. The period from the day of prion inoculation until the day the mice were sacrificed is defined as incubation time plotted in the Kaplan-Meier curve (Fig. 4a). The incubation time of scrapie-infected transgenic mice expressing LRP102-295::FLAG (153±3 days, p-value < 0.05, student´s t-test, squares) was significantly prolonged in comparison to scrapie-infected wild-type mice (148±5 days, triangles) (Fig. 4a). The results in Figure 4b are mean values of 11 transgenic mice and 10 wild-type mice, respectively. Statistical evaluations of the incubation times according to the non-parametric Wilcoxon-test (p-value = 0.01367) and the log rank-test (p-value = 0.0215) confirmed that the reductions of the incubation times of scrapie-infected transgenic mice, hyper-expressing the LRP102-295::FLAG mutant, compared to wild-type mice are significant.

Brain homogenates of mice at the terminal stage of the disease were digested with Proteinase K (PK) and subjected to SDS-PAGE followed by Western blotting. The brains of scrapie-infected transgenic mice revealed a significant reduction of PK-resistant PrPres content by 71% (p-value < 0.05, student´s t-test) in comparison to scrapie-infected wild-type mice (average of eleven transgenic and six wild-type mice, analyzed three times by imunoblotting) (Fig. 5a, 5b). Additionally, total PrP-levels (without PK-digestion) of the

brains of scrapie-infected mice were assessed by immunoblotting (Fig. 5c) and densitometric quantification, revealing no difference between the brains of transgenic (set to 100%) and wild-type mice (99%, p-value > 0.05, student´s t-test) (Fig. 5d).

Discussion

Despite numerous attempts to prevent, treat or cure prion diseases, TSEs are still incurable. Medications are normally associated with side effects to a grater or lesser extent. Thus, great efforts have been made to develop other strategies e.g. targeting prion protein expression via RNA interference technology[29] or generation of transdominant negative PrP mutants[26] to reduce PrP^{Sc} formation in vivo. Here, we describe the successful generation of transgenic mice expressing a transdominant negative laminin receptor mutant LRP102-295::FLAG. Encompassing the extracellular domain only, the LRP mutant is secreted into the extracellular matrix where it traps PrP^{Sc} molecules, preventing them from binding and internalization[17]. To generate transgenic mice expressing LRP102-295::FLAG, the encoding vector was microinjected into pronuclei of mouse zygotes. Injection before the first doubling of the chromosomes is necessary to avoid mosaic expression of the transgene. Since fundamental prion propagation is thought to occur in the CNS, expression of the laminin receptor decoy mutant in the brain was aimed with the NSE-promoter. Indeed, LRP102-295::FLAG expression was achieved in the brain of hemizygous transgenic (+/-) mice, but not in the spleen, an organ of the lymphoreticular system (Fig. 2a). The ratio of LRP102-295::FLAG and endogenous LRP expression in hemizygous transgenic mice was calculated with approx. 1 to 1.8 (Fig. 2b and c). Homozygous transgenic mice might therefore express equal

amounts of the LRP-mutant and endogenous LRP, which might result in an increased decoy effect.

To further characterize the transgenic mice of the F3-generation, PrP^c levels in the brains and spleens were investigated and western blot data were quantified by densitometric analysis. Transgenic mice show no siginificant alterations in the PrP^c expression levels neither in the brain nor in splenic tissue in comparison to wild-type mice, suggesting that the expression of the LRP102-295::FLAG mutant does not influence PrP^c levels. The fact that the levels of neither the diglycosylated nor the non-glycosylated form of PrP^c are significantly altered in the brain of transgenic compared to wild-type mice suggests that LRP102-295::FLAG is not significantly impeding glycosylation and/or secretion of PrP.

To analyze the influence of laminin receptor mutant expression on disease progression in vivo, heterozygous transgenic mice (+/-) of the F3-generation, genotyped by PCR and proven to express LRP102-295::FLAG in the brain, were intracerebrally inoculated with the RML scrapie strain and monitored for TSE relevant symptoms[28]. The incubation time of scrapie-infected hemizygous transgenic mice (+/-) was slightly but significantly prolonged (153±3 days, p-value < 0.05) in comparison to scrapie-infected wild-type (FVB) mice (148±5 days).

Transgenic mice expressing LRP102-295::FLAG revealed a significant reduction of the PK-resistant PrP (PrPres) level in the brain by 71% compared to wild-type mice at the terminal stage (the time point mice were sacrificed when two of the TSE-relevant symptoms[28] appeared over three consecutive days).

The analysis of the total PrP content of brain-homogenates of scrapie-infected transgenic and wild-type mice without PK-digestion revealed no difference between the PrP-levels.

Assuming that only PK-digestion of PrP^{Sc} results in formation of PrPres, one might reason that PrPres levels after PK-digestion also represent PrP^{Sc} levels. However, one would expect that total PrP levels in the brains of scrapie-infected transgenic mice would be decreased in correlation to decreased PrPres. However, the fact that PrP^{c} levels in healthy mice and total PrP levels in scrapie infected mice remain unaltered upon transgenicity, may suggest that PK-resistance of PrP^{Sc} might be decreased in transgenic mice by a so far unknown mechanism. To definitely compare PrP^{Sc} levels of scrapie infected transgenic and wild-type mice, however, a PrP^{Sc}-specific antibody is required. Thus, expression of the LRP102-295::FLAG mutant in the brain of transgenic animals has a considerable effect on the reduction of the PrPres load in the terminal stage, but only a slight effect on the prolongation of the incubation time (5 days) in comparison to wild-type mice, suggesting that the PrP^{Sc} load does not necessarily correlate with disease progression or infectivity. Lasmezas and colleagues demonstrated that mice, infected with brain homogenates from BSE-infected cattle, reveal disease progression without depositing proteinase K-resistant PrP (PrPres)[30]. Furthermore, a rapid TSE transmission has been observed in mice, despite extremely low levels of disease-associated PrP[31]. Recently, high titers of TSE infectivity were associated with minimal levels of PrP^{Sc} in the brains of animals showing clinical and vacuolar signs of TSE[32]. Thus, to predict incubation times or life spans in vivo in TSEs, further analyses are necessary to elucidate the correlation of infectivity, deposition of disease-associated PrP, disease progression and other hallmarks of TSEs.

Our previous studies revealed that scrapie-infected rodents showed increased LRP levels in the brain in comparison to non-infected rodents[33], suggesting that increased

endogenous laminin receptor levels in the transgenic LRP102-295::FLAG expressing mice might compete with the secreted LRP102-295::FLAG for PrP^{Sc} binding. Increased LRP levels on the cell surface might reduce the decoy effect of the secreted mutant, trapping less infectious PrP molecules in the ECM, which might explain the only slight increase in incubation times of scrapie-infected transgenic LRP102-295::FLAG expressing mice. In contrast, the reduction of the PrPres levels in the brain of the transgenic mice at the terminal stage is considerable and accounts for a strong trapping effect, which might be even enforced in homozygous transgenic mice expressing LRP102-295::FLAG from both alleles. Our attempts, however, to establish a homozygous LRP102-295::FLAG expressing mouse line failed due to the infertility of homozygous transgenic mice (determined by back-crossing experiments), suggesting that expression of the LRP102-295::FLAG mutant from both alleles might lead to infertility. Beside the deficiency in reproduction, the transgenic mice showed non phenotypic alterations compared to wild-type mice and were clinically and physiologically normal.

In conclusion, the expression of a LRP decoy mutant in the brain, resulting in a significantly reduced PrP^{res} level in the terminal stage, concomitant with a significant prolonged incubation time, displays an alternative promising approach in TSE therapy.

Materials and Methods

Plasmid generation and microinjection

NheI and *NotI* restriction sites were used to subclone the sequence encoding for LRP102-295::FLAG[17] into the pCIneoNSE-asLRP plasmid[25] to generate the vector

pCIneoNSE_LRP102-295FLAG. The correctness of the plasmid sequence was verified by dideoxy sequencing. The vector pCIneoNSE_LRP102-295FLAG was linearized by digestion with the restriction enzymes *BglII* and *BamHI* (NEB), purified by gel extraction (Genomed) followed by microinjection into pronuclei of mouse zygotes (FVB) and transferred into oviducts of female recipient mice (NMRI). The offspring were routinely screened by PCR.

Genotyping of transgenic mice by polymerase chain reaction (PCR) analysis

Genomic DNA of mice was extracted from mouse tail tips using a Tissue DNA Mini Kit (PeqLab) according to the manufacturer`s instructions. PCR reaction of the genomic DNA was performed employing recombinant Taq Polymerase Reaction Kit (Invitrogen) and PCR products were analyzed by 1% agarose gel electrophoresis containing GelStar® Nucleic Acid Gel Stain (Lonza). The primers used for DNA amplification are directed against the backbone sequence of pCIneoNSE_LRP102-295FLAG, directly before and after the sequence encoding for LRP102-295FLAG (primer sequence forward: 5´ TCA ATT ACA GCT CTT AAG GCT A 3´; primer sequence backward: 5` TTA TCA TGT CTG CTC GAA GC 3`). As positive control to verify the correct fragment size of the PCR products of the genomic DNA, the plasmid pCIneoNSE_LRP102-295FLAG was employed. Genomic DNAs of tg and non-tg mice, respectively, served as positive and negative controls, respectively. A DNA-free sample verified a contamination-free PCR analysis.

Analysis of protein expression

To analyze the expression of LRP102-295::FLAG protein in the brain and spleen, organ homogenates from transgenic mice of the F3-generation were prepared and 100µg of total protein amount was subjected to 12% SDS-PAGE followed by blotting onto a polyvinylidenedifluoride (PVDF) membrane (Amersham). Membranes were blocked with 20% horse serum in PBS/0.2% Tween20 and probed with anti-FLAG M2 antibody (1:5000, Sigma) and anti-mouse IgG POD (1:5000, Sigma), stripped and reprobed with anti-β-actin (1:5000, Sigma) and anti-mouse IgG POD (1:5000, Sigma). For PrPres detection 200µg of total protein amount of each brain homogenate were adjusted in volume, digested with 20µg/ml PK for 30 minutes at 37°C, precipitated in 100% methanol and subjected to 12% SDS-PAGE. After blotting and blocking as described above the membranes were probed with 4H11 (1:1000, kindly provided by H. Schätzl) and anti-mouse IgG POD (1:5000, Sigma). Directly before PK-digestion 10µg of each sample were separated and analyzed for β-actin content as loading control as described above. Blots were developed using an enhanced chemiluminescence system (Perkin Elmer) and exposed on chemiluminescence films (RP New, CEA). Stripping of the membranes was performed for 30 minutes using stripping buffer (100 mM glycine, 1% SDS, 0.1% Nonidet P40; pH 2.2). For total PrP detection in scrapie-infected mice and for PrP^c detection in non-infected mice 100µg of total protein amount were treated as described above in the absence of PK-digestion. For endogenous LRP and LRP102-295::FLAG detection 100µg of total protein amount were analyzed by 12% SDS-PAGE, followed by incubation with the LRP/LR-specific antibody IgG1 iS18 [21] (1:10000) and the corresponding anti-human IgG POD (1:10000, Jackson Immunoresearch). After stripping, the membrane was reprobed for β-actin detection.

PrP^{Sc} inoculation of mice and monitoring

Six weeks old hemizygous transgenic mice expressing LRP102-295::FLAG (11 animals) and wild-type mice (FVB, 10 animals), respectively, were narcotized (ether inhalation) followed by intracerebral inoculation with 30µl of a 1% RML brain homogenate. Mice were monitored for the appearance of TSE-relevant symptoms [28] and the animals were sacrificed at the day they reveal two TSE-relevant symptoms over a period of three consecutive days.

Statistical analyses

Statistical analysis were performed employing a Student's t test with two tailed distribution and two-sample unequal variance. For the statistical evaluation of the incubation times, the non-parametric Wilcoxon-test and the log rank-test were performed additionally.

Acknowledgements

We thank Tina Hallas, Alessandro Tosi, Tamara Holy and Jennifer Hentrich for excellent technical assistance. We were grateful to Hermann Schätzl from the Institute of Virology of the Technical University Munich for kindly providing the 4H11 antibody. The work was supported by the Bundesministerium für Bildung und Forschung (grant: 01-KO-0514), the European Commission (grant: NoE-NeuroPrion FOOD-CT-2004-506579) and the Deutsche Forschungsgemeinschaft (grant: WE 2664/2-1). Animal experiments were approved by the Bavarian government (Az: 55.2-1-54-2531-45-05).

References

1. Aguzzi, A. & Weissmann, C. (1998). Prion diseases. *Haemophilia* **4**, 619-27.
2. Prusiner, S. B., Scott, M. R., DeArmond, S. J. & Cohen, F. E. (1998). Prion protein biology. *Cell* **93**, 337-48.
3. Weissmann, C. (1999). Molecular genetics of transmissible spongiform encephalopathies. *J Biol Chem* **274**, 3-6.
4. McKinley, M. P., Meyer, R. K., Kenaga, L., Rahbar, F., Cotter, R., Serban, A. & Prusiner, S. B. (1991). Scrapie prion rod formation in vitro requires both detergent extraction and limited proteolysis. *J Virol* **65**, 1340-51.
5. Fraser, H. & Dickinson, A. G. (1967). Distribution of experimentally induced scrapie lesions in the brain. *Nature* **216**, 1310-1.
6. Gauczynski, S., Peyrin, J. M., Haik, S., Leucht, C., Hundt, C., Rieger, R., Krasemann, S., Deslys, J. P., Dormont, D., Lasmezas, C. I. & Weiss, S. (2001). The 37-kDa/67-kDa laminin receptor acts as the cell-surface receptor for the cellular prion protein. *Embo J* **20**, 5863-75.
7. Hundt, C., Peyrin, J. M., Haik, S., Gauczynski, S., Leucht, C., Rieger, R., Riley, M. L., Deslys, J. P., Dormont, D., Lasmezas, C. I. & Weiss, S. (2001). Identification of interaction domains of the prion protein with its 37-kDa/67-kDa laminin receptor. *Embo J* **20**, 5876-86.
8. Gauczynski, S., Nikles, D., El-Gogo, S., Papy-Garcia, D., Rey, C., Alban, S., Barritault, D., Lasmezas, C. I. & Weiss, S. (2006). The 37-kDa/67-kDa laminin receptor acts as a receptor for infectious prions and is inhibited by polysulfated glycanes. *J Infect Dis* **194**, 702-9.
9. Morel, E., Andrieu, T., Casagrande, F., Gauczynski, S., Weiss, S., Grassi, J., Rousset, M., Dormont, D. & Chambaz, J. (2005). Bovine Prion Is Endocytosed by Human Enterocytes via the 37 kDa/67 kDa Laminin Receptor. *Am J Pathol* **167**, 1033-1042.
10. Ludewigs, H., Zuber, C., Vana, K., Nikles, D., Zerr, I. & Weiss, S. (2007). Therapeutic approaches for prion disorders. *Expert Rev Anti Infect Ther* **5**, 613-30.
11. Vana, K., Zuber, C., Nikles, D. & Weiss, S. (2007). Novel aspects of prions, their receptor molecules, and innovative approaches for TSE therapy. *Cell Mol Neurobiol* **27**, 107-28.
12. Vana, K., Zuber, C., Pflanz, H., Kolodiejczak, D., Zemora, G., Bergmann, A.-K. & Weiss, S. (2009). LRP/LR as an alternative promising target in therapy of prion diseases, Alzheimer's Disease and cancer. *Infectious Disorders - Drug Targets* **90**, 69-80.
13. Zuber, C., Ludewigs, H. & Weiss, S. (2007). Therapeutic approaches targeting the prion receptor LRP/LR. *Vet Microbiol* **123**, 387-93.
14. Zuber, C., Knackmuss, S., Rey, C., Reusch, U., Rottgen, P., Frohlich, T., Arnold, G. J., Pace, C., Mitteregger, G., Kretzschmar, H. A., Little, M. & Weiss, S. (2008). Single chain Fv antibodies directed against the 37 kDa/67 kDa laminin receptor as therapeutic tools in prion diseases. *Mol Immunol* **45**, 144-51.

15. Zuber, C., Mitteregger, G., Pace, C., Zerr, I., Kretzschmar, H. A. & Weiss, S. (2007). Anti-LRP/LR antibody W3 hampers peripheral PrPSc propagation in scrapie infected mice. *Prion* **1**, 207-212.
16. Zuber, C., Mitteregger, G., Schuhmann, N., Rey, C., Knackmuss, S., Rupprecht, W., Reusch, U., Pace, C., Little, M., Kretzschmar, H. A., Hallek, M., Büning, H. & Weiss, S. (2008). Delivery of single-chain antibodies scFvs directed against the 37 kDa/67 kDa laminin receptor into murine brain via recombinant Adeno-associated viral vectors for prion disease gene therapy. *J Gen Virol* **89**, 2054-2060.
17. Vana, K. & Weiss, S. (2006). A trans-dominant negative 37kDa/67kDa laminin receptor mutant impairs PrP(Sc) propagation in scrapie-infected neuronal cells. *J Mol Biol* **358**, 57-66.
18. Leucht, C., Simoneau, S., Rey, C., Vana, K., Rieger, R., Lasmezas, C. I. & Weiss, S. (2003). The 37 kDa/67 kDa laminin receptor is required for PrP(Sc) propagation in scrapie-infected neuronal cells. *EMBO Rep* **4**, 290-5.
19. Pflanz, H., Vana, K., Mitteregger, G., Pace, C., Messow, D., Nikles, D., Kretzschmar, H. A. & Weiss, S. (2009). Microinjection of lentiviral vectors directed against laminin receptor precursor mRNA prolongs the pre-clinical phase in scrapie-infected mice. *J Gen Virol* **90**, 269-274.
20. Susantad, T. & Smith, D. R. (2008). siRNA-mediated silencing of the 37/67-kDa high affinity laminin receptor in Hep3B cells induces apoptosis. *Cell Mol Biol Lett* **13**, 452-64.
21. Zuber, C., Knackmuss, S., Zemora, G., Reusch, U., Vlasova, E., Diehl, D., Mick, V., Hoffmann, K., Nikles, D., Frohlich, T., Arnold, G. J., Brenig, B., Wolf, E., Lahm, H., Little, M. & Weiss, S. (2008). Invasion of tumorigenic HT1080 cells is impeded by blocking or downregulating the 37-kDa/67-kDa laminin receptor. *J Mol Biol* **378**, 530-9.
22. Simoneau, S., Haik, S., Leucht, C., Dormont, D., Deslys, J. P., Weiss, S. & Lasmezas, C. (2003). Different isoforms of the non-integrin laminin receptor are present in mouse brain and bind PrP. *Biol Chem* **384**, 243-6.
23. Baloui, H., von Boxberg, Y., Vinh, J., Weiss, S., Rossier, J., Nothias, F. & 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**, 2605-16.
24. Baloui, H., Stettler, O., Weiss, S., Nothias, F. & von Boxberg, Y. (2009). Upregulation in rat spinal cord microglia of the nonintegrin laminin receptor 37 kDa-LRP following activation by a traumatic lesion, or peripheral injury. *J Neurotrauma* **26**, 195-207.
25. Leucht, C., Vana, K., Renner-Muller, I., Dormont, D., Lasmezas, C. I., Wolf, E. & 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**, 81-5.
26. Perrier, V., Kaneko, K., Safar, J., Vergara, J., Tremblay, P., DeArmond, S. J., Cohen, F. E., Prusiner, S. B. & Wallace, A. C. (2002). Dominant-negative inhibition of prion replication in transgenic mice. *Proc Natl Acad Sci U S A* **99**, 13079-84.

27. Toupet, K., Compan, V., Crozet, C., Mourton-Gilles, C., Mestre-Frances, N., Ibos, F., Corbeau, P., Verdier, J. M. & Perrier, V. (2008). Effective gene therapy in a mouse model of prion diseases. *PLoS ONE* **3**, e2773.
28. Sethi, S., Lipford, G., Wagner, H. & Kretzschmar, H. (2002). Postexposure prophylaxis against prion disease with a stimulator of innate immunity. *Lancet* **360**, 229-30.
29. Pfeifer, A., Eigenbrod, S., Al-Khadra, S., Hofmann, A., Mitteregger, G., Moser, M., Bertsch, U. & Kretzschmar, H. (2006). Lentivector-mediated RNAi efficiently suppresses prion protein and prolongs survival of scrapie-infected mice. *J Clin Invest* **116**, 3204-10.
30. Lasmezas, C. I., Deslys, J. P., Robain, O., Jaegly, A., Beringue, V., Peyrin, J. M., Fournier, J. G., Hauw, J. J., Rossier, J. & Dormont, D. (1997). Transmission of the BSE agent to mice in the absence of detectable abnormal prion protein. *Science* **275**, 402-5.
31. Manson, J. C., Jamieson, E., Baybutt, H., Tuzi, N. L., Barron, R., McConnell, I., Somerville, R., Ironside, J., Will, R., Sy, M. S., Melton, D. W., Hope, J. & Bostock, C. (1999). A single amino acid alteration (101L) introduced into murine PrP dramatically alters incubation time of transmissible spongiform encephalopathy. *Embo J* **18**, 6855-64.
32. Barron, R. M., Campbell, S. L., King, D., Bellon, A., Chapman, K. E., Williamson, R. A. & Manson, J. C. (2007). High titers of transmissible spongiform encephalopathy infectivity associated with extremely low levels of PrPSc in vivo. *J Biol Chem* **282**, 35878-86.
33. Rieger, R., Edenhofer, F., Lasmezas, C. I. & Weiss, S. (1997). The human 37-kDa laminin receptor precursor interacts with the prion protein in eukaryotic cells. *Nat Med* **3**, 1383-8.

Figure legends

Fig. 1. (a) PCR analysis of genomic DNA derived from a founder animal (TgN(NSE_LRP102-295::FLAG)3) and an age-matched wild-type mouse in comparison with the plasmid pCIneoNSE_LRP102-295FLAG. A 711bp DNA fragment was visualized by 1% agarose gel electrophoresis. Fragment size markers in basepairs (bp) are indicated on the left. (b) PCR analysis of genomic DNA of mice of the F3-generation derived from the founder animal (TgN(NSE_LRP102-295::FLAG)3) (Fig. 1a). The vector pCIneoNSE_LRP102-295FLAG served as positive control, genomic DNAs of tg mice of the F2-generation and of non-tg mice from another litter of the F3-generation, respectively, served as positive and negative controls, respectively. A DNA-free sample (H_2O) was used as a control. Fragment size markers in basepairs (bp) are indicated on the right.

Fig. 2. Transgenic hemizygous (+/-) mice of the F3 generation express LRP102-295::FLAG in the brain. (a) Representative western blot analysis of brain and spleen homogenates of transgenic mice of the F3-generation and wild-type (FVB) mice, matching in age. Using an anti-FLAG M2 antibody, LRP102-295::FLAG expression was monitored in the brain and spleen of transgenic mice and wild-type mice (upper panel). LRP102-295::FLAG revealed a molecular weight of approx. 32kDa[17]. An anti-β-actin antibody was used to verify equal amounts of loaded protein (lower panel). Molecular weight markers in kilodalton (kDa) are indicated on the left. (b) Representative western blot analysis of brain homogenates of wild-type (FVB) mice and transgenic mice of the F3-generation. Endogenously expressed LRP and LRP102-295::FLAG was detected

using IgG1 iS18 directed against the C-terminus of LRP/LR [21]. Wild-type mice show one specific band for endogenous LRP and transgenic mice reveal an additional band for LRP102-295::FLAG. The lower panel represents β-actin detection to verify equal protein amounts. Molecular weight markers in kilodalton (kDa) are indicated on the left. (c) Densitometric analysis of western blot data of endogenous LRP and LRP102-295::FLAG of Fig. 2B using the NIH ImageJ software. Endogenous LRP-levels do not differ significantly between wild-type (117% ± 9%) and transgenic mice (100% ± 7%, p-value > 0.05, student´s t-test, LRP-level set to 100%). The ratio of LRP102-295::FLAG to endogenous LRP in the brains of transgenic mice is 1 to 1.77. Results are calculated from three analyses of three mice each.

Fig. 3. PrP^c expression levels in brain and spleen, respectively, are similar in wild-type (FVB) and hemizygous transgenic mice (+/-). (a) Representative western blot analysis of brain and spleen homogenates of transgenic mice of the F3-generation and wild-type (FVB) mice, matching in age. PrP^c was detected using anti PrP-antibody 4H11. Equally loaded amounts of protein were verified by β-actin detection. Molecular weight markers in kilodalton (kDa) are indicated on the left. (b) Densitometric analysis of western blot data of PrP^c of Fig. 3A using the NIH ImageJ software. Splenic PrP^c levels do not differ significantly between transgenic (100% ± 11%, PrP^c level set to 100%) and wild-type mice (82% ± 10%, p-value > 0.05, student´s t-test). Similarly, PrP^c levels in the brains of transgenic (100% ± 46%, PrP^c level set to 100%) and wild-type mice (95% ± 21%, p-value > 0.05, student´s t-test) do not differ significantly. Please note that the unglycosylated and diglycosylated forms of PrP have been added and statistically

evaluated. Levels of the diglycosylated and unglycosylated forms of PrP^c (analyzed separately) from the brain of transgenic and non-transgenic mice do not differ significantly. Results are calculated from three analyses of three mice each.

Fig. 4. (a) Kaplan-Meier curve of scrapie-infected hemizygous (+/-) transgenic mice of the F3-generation and wild-type mice. Scrapie-infected animals were sacrificed after showing two TSE-relevant symptoms over a period of three days. The incubation times of transgenic mice expressing LRP102-295::FLAG (squares), and wild-type mice (triangles) are displayed on the abscissa as days post scrapie inoculation. Numbers of mice are displayed as percentage of total mice number on the ordinate. Incubation time is defined as the time period from the day of RML inoculation until the animals show two of the TSE-relevant symptoms according to[28] over a period of three consecutive days. This time point is defined as the terminal stage. (b) Table displaying mean values of the incubation time in days post scrapie inoculation (dpi). Statistical evaluations of the reductions of incubation time of transgenic and wild-type mice revealed p-values of 0.027 (student´s t-test), 0.01367 (non-parametric Wilcoxon-test) and 0.0215 (log rank-test).

Fig. 5. Western blot analysis of brain-homogenates of scrapie-infected hemizygous (+/-) transgenic mice of the F3 generation and wild-type mice (FVB) at the terminal stage of disease. (a) PK-digested brain-homogenates of scrapie-infected transgenic and wild-type mice were analyzed for PrPres with the anti-PrP antibody 4H11 (upper panel). Prior to PK-digestion 10µg of each sample was separated and analyzed for equal amounts of

protein employing an anti-β-actin antibody (lower panel). Molecular weight markers in kilodalton (kDa) are indicated on the left. (b) Densitometric quantification of the western blot data of Fig. 4a using the NIH ImageJ software. PK-resistant PrP in the brains of scrapie-infected transgenic mice was significantly reduced by 71% (p-value < 0.05; student´s t-test) in comparison to wild-type mice (set to 100%; average of eleven transgenic and six wild-type mice, analyzed three times by imunoblotting) (Fig. 5a, 5b). (c) Brain-homogenates of scrapie-infected transgenic and wild-type mice were analyzed for total PrP content without PK-digestion with the anti-PrP antibody 4H11. β-actin detection verified equal amounts of protein. Molecular weight markers in kilodalton (kDa) are indicated on the left. (d) Densitometric quantification of the western blot data of Fig. 5C using the NIH ImageJ software. The total PrP content is equal in the brains of scrapie-infected transgenic (100% ± 6%, PrP-level set to 100%) and wild-type mice (99% ± 14%, p-value > 0.05, student´s t-test).

Figure 1

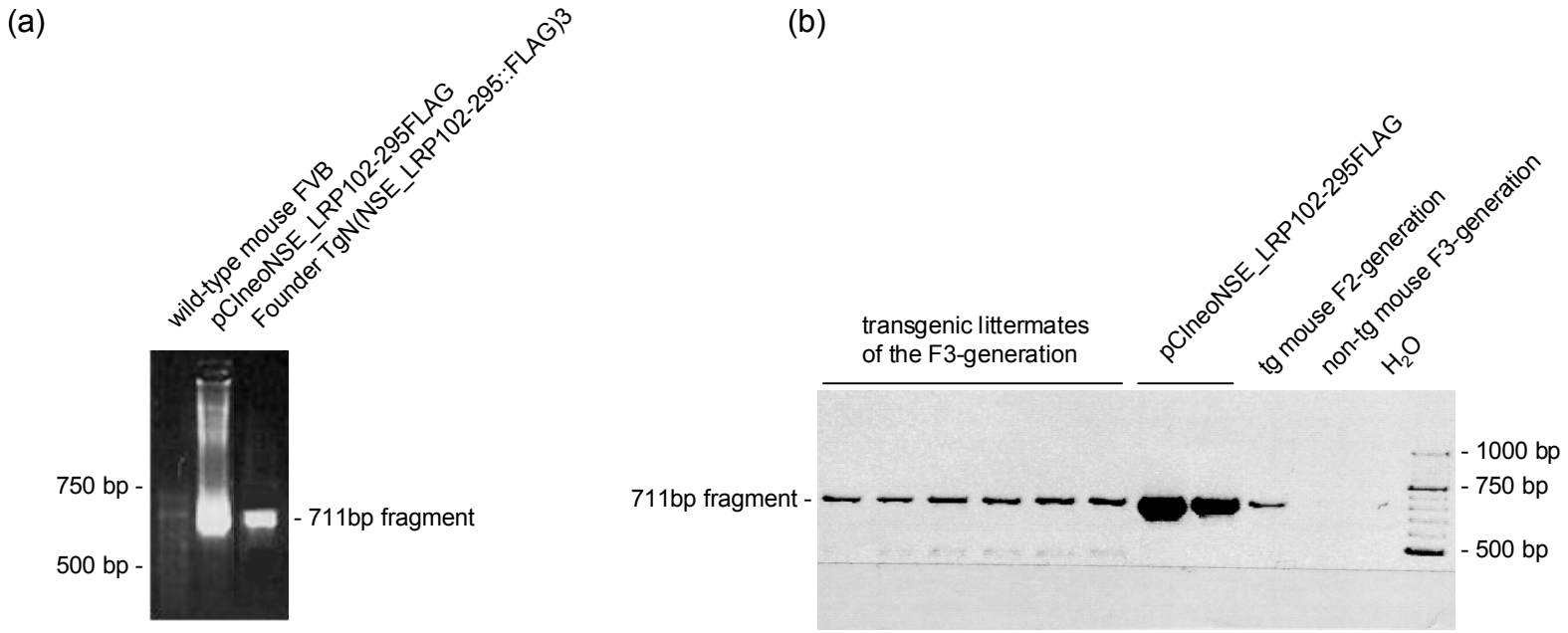
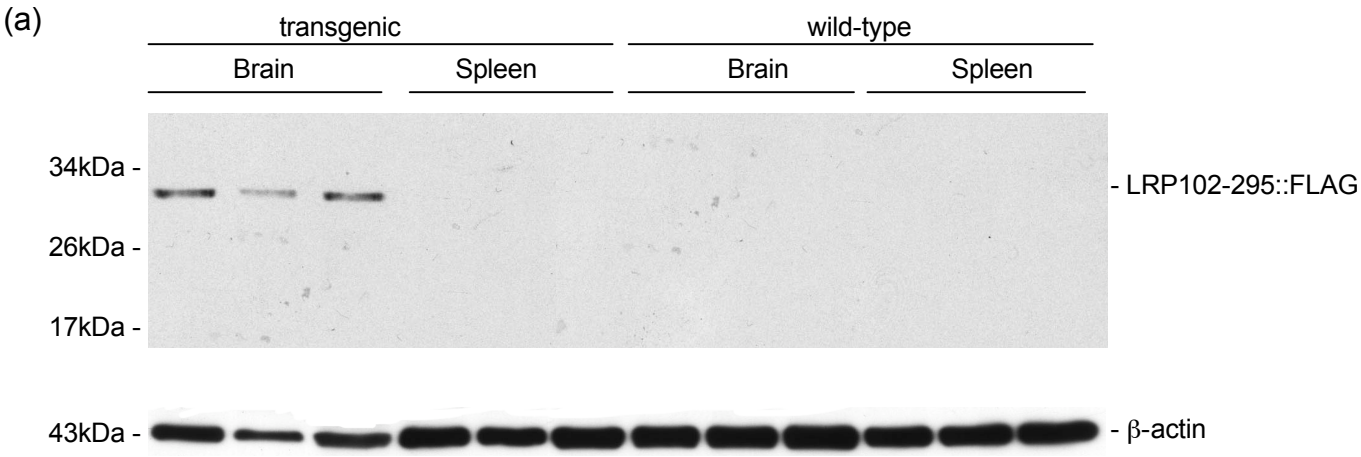
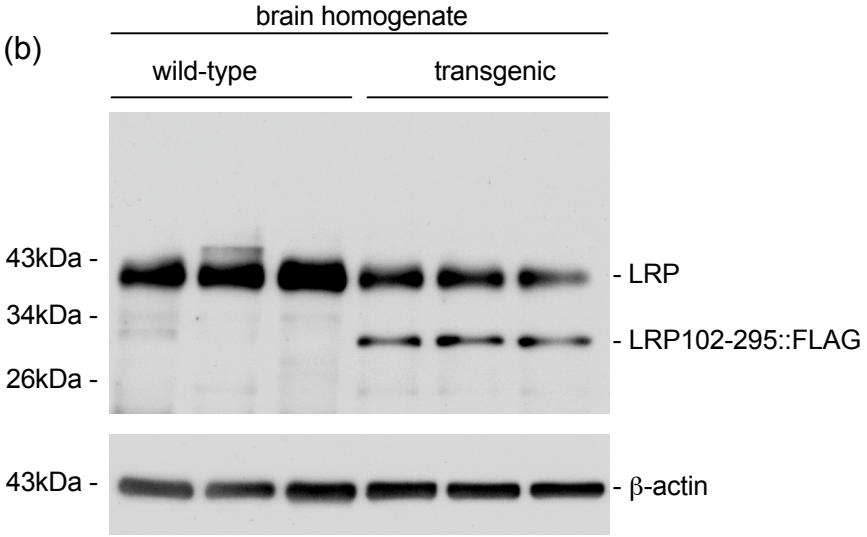

(a)
wild-type mouse FVB
pCIneoNSE_LRP102-295FLAG
Founder TgN(NSE_LRP102-295::FLAG)3
750 bp -
500 bp -
- 711bp fragment
(b)
transgenic littermates
of the F3-generation
pCIneoNSE_LRP102-295FLAG
tg mouse F2-generation
non-tg mouse F3-generation
H2O
711bp fragment -
- 1000 bp
- 750 bp
- 500 bp

Figure 2


(a)
transgenic
wild-type
Brain
Spleen
Brain
Spleen
34kDa -
26kDa -
17kDa -
- LRP102-295::FLAG
43kDa -
- β-actin


(b)
brain homogenate
wild-type
transgenic
43kDa -
34kDa -
26kDa -
- LRP
- LRP102-295::FLAG
43kDa -
- β-actin

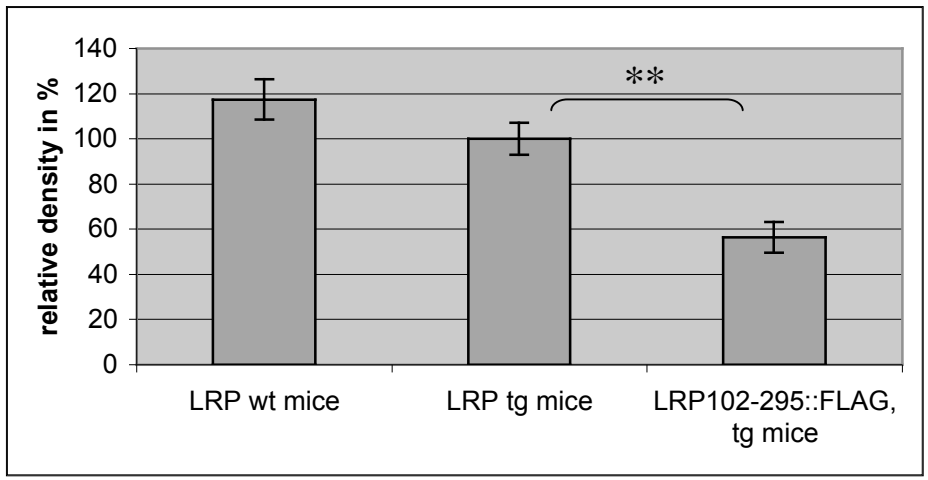
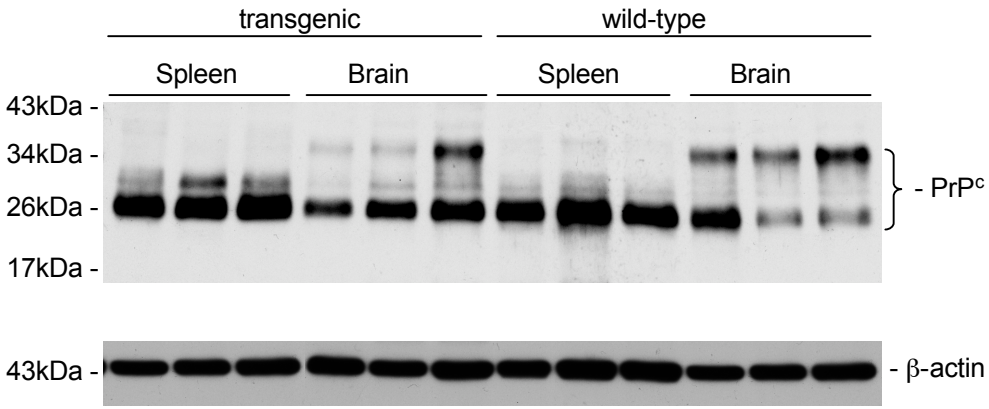

(c)
relative density in %
140
120
100
80
60
40
20
0
**
LRP wt mice
LRP tg mice
LRP102-295::FLAG, tg mice

Figure 3

(a)


transgenic
wild-type
Spleen
Brain
Spleen
Brain
43kDa -
34kDa -
26kDa -
17kDa -
- PrPc
43kDa -
- β-actin

(b)

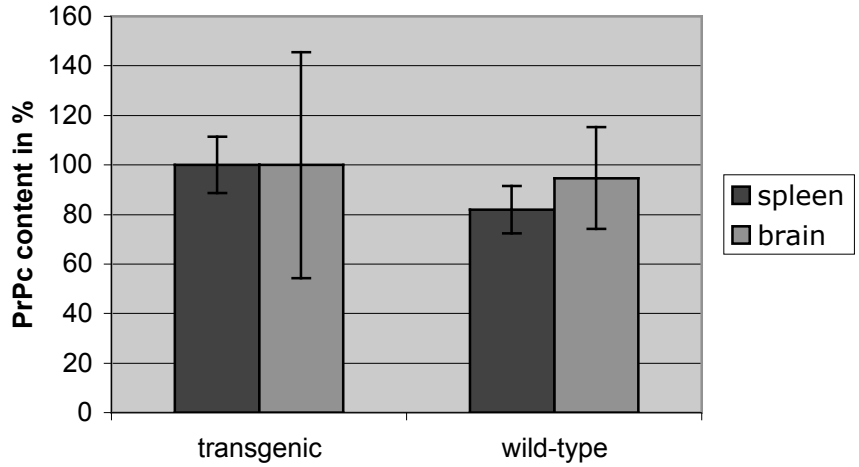
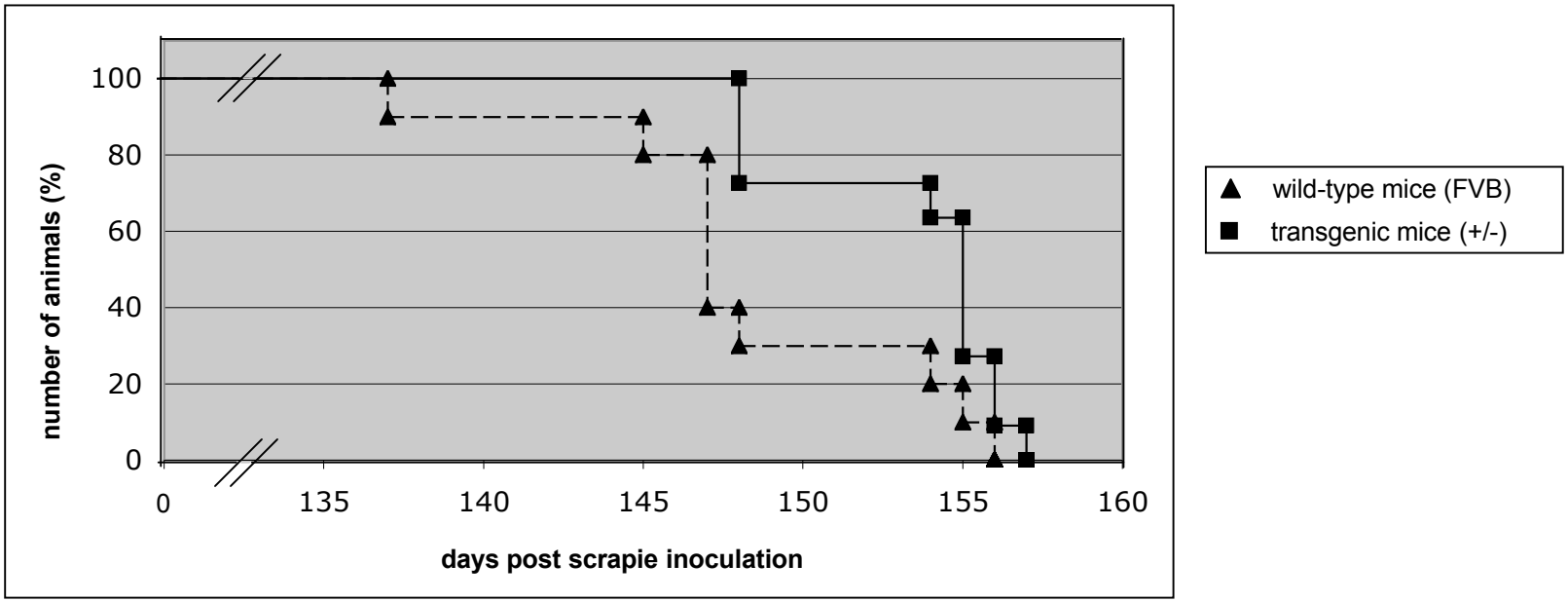

160
140
120
100
80
60
40
20
0
PrPc content in %
transgenic
wild-type
spleen
brain

Figure 4

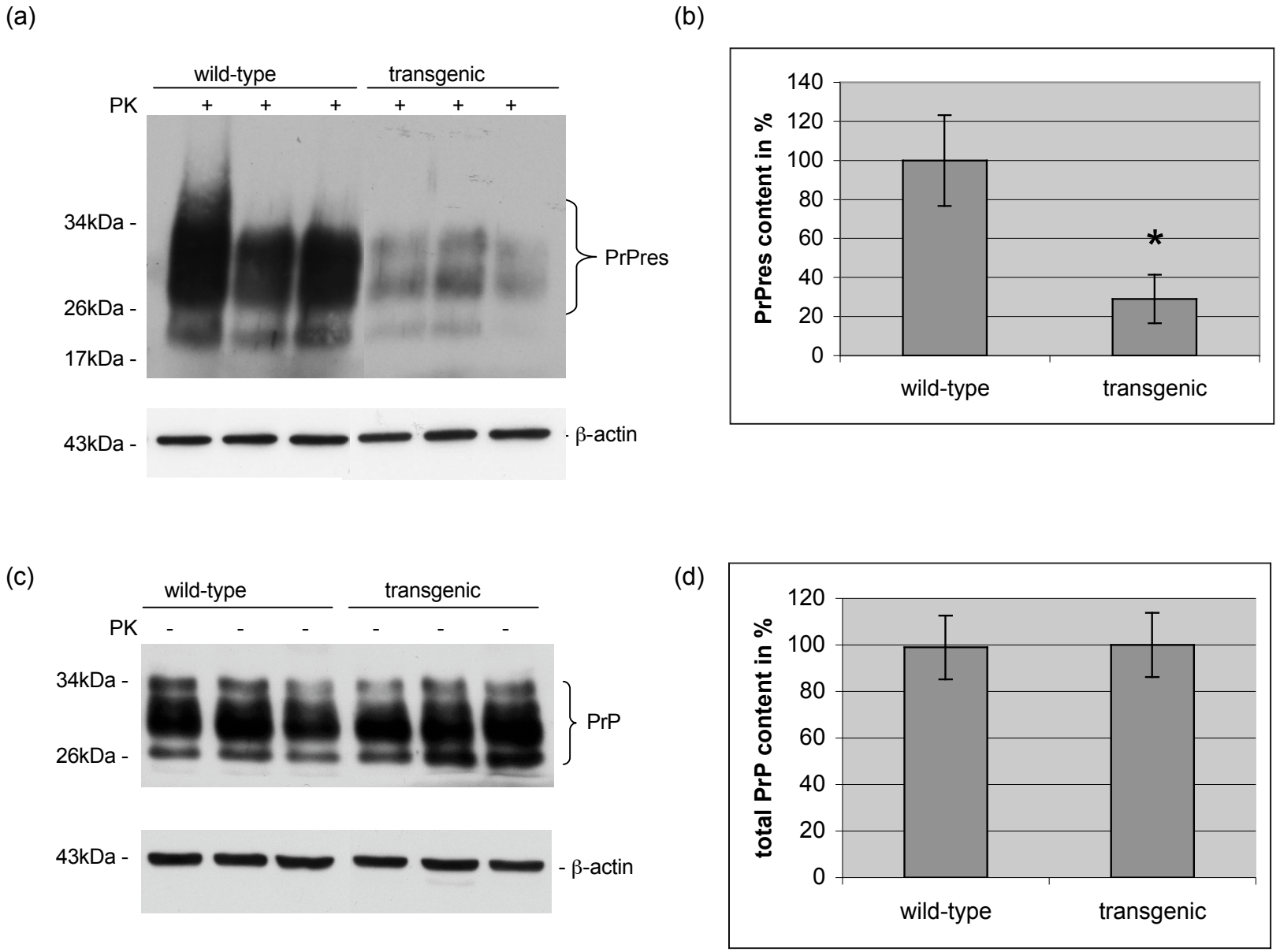
(a)



(b)

	no. of mice	mean incubation time (dpi)
wild-type mice (FVB)	10	148 ± 5
transgenic mice (+/-)	11	153 ± 3 (p< 0,05)

Figure 5


(a)
wild-type
transgenic
PK
+ + + + + +
34kDa -
26kDa -
17kDa -
PrPres
43kDa -
β-actin
(b)
PrPres content in %
140
120
100
80
60
40
20
0
*
wild-type
transgenic
(c)
wild-type
transgenic
PK
- - - - - -
34kDa -
26kDa -
PrP
43kDa -
β-actin
(d)
total PrP content in %
120
100
80
60
40
20
0
wild-type
transgenic

Chapter V

Microinjection of lentiviral vectors expressing small interfering RNAs directed against laminin receptor precursor mRNA prolongs the pre-clinical phase in scrapie-infected mice

published as:

Pflanz, H., Vana, K., Mitteregger, G., Pace, C. Messow, D., Sedlaczek, C., Nikles, D., Kretzschmar, H. A. and Weiss, S. F., Microinjection of lentiviral vectors expressing small interfering RNAs directed against laminin receptor precursor mRNA prolongs the pre-clinical phase in scrapie-infected mice. *J Gen Virol* 2009. 1: 269-274.

Journal of General Virology (2009), **90**, 269–274 DOI 10.1099/vir.0.004168-0

Short Communication

Microinjection of lentiviral vectors expressing small interfering RNAs directed against laminin receptor precursor mRNA prolongs the pre-clinical phase in scrapie-infected mice

Heike Pflanz,[1]† Karen Vana,[1]† Gerda Mitteregger,[2]† Claudia Pace,[2] Diana Messow,[1] Cilli Sedlaczek,[1] Daphne Nikles,[1] Hans A. Kretzschmar[2] and Stefan F. T. Weiss[1]

Correspondence
Stefan Weiss
weiss@lmb.uni-muenchen.de

[1]Laboratorium für Molekulare Biologie - Genzentrum - Institut für Biochemie der LMU München, Feodor-Lynen Str. 25, D-81377 München, Germany

[2]Zentrum für Neuropathologie und Prionforschung der LMU München, Feodor-Lynen-Str. 23, D-81377 München, Germany

We examined therapeutic *in vitro* and *in vivo* approaches using lentivirus-based packaging of small interfering RNAs (siRNAs) targeting the non-integrin laminin receptor mRNA for treatment and prevention of prion disorders. Transfection of N2aSc$^+$ cells with recombinant plasmids expressing three different siRNAs, significantly reduced both the LRP (laminin receptor precursor) and PrP^{Sc} levels by approximately 40–60 %. Stereotactic intracerebral microinjection of recombinant lentiviral vectors LVsiRNA-LRP 7 and 9 into the cortex of C57BL/6 wild-type mice resulted in a significant reduction of the LR levels in the cortex 15 days post-injection by 62 and 82 %, respectively. Intracerebral RML inoculation of C57BL/6 mice after microinjection with recombinant lentiviral vector LVsiRNA-LRP 7 into the hippocampus resulted in a significant reduction of both LRP and PrP^{Sc} levels by 36 and 41 %, respectively, concomitant with a significant prolongation of the pre-clinical phase. Lentiviral vectors expressing siRNAs targeting LRP mRNA represent a novel delivery system for the treatment of transmissible spongiform encephalopathies.

Received 21 May 2008
Accepted 8 September 2008

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 aetiology of TSEs (Prusiner *et al.*, 1990). Since prion diseases are lethal disorders, great endeavours have been undertaken to develop an efficient anti-prion therapy (for reviews see Ludewigs *et al.*, 2007; Trevitt & Collinge, 2006; Weissmann & Aguzzi, 2005).

The 37/67 kDa laminin receptor (LRP/LR) was identified as the receptor for prions (Gauczynski *et al.*, 2006, 2001b) and is localized on the cell surface, in the cytoplasm and the nucleus, respectively (for reviews see Gauczynski *et al.*, 2001a; Ludewigs *et al.*, 2007; Vana *et al.*, 2007, 2008, Zuber *et al.*, 2007a). Furthermore, LRP/LR colocalizes with PrP on the cell surface (Gauczynski *et al.*, 2001b) and in the perinuclear compartment (Nikles *et al.*, 2008). LRP/LR binding to laminin, elastin and carbohydrates accounts for its fundamental role in cell adhesion, cell movement and growth (for review see Gauczynski *et al.*, 2001a; Rieger *et al.*, 1999). LRP/LR plays an important role in the invasive and metastatic potential of neoplastic cells (Givant-Horwitz *et al.*, 2005; Landowski *et al.*, 1995) and tumour angiogenesis (Tanaka *et al.*, 2000) (for reviews see Castronovo, 1993; Menard *et al.*, 1997; Vana *et al.*, 2008). Blocking or downregulating LRP/LR hampers the invasive potential of tumorigenic fibrosarcoma cells, by blocking the laminin-1–LRP/LR interaction on the cell surface (Zuber *et al.*, 2008b). LRP/LR further supports maintenance of nuclear structures (Kinoshita *et al.*, 1998), supports the translation process in the cytosol (Auth & Brawerman, 1992) and plays a critical role in maintaining cell viability by preventing apoptosis (Susantad & Smith, 2008). On the cell surface LRP/LR acts as a receptor for viruses such as adeno-associated virus (AAV), dengue virus and alpha-viruses (for reviews see Ludewigs *et al.*, 2007; Vana *et al.*, 2007, 2008; Zuber *et al.*, 2007a). However, the LRP/LR

†These authors contributed equally to this work.

A figure showing the siRNA target sequences within the murine LRP mRNA is available with the online version of this paper.

polymorphism seems to be enigmatic. The 37 kDa LRP is thought to be the precursor of the 67 kDa high affinity laminin receptor. In mammalian cells, both LRP and LR are present in plasma membrane fractions (Gauczynski *et al.*, 2001a). In mouse brain both isoforms exist (Simoneau *et al.*, 2003). In adult rat brain the LR represents the major isoform, whereas LRP is expressed in the post-natal central nervous system (Baloui *et al.*, 2004). LRP is upregulated in spinal cord microglia after activation by a traumatic lesion or peripheral injury (Baloui *et al.*, 2008). The identification of LRP/LR as the receptor for prions conducts the idea of an alternative strategy for a TSE therapy. Application of either LRP-specific small interfering RNAs (siRNAs), the polyclonal anti-LRP antibody W3 (Leucht *et al.*, 2003) as well as a *trans*-dominant-negative LRP mutant (Vana & Weiss, 2006) interferes with PrP^{Sc} propagation *in vitro*. Passive immunotransfer of W3 into scrapie-infected mice significantly reduced the peripheral PrP^{Sc} level concomitant with a prolongation of survival (Zuber *et al.*, 2007b). Delivery of single-chain (sc) Fv antibodies directed against LRP/LR into mice by either passive immunotransfer (Zuber *et al.*, 2008a) or microinjection of recombinant AAV (Zuber *et al.*, 2008c) both resulted in a significant reduction of peripheral PrP^{Sc} propagation.

A series of chemical compounds have been identified as therapeutic agents efficiently interfering with PrP^{Sc} propagation. Among these, only some are efficient both *in vitro* and *in vivo* (for review see Ludewigs *et al.*, 2007). Gene therapy approaches evolved 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.

RNA interference (RNAi) technology has been described as one of the most powerful systems to knock-down an endogenous gene at the post-transcriptional level (Tuschl *et al.*, 1999). siRNA-mediated gene transfer by lentiviral vectors leads to a long-lasting silencing effect due to an integration of lentiviral DNA into the genome of the target cells (Naldini *et al.*, 1996a).

In this study, human immunodeficiency virus (HIV)-derived vectors that enable the expression of short hairpin RNA (shRNA) directed against murine LRP mRNA were generated. To examine the efficiency of siRNA target sequences within the LRP mRNA (Supplementary Fig. S1 available in JGV Online), transfections of siRNA-encoding plasmids (pENTR) were performed using chronically scrapie-infected mouse neuronal cells ($N2aSc^+$) (Fig. 1). Cells were cultured in Dulbecco's modified Eagle's medium with GlutaMax-I (Gibco) containing 10 % fetal calf serum, 1 × minimal essential medium non-essential amino acids, 100 U penicillin ml^{-1}, 100 µg streptomycin ml^{-1} and 0.5 mg geneticin ml^{-1} at 37 °C in 5 % CO_2. Post-transfection (72 h), cell lysates were subjected to

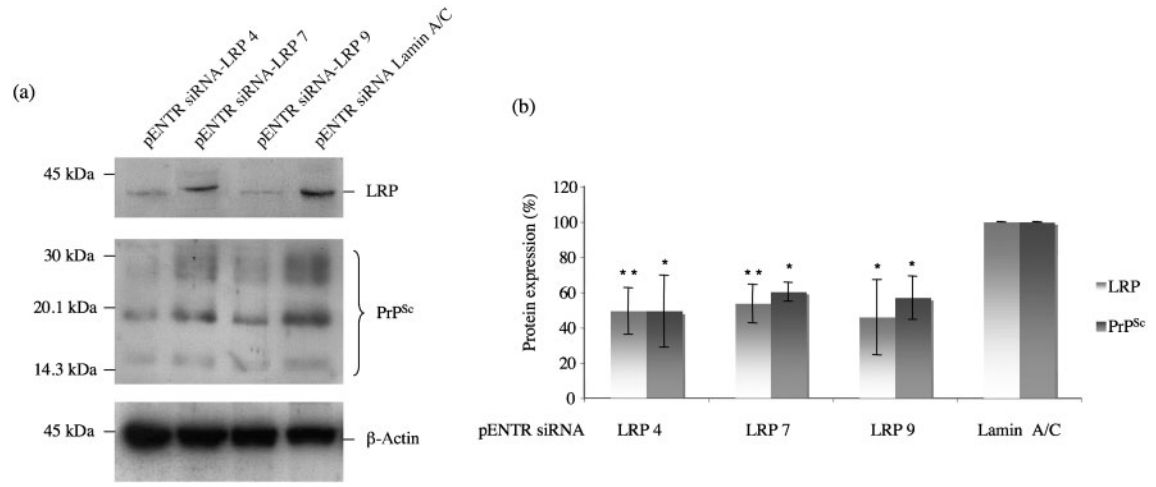


Fig. 1. Influence of pENTR vectors encoding LRP-specific siRNAs on both the LRP/LR level and PrP^{Sc} propagation in scrapie-infected neuronal cells. (a) Post-transfection (72 h) crude cell lysates of $N2aSc^+$ cells were subjected to SDS-PAGE following Western blotting. Cellular LRP/LR levels were detected using the single-chain anti-LRP antibody N3 (upper panel). As loading control anti-β-actin antibody (Sigma) was used (lower panel). PrP^{Sc} detection was carried out in proteinase K-digested cell lysates using anti-PrP SAF83 antibody (middle panel). Molecular mass markers in kilodalton (kDa) are indicated on the left. (b) Quantitative analysis using the NIH Image software revealed reduced endogenous LRP levels by 50 % for pENTR siRNA-LRP 4- ($P<0.005$), 47 % for pENTR siRNA-LRP 7- ($P<0.005$) and 54 % for pENTR siRNA-LRP 9- ($P<0.05$) transfected cells, respectively, in comparison to pENTR siRNA-Lamin A/C-transfected cells (LRP level set to 100 %). PrP^{Sc} levels were reduced by 50 % for pENTR siRNA-LRP 4- ($P<0.05$), 40 % for pENTR siRNA-LRP 7- ($P<0.05$) and 43 % for pENTR siRNA-LRP 9- ($P<0.05$) transfected cells, respectively, in comparison to pENTR siRNA-Lamin A/C-transfected cells (PrP^{Sc} level set to 100 %). The results of three individual experiments are shown and expressed as percentages of control levels ± SD.

SDS-PAGE and Western blotting. Employment of the anti-LRP single-chain antibody N3 predominantly recognizing the 37 kDa LRP form (Zuber *et al.*, 2008a) and the PrP-specific SAF83 antibody revealed that both LRP and PrP^{Sc} levels were significantly reduced, when using pENTR siRNA-LRP 4, 7 and 9 comparing these with the control pENTR siRNA-Lamin A/C (Fig. 1a, upper and middle panel). Quantitative analysis (for all quantitative analyses the Student's *t* test was used) of three independent experiments revealed a significant reduction of the LRP and PrP^{Sc} content by 50 % with pENTR siRNA-LRP 4, by 47/40 % using pENTR siRNA-LRP 7 and by 54/43 % using pENTR siRNA-LRP 9, respectively (Fig. 1b), confirming an important role for LRP in prion propagation.

Using the Block-it Lentiviral RNAi Expression System (Invitrogen), lentiviral RNAi vectors encoding siRNAs directed against the LRP mRNA were generated according to the manufacturer's instructions. After integration of lentiviral vector DNAs LVsiRNA-LRP 7 and 9 into the genome of mouse neuroblastoma cells (N2a) (data not shown), Western blot analysis of cell lysates revealed a significant LRP/LR downregulation when comparing N2a cells with integrated LVsiRNA-Lamin A/C DNA (data not shown).

To investigate the effect of the LRP-specific siRNAs *in vivo*, lentiviral particles [1×10^4 transducing units (TU) per mouse] were microinjected via a stereotactic device into the cortex of 6-week-old anaesthetized C57BL/6 mice (four mice per group) using a Hamilton syringe connected with a 27-gauge needle with a flux of 1 µl min^{-1}. Cortex samples (10 % homogenates) were prepared 15 days post-injection, followed by Western blot analysis of the LRP/LR levels (Fig. 2a). Quantitative analysis revealed a significant reduction of the cortical LR level by 62 and 82 %, respectively, after microinjection of lentiviral vectors LVsiRNA-LRP 7 and 9, respectively, compared with mice treated with LVsiRNA-Lamin A/C. Animals treated with LVsiRNA-LRP 4 showed a not significant reduction of the LR level by 45 % ($P<0.5$) (Fig. 2b), suggesting a diminished effectiveness *in vivo*.

Due to the significant LRP-specific silencing effects of LVsiRNA-LRP 7 and 9, respectively, both lentiviral vectors were selected for further studies in scrapie-infected mice. Two weeks after microinjection of the lentiviral particles (2.1×10^6 TU per mouse) into the hippocampus of the right hemisphere, mice were inoculated with 20 µl 1 % RML brain homogenate into the same brain region. To investigate whether lentiviral particles expressing siRNAs directed against LRP mRNA have the capacity to influence the etiopathology of prion diseases, mice were monitored for the occurrence of TSE-relevant symptoms (Sethi *et al.*, 2002) (Fig. 3a). Statistical evaluation using Kaplan–Meier survival curves demonstrated a significant prolongation of the pre-clinical phase in mice microinjected with LVsiRNA-LRP 7 (145 ± 0 days, $P<0.005$) and LVsiRNA-LRP 9 (143 ± 3 days, $P<0.05$), respectively, in comparison

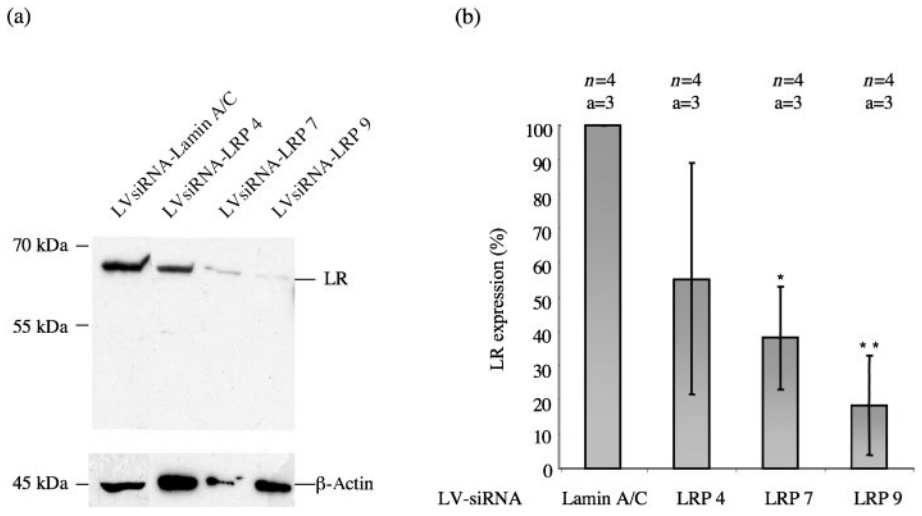
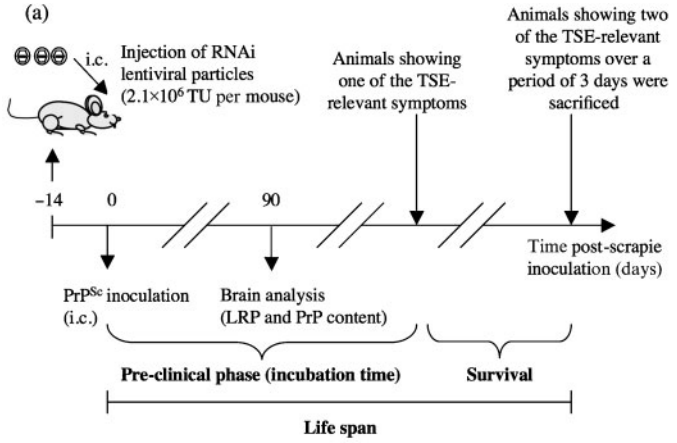


Fig. 2. *In vivo* effect of lentiviral particles expressing siRNAs directed against the LRP mRNA. (a) Lentiviral particles encoding siRNAs targeting LRP mRNA were microinjected into the cortex of C57BL/6 mice. Cortex homogenates (10 %) were prepared 15 days post-injection and analysed by SDS-PAGE followed by Western blotting. Single-chain antibody scFv S18 (Zuber *et al.*, 2008a) was used for LR detection (upper panel) and equally loaded protein amounts were verified with the anti-β-actin antibody (Sigma) (lower panel). Molecular mass markers in kilodalton (kDa) are indicated on the left. (b) Quantification of the Western blot signals revealed reduced LR levels for LVsiRNA-LRP 4-, LVsiRNA-LRP 7- and LVsiRNA-LRP 9-treated animals by 45 ($P<0.5$), 62 ($P<0.05$) and 82 % ($P<0.005$), respectively, in comparison to siRNA Lamin A/C-treated animals (LR level set to 100 %). Experiments were performed in triplicate (four animals per group) and results were expressed as percentages of control levels ± SD. a=number of animals.

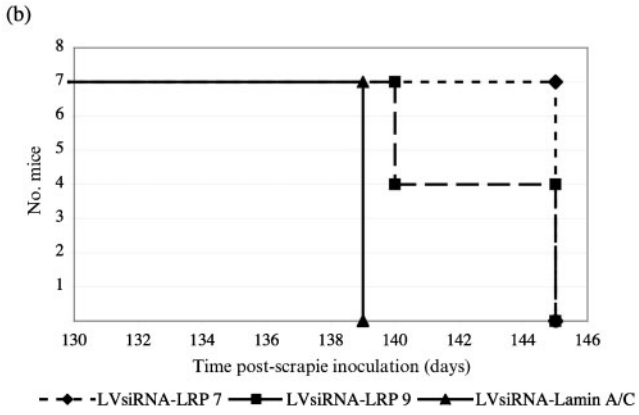
to mice microinjected with the control LVsiRNA-Lamin A/C (139±0 days) (Fig. 3b, c).

To analyse the protein levels, hippocampal samples were extracted 90 days post-scrapie inoculation, digested with 20 μg proteinase K ml^{-1} of total protein for 30 min at 37 °C following methanol precipitation and subjected to SDS-PAGE and Western blotting. Quantification of the data revealed a significant reduction of the endogenous LRP and PrP^{Sc} levels by 36 and 41 %, respectively, using LVsiRNA-LRP 7 (Fig. 3d). While intracerebral microinjection of LVsiRNA-LRP 9 resulted in a significant reduction of the LR level in the cortex of non-infected mice, the same lentiviral vector failed to significantly reduce the LRP level in scrapie-infected animals (Fig. 3d), although the PrP^{Sc} level was significantly reduced by 38 %. We hypothesize



(c)

	Pre-clinical phase (mean days p.i.±SD)	Survival (mean days±SD)	Life span (mean days p.i.±SD)
LVsiRNA-Lamin A/C	139 ± 0	16 ± 4	155 ± 4
LVsiRNA-LRP 7	145 ± 0 (P<0.005)	15 ± 4 (P<0.5)	160 ± 4 (P<0.05)
LVsiRNA-LRP 9	143 ± 3 (P<0.05)	18 ± 8 (P<0.5)	161 ± 10 (P<0.5)



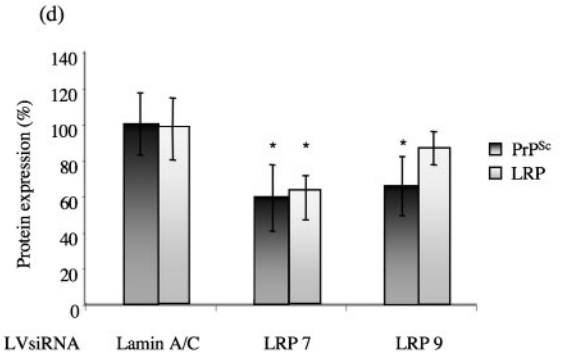


Fig. 3. Analysis of scrapie-infected C57BL/6 mice after microinjection of lentiviral vectors expressing siRNAs directed against the LRP mRNA into the hippocampus. (a) Mice were microinjected with lentiviral particles 2 weeks prior to intracerebral inoculation with 1 % RML homogenate. The first group of mice was sacrificed 90 days post-scrapie-infection to analyse the LRP and PrP content, respectively. Remaining mice were monitored and sacrificed at the day when two of the TSE-relevant symptoms (Sethi *et al.*, 2002) appeared over 3 days. The pre-clinical phase (incubation time) of the disease is defined as the time from the day of scrapie inoculation until the day where the first TSE-relevant symptom (Sethi *et al.*, 2002) occurred, whereas survival is the time from the day the first symptom occurred until the day where two symptoms (Sethi *et al.*, 2002) are manifested on 3 consecutive days. (b) Kaplan–Meier curve of 1 % RML-infected mice (seven animals per group) microinjected with LVsiRNA-LRP 7 (diamond), LVsiRNA-LRP 9 (square) and LVsiRNA-Lamin A/C (triangle), respectively. (c) Statistical evaluation of the pre-clinical phase, survival and life span in scrapie-infected mice after injection of lentiviral vectors LVsiRNA-LRP 7, 9 and Lamin A/C, respectively. Pre-clinical phase (incubation time) and survival are defined in (a). Life span is defined as pre-clinical phase plus survival. Days p.i.=days post-RML inoculation. (d) Western blot data of hippocampus homogenates (10 %) from scrapie-infected mice after injection of lentiviral vectors LVsiRNA-LRP 7, 9 and Lamin A/C, respectively, 90 days post-scrapie inoculation were quantified by NIH Image software. Antibodies scFv S18 (Zuber *et al.*, 2008a) and SAF83 were used for LRP and PrP^{Sc} detection, respectively. An anti-β-actin antibody (Sigma) was used for β-actin detection (control). LRP and PrP^{Sc} levels of LVsiRNA-LRP 7 and LVsiRNA-LRP 9-treated mice were reduced by 36 (P<0.05)/41 % (P<0.05) and 12 (P<0.5)/38 % (P<0.05), respectively, in comparison to LVsiRNA-Lamin A/C-treated animals (LRP/PrP^{Sc} levels were set to 100 %). Experiments were performed in triplicate (seven animals per group) and expressed as percentages of control levels ± SD.

that due to the different distribution of LRP/LR isoforms in the brain (Baloui *et al.*, 2004), scFv S18 used for LRP/LR detection might preferentially recognize LR in the cortex and LRP in the hippocampus, respectively. Furthermore, it has been demonstrated that LRP levels in the brains of scrapie-infected hamsters are increased in comparison to uninfected animals (Rieger *et al.*, 1997). The silencing effect of lentiviral vectors expressing siRNAs directed against LRP mRNA might be weakened by so far unknown mechanisms in the cell enhancing LRP expression in response to a scrapie infection.

In the present study, we employed a lentiviral LRP-specific siRNA approach and demonstrated a downregulation of LRP/LR gene expression concomitant with a decrease of PrP^{Sc} levels in both a scrapie cell culture and animal model, confirming an essential requirement of LRP/LR for prion propagation (Leucht *et al.*, 2003). In addition, in scrapie-infected mice injected with lentiviral vectors expressing LRP-specific siRNAs, a significant prolongation of the pre-clinical phase has been observed.

Thus, lentiviral-mediated siRNA delivery represents a promising therapeutic tool to specifically knock-down disease-relevant genes. The advantage of lentiviral vectors lies in their ability to transduce dividing cells such as embryonic stem cells (Pfeifer *et al.*, 2006), and non-dividing and terminally differentiated cells such as neurons (Blomer *et al.*, 1997; Naldini *et al.*, 1996b). 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, lentivector-mediated RNAi was used to generate chimeric mice that express lower PrP levels depending on the degree of chimerism. In highly chimeric mice, survival was significantly prolonged after scrapie infection (Pfeifer *et al.*, 2006). These data show that the application of the RNAi technology is a highly effective therapeutic approach against various neurological diseases.

Very recently, we developed an AAV-based gene delivery system targeting LRP/LR by transferring anti-LRP/LR single-chain antibody into scrapie-infected mice (Zuber *et al.*, 2008c). This approach resulted in a significant reduction of peripheral PrP^{Sc} propagation without a significant prolongation of incubation times and survival (Zuber *et al.*, 2008c). In contrast, the lentiviral siRNA delivery system presented here resulted in a significant prolongation of the pre-clinical phase in scrapie-infected mice, pointing towards an alternative therapeutic tool for the treatment of prion disorders.

Acknowledgements

The authors thank C. I. Lasmézas for providing the anti-PrP antibody SAF83 and fruitful discussions, T. Hallas, M. Ruhmann and C. Rank for excellent technical assistance. Scrapie-infected mouse neuroblastoma cells ($N2aSc^{+}$) were kindly provided by S. Lehmann. All animal experiments were approved by the Bavarian Government (AZ: 55.2-1-54-44/05). This work was supported by the Bundesministerium für Bildung und Forschung (grant 01-KO-0514), the European Commission (grant no. NoE NeuroPrion FOOD-CT 2004-506579) and the Deutsche Forschungsgemeinschaft (grant no. WE 2664/2-1).

References

Auth, D. & 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.

Baloui, H., Stettler, O., Weiss, S., Nothias, F. & von Boxberg, Y. (2008). Upregulation in rat spinal cord microglia of the nonintegrin laminin receptor 37 kDa-LRP following activation by a traumatic lesion, or peripheral injury. *J Neurotrauma* (in press).

Baloui, H., von Boxberg, Y., Vinh, J., Weiss, S., Rossier, J., Nothias, F. & 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.

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

Castronovo, V. (1993). Laminin receptors and laminin-binding proteins during tumor invasion and metastasis. *Invasion Metastasis* **13**, 1–30.

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

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

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

Gauczynski, S., Nikles, D., El-Gogo, S., Papy-Garcia, D., Rey, C., Alban, S., Barritault, D., Lasmezas, C. I. & Weiss, S. (2006). The 37-kDa/67-kDa laminin receptor acts as a receptor for infectious prions and is inhibited by polysulfated glycanes. *J Infect Dis* **194**, 702–709.

Givant-Horwitz, V., Davidson, B. & Reich, R. (2005). Laminin-induced signaling in tumor cells. *Cancer Lett* **223**, 1–10.

Kinoshita, K., Kaneda, Y., Sato, M., Saeki, Y., Wataya, K. M. & Hoffmann, A. (1998). LBP-p40 binds DNA tightly through associations with histones H2A, H2B, and H4. *Biochem Biophys Res Commun* **253**, 277–282.

Landowski, T. H., Uthayakumar, S. & Starkey, J. R. (1995). Control pathways of the 67 kDa laminin binding protein: surface expression and activity of a new ligand binding domain. *Clin Exp Metastasis* **13**, 357–372.

Leucht, C., Simoneau, S., Rey, C., Vana, K., Rieger, R., Lasmezas, C. I. & 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.

Ludewigs, H., Zuber, C., Vana, K., Nikles, D., Zerr, I. & Weiss, S. (2007). Therapeutic approaches for prion disorders. *Expert Rev Anti Infect Ther* **5**, 613–630.

Menard, S., Castronovo, V., Tagliabue, E. & Sobel, M. E. (1997). New insights into the metastasis-associated 67 kD laminin receptor. *J Cell Biochem* **67**, 155–165.

Naldini, L., Blomer, U., Gage, F. H., Trono, D. & 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. & Trono, D. (1996b). In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272**, 263–267.

Nikles, D., Vana, K., Gauczynski, S., Knetsch, H., Ludewigs, H. & Weiss, S. (2008). Subcellular localization of prion proteins and the 37 kDa/67 kDa laminin receptor fused to fluorescent proteins. *Biochim Biophys Acta* **1782**, 335–340.

Pfeifer, A., Eigenbrod, S., Al-Khadra, S., Hofmann, A., Mitteregger, G., Moser, M., Bertsch, U. & Kretzschmar, H. (2006). Lentivector-mediated RNAi efficiently suppresses prion protein and prolongs survival of scrapie-infected mice. *J Clin Invest* **116**, 3204–3210.

Prusiner, S. B., Scott, M., Foster, D., Pan, K. M., Groth, D., Mirenda, C., Torchia, M., Yang, S. L., Serban, D. & other authors (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. & Cohen, F. E. (1998). Prion protein biology. *Cell* **93**, 337–348.

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

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

Sethi, S., Lipford, G., Wagner, H. & Kretzschmar, H. (2002). Postexposure prophylaxis against prion disease with a stimulator of innate immunity. *Lancet* **360**, 229–230.

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

Susantad, T. & Smith, D. R. (2008). siRNA-mediated silencing of the 37/67-kDa high affinity laminin receptor in Hep3B cells induces apoptosis. *Cell Mol Biol Lett* **13**, 452–464.

Tanaka, M., Narumi, K., Isemura, M., Abe, M., Sato, Y., Abe, T., Saijo, Y., Nukiwa, T. & Satoh, K. (2000). Expression of the 37-kDa laminin binding protein in murine lung tumor cell correlates with tumor angiogenesis. *Cancer Lett* **153**, 161–168.

Trevitt, C. R. & Collinge, J. (2006). A systematic review of prion therapeutics in experimental models. *Brain* **129**, 2241–2265.

Tuschl, T., Zamore, P. D., Lehmann, R., Bartel, D. P. & Sharp, P. A. (1999). Targeted mRNA degradation by double-stranded RNA in vitro. *Genes Dev* **13**, 3191–3197.

Vana, K. & Weiss, S. (2006). A trans-dominant negative 37kDa/67kDa laminin receptor mutant impairs PrP^{Sc} propagation in scrapie-infected neuronal cells. *J Mol Biol* **358**, 57–66.

Vana, K., Zuber, C., Nikles, D. & Weiss, S. (2007). Novel aspects of prions, their receptor molecules, and innovative approaches for TSE therapy. *Cell Mol Neurobiol* **27**, 107–128.

Vana, K., Zuber, C., Pflanz, H., Kolodziejczak, D., Zemora, G., Bergmann, A.-K. & Weiss, S. (2008). LRP/LR as an alternative promising target in therapy of prion diseases. *Infect Disord Drug Targets* (in press).

Weissmann, C. & Aguzzi, A. (2005). Approaches to therapy of prion diseases. *Annu Rev Med* **56**, 321–344.

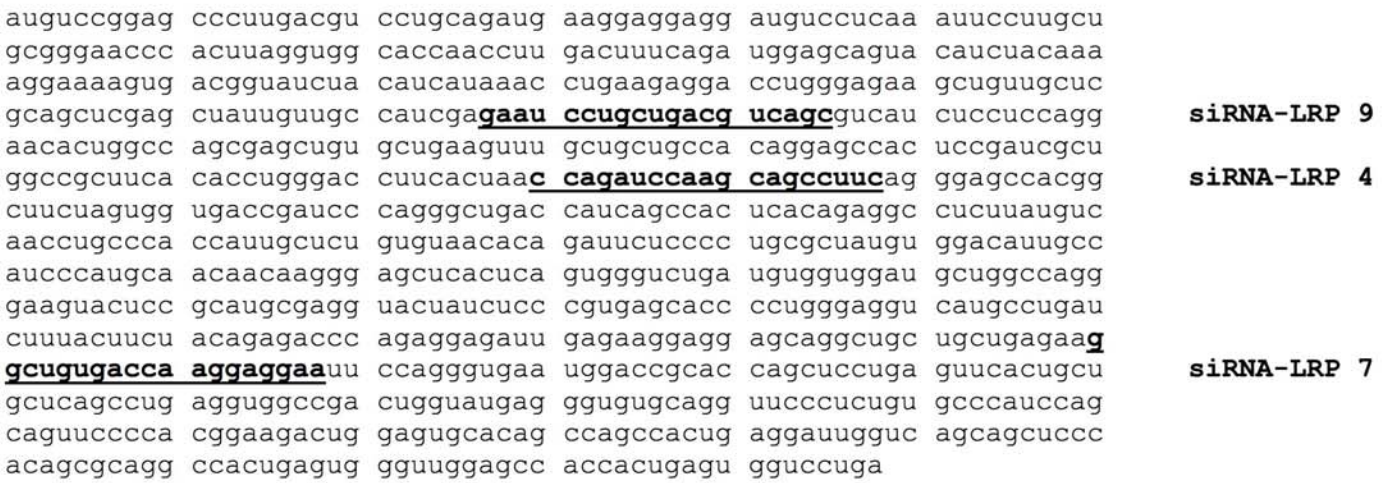
Zuber, C., Ludewigs, H. & Weiss, S. (2007a). Therapeutic approaches targeting the prion receptor LRP/LR. *Vet Microbiol* **123**, 387–393.

Zuber, C., Mitteregger, G., Pace, C., Zerr, I., Kretzschmar, H. A. & Weiss, S. (2007b). Anti-LRP/LR antibody W3 hampers peripheral PrPSc propagation in scrapie infected mice. *Prion* **1**, 207–212.

Zuber, C., Knackmuss, S., Rey, C., Reusch, U., Rottgen, P., Frohlich, T., Arnold, G. J., Pace, C., Mitteregger, G. & other authors (2008a). Single chain Fv antibodies directed against the 37 kDa/67 kDa laminin receptor as therapeutic tools in prion diseases. *Mol Immunol* **45**, 144–151.

Zuber, C., Knackmuss, S., Zemora, G., Reusch, U., Vlasova, E., Diehl, D., Mick, V., Hoffmann, K., Nikles, D. & other authors (2008b). Invasion of tumorigenic HT1080 cells is impeded by blocking or downregulating the 37-kDa/67-kDa laminin receptor. *J Mol Biol* **378**, 530–539.

Zuber, C., Mitteregger, G., Schuhmann, N., Rey, C., Knackmuss, S., Rupprecht, W., Reusch, U., Pace, C., Little, M. & other authors (2008c). Delivery of single-chain antibodies (scFvs) directed against the 37/67 kDa laminin receptor into mice via recombinant adeno-associated viral vectors for prion disease gene therapy. *J Gen Virol* **89**, 2055–2061.



Supplementary Fig. S1. mRNA sequence of the murine laminin receptor precursor. Target RNA sequences for the three LRP-specific siRNAs used are indicated. siRNA-LRP 9, siRNA-LRP 4 and siRNA-LRP 7 target bases 207–225, 330–348 and 660–678, respectively.

Pflanz, H., Vana, K., Mitteregger, G., Pace, C., Messow, D., Sedlaczek, C., Nikles, D., Kretzschmar, H. A. and Weiss, S. F. T. (2009). Microinjection of lentiviral vectors expressing small interfering RNAs directed against laminin receptor precursor mRNA prolongs the pre-clinical phase in scrapie-infected mice. *J Gen Virol* **90**, 269–274.

Chapter VI

The laminin receptor specific antibody IgG1-iS18 impedes amyloid β release from human embryonic kidney cells

submitted as:

Pflanz, H., Bergmann, A.-K., Dimitriadis, C., Knackmuss, S., Reusch, U., Zuber, C., Haass, C., Little, M., Weiss, S., The laminin receptor specific antibody IgG1-iS18 impedes amyloid β release from human embryonic kidney cells. *Nature* submitted

The laminin receptor specific antibody IgG1-iS18 impedes amyloid ß release from human embryonic kidney cells

Heike Pflanz[1], Ann-Katrin Bergmann[1], Christoph Dimitriadis[1], Stefan Knackmuss[2], Uwe Reusch[2], Chantal Zuber[1], Christian Haass[3], Melvyn Little[2] & Stefan Weiss[1]

[1]Laboratorium für Molekulare Biologie – Genzentrum - Institut für Biochemie der LMU München, Feodor-Lynen-Strasse 25, D-81377 München, Germany

[2]Affimed Therapeutics AG – Technologiepark, Im Neuheimer Feld 582, D-69120 Heidelberg, Germany

[3]Butenandt-Institut – Institut für Biochemie der LMU München, Schillerstrasse 44, D-80336 München, Germany

Alzheimer´s disease (AD) and Creutzfeldt-Jakob disease (CJD) are neurodegenerative diseases sharing a characteristic pathological feature. Both show the deposition of amyloid plaques in the brains of affected individuals[1]. The prion protein, associated with Creutzfeldt-Jakob disease and other transmissible spongiform encephalopathies, regulates the formation of amyloid β, contributing to Morbus Alzheimer[2]. Recently, the non-integrin 37kDa/67kDa laminin receptor (LRP/LR) was identified as a receptor for prions[3, 4]. Furthermore, the amyloid β precursor protein (APP), LRP/LR and the prion protein localize at the cell surface, where the proteolytic cleavage of APP and the generation of amyloid β takes place. A possible influence of LRP/LR on APP processing and amyloid β generation has not been investigated. Here we show that incubation of human embryonic kidney cells, endogenously expressing APP and generating amyloid β with the LRP-specific monoclonal humanized antibody IgG1 iS18 resulted in a significant reduction of the amyloid β generation by 99%. Furthermore, levels of the β-secretase shedding product soluble APPβ (sAPPβ) are significantly decreased upon IgG1 iS18 antibody treatment by 99% concomitant with not significantly altered APP expression levels, implicating that LRP/LR regulates β-secretase activity by promoting Aβ secretion. Our results reveal that LRP/LR might play a possible regulatory role in the enzymatic cleavage process of APP, supporting amyloidogenesis. LRP/LR specific pharmaceuticals such as the IgG1-iS18 antibody may represent new and promising therapeutic tools for the treatment of Alzheimer´s disease.

Alzheimer´s disease (AD) is the most common dementia and is still incurable. It is characterized by a loss of neurons and synapses in the cerebral cortex and certain subcortical regions, accompanied by atrophy of the affected regions[5]. The brains of patients suffering from Alzheimer´s disease show amyloid plaque deposition and neurofibrillary tangles (NFT). The plaque deposits mainly consist of extracellularly accumulated amyloid β (Aβ), which is a product of the proteolytic cleavage of amyloid precursor protein (APP) (for review see[6]). The neurofibrillary tangles are intraneural lesions consisting of hyperphosphorylated tau protein[7], which is a microtubule stabilizing protein. An accumulation of Aβ in the extracellular matrix (ECM) might be due to a reduced degradation of Aβ or an increased proteolytic cleavage of APP. In the non-amyloidogenic pathway APP is cleaved by the α-secretase releasing the soluble N-terminal ectodomain of APP (sAPPα) into the ECM, followed by γ-secretase cleavage, which releases a small peptide (p3) into the extracellular space. The α-secretase cleavage is most likely performed by ADAM10 (a disintegrin and metalloproteinase) and ADAM17, although other candidates have been identified (for review see[8]). The α-secretase cleavage site is located within the Aβ peptide sequence and thus, α-cleavage precludes Aβ peptide generation. The γ-secretase is a protease complex and consists of four integral membrane proteins (for review see[9]). In case of APP processing within the amyloidogenic pathway, β-secretase cleavage releases a soluble N-terminal part of APP (sAPPβ) into the ECM. Beta-site-APP-cleaving-enzyme (BACE-1) has been identified as the β-secretase homologue (for review see[10]). β-cleavage is followed by γ-secretase cleavage, producing predominantly Aβ isoforms Aβ1-40 and Aβ1-42. Aβ1-42 is particularly prone to precipitation and aggregation[11]. The physiological function of APP is still poorly understood and remains under investigation. It has been suggested that APP might act as a receptor or a growth factor due to its growth factor like domain at the N-terminus[12]. APP has further been proposed to

play a role in cell adhesion, migration and proliferation[13] as well as cell movement regulation[14].

Recently, a regulatory effect of the cellular prion protein (PrP^c) on the β-secretase cleavage of APP has been described[2]. PrP^c directly interacts with the β-secretase and inhibits its enzymatic activity, thereby preventing the generation of Aβ peptides[2]. The misfolded infectious isoform of the host encoded cellular prion protein is called PrP^{Sc} and is supposed to act as the causative agent of transmissible spongiform encephalopathies (TSE), which include Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), Kuru and fatal familial insomnia (FFI) in humans, bovine spongiform encephalopathy (BSE) in cattle and scrapie in sheep. Although the function of the prion protein has to be further elucidated it has been suggested that PrP^c plays a role in e.g. copper trafficking, T-cell function and oxidative stress homeostasis (for review see[15]). The 37kDa/67kDa laminin receptor (LRP/LR) was identified as a receptor for PrP^c[4] and PrP^{Sc}[3]. LRP/LR and PrP co-localize in the perinuclear compartent and at the cell membrane of baby hamster kidney (BHK) cells[16]. LRP/LR mediates bovine prion endocytosis by human enterocytes[17], suggesting an important role of the receptor in the species barrier of prion diseases. LRP/LR represents a promising alternative target in prion disorders (for review see[18-20]). The laminin receptor is involved in the translational machinery[21,22] and the maintanance of nuclear structures[23]. LRP/LR is a receptor for elastin, carbohydrates and a series of viruses such as Venezuelean equine encephalitis virus (VEE), Sindbis virus and Dengue virus, and certain AAV subtypes (for review see[19]). LRP/LR is involved in cell adhesion and tumor metastasis formation[24]. Tools downregulating or blocking LRP/LR such as the monoclonal antibody IgG1-iS18 impede the two key components of metastatic tumors: invasion and adhesion[25].

The finding that the cellular prion protein regulates Amyloid β secretion[2], together with the fact that LRP/LR acts as a receptor for prions[3, 4], encouraged us to investigate a possible influence of the laminin receptor on the generation of Aβ. Additionally, as integral

membrane proteins APP and LRP/LR share the same subcellular localization with the glycosyl-phosphatidylinositol (GPI) anchored PrP^c, a direct interaction partner of LRP/LR and the APP-processing β-secretase, suggesting a possible linkage between both regulatory networks. We investigated the influence of the anti-LRP/LR specific antibody IgG1-iS18 on the release of amyloid β in human embryonic kidney cells (HEK293FT), selected as the model cell line due to endogenous expression of APP[26]. HEK293FT cells indeed express moderate levels of APP (Fig. 1a, no antibody treatment) and release considerable amounts of amyloid β into the conditioned medium (Fig.1e, no antibody treatment). Treatment of HEK293FT cells with the LRP/LR-specific antibody IgG1-iS18 significantly reduced Aβ levels in contrast to IgG1-HD37 directed against CD-19 on human B-lymphocytes which had no significant effect on Aβ-levels (Fig.1e). Densitometric analysis of the amyloid β signals revealed a significant reduction of amyloid β levels in the conditioned media of cells treated with IgG1-iS18 by 99% ($p\text{-value}<5*10^{-8}$, two-tailed t-test, n=6) in comparison to non-treated cells (Fig. 2). APP expression levels were not significantly altered in the presence of IgG1-iS18 (Fig.1a; Fig. 3), suggesting that decreased amyloid β levels by IgG1-iS18 are not due to decreased APP levels. IgG1-HD37 had no significant effect on the release of amyloid β from HEK293FT cells (Fig. 1e; Fig. 2) and no significant influence on endogenous APP and LRP levels (Fig. 1a, b; Fig. 3). LRP levels remain unchanged upon treatment with IgG1-iS18 (Fig. 3) indicating that the significant reduction of amyloid β levels by IgG1-iS18 is not due to an altered LRP expression level. Shedding of sAPPβ is due to the enzymatic processing of APP by β-secretases during the amyloidogenic pathway. Thus, sAPPβ levels in the conditioned media of HEK293FT cells were analyzed to investigate whether the reduction of Aβ levels might be due to an impairment of the β-secretase activity. Statistical evaluations of the sAPPβ levels in the media of IgG1-iS18 treated HEK293FT cells (Fig. 1d) revealed a significant reduction by 99% (Fig. 4) compared to non-treated cells ($p\text{-value}<0.0005$, two-tailed t-test,

n=6). The decrease of amyloid β levels (Fig. 2) correlates with the reduction of sAPPβ levels (Fig. 4) indicating that treatment with the LRP-specific antibody IgG1-iS18 impairs β-secretase activity and thereby prevents the release of sAPPβ.

Prion diseases in humans, such as Creutzfeldt-Jakob disease (CJD) and AD might be connected regarding the co-existence of amyloid β plaques and PrP^{Sc} deposition or further pathogenic and clinical similarities[27, 28]. A regulatory influence of prion protein levels on amyloid β formation has been shown[2, 29], although leading to contradictory results. Most recently, it was demonstrated that PrP antibodies prevent amyloid β-oligomer binding to PrP^{c} and rescue synaptic plasticity in hippocampal slices from oligomeric amyloid β suggesting that PrP^{c} is a mediator of amyloid β-oligomer-induced synaptic dysfunction[30]. The discovery of LRP/LR as a receptor for prions might represent a link to the regulatory network of proteins and enzymes related to amyloid β generation, especially since APP, APP processing enzymes, PrP^{c} and its receptor LRP/LR are all localized on the cell surface. Here, we report on the *in vitro* effect of the LRP/LR specific monoclonal antibody IgG1-iS18 on the amyloid β levels secreted from human embryonic kidney cells. Amyloid β levels in the conditioned media of HEK293FT cells were significantly reduced by 99% after pre-incubation of the cells with IgG1-iS18. APP and LRP levels were not altered upon IgG1-iS18 treatment, suggesting that the significant reduction of amyloid β levels is not a consequence of reduced APP or LRP levels. To elucidate whether the β-secretase as the direct interaction partner of PrP^{c2} might be associated with the regulatory effect of LRP/LR, the sAPPβ levels of the conditioned media of the IgG1-iS18 treated cells were analyzed. After treatment of HEK293FT cells with IgG1-iS18, sAPPβ levels were significantly decreased by 99%, which correlates with the significant reduction of amyloid β levels by 99%. These results indicate that LRP/LR might regulate β-secretase activity, increasing sAPPβ shedding and amyloid β secretion. PrP^{c} and its receptor LRP/LR therefore encompass oppose effects within the amyloidogenic pathway: PrP^{c}

decreases β-secretase activity, impedes sAPPβ shedding and Aβ-production[2], whereas LRP/LR seems to have oppositional effects since the LRP/LR specific antibody IgG1-iS18 significantly reduces amyloid β and sAPPβ levels. Our results recommend the humanized monoclonal antibody IgG1-iS18 which renders it suitable for a possible clinical applicability in humans as a new and promising therapeutic tool for the treatment of Alzheimer's disease.

METHODS

Human embryonic kidney (HEK293FT) cells were cultured in Dulbecco's Modified Eagle Medium with 4.5g/l glucose (DMEM, Pan Biotech) supplemented with 10% fetal calf serum (FCS, Gibco) and 1% PenStrep (10000U/ml Penicilline and Streptomycine, Gibco) at 37°C and 5% CO_2. The cells were plated to 30% confluency. After 24 hours, culture medium was exchanged now containing 50µg/ml anti-LRP/LR antibody IgG1-iS18, control antibody IgG1-HD37 or no antibody. The conditioned media and the cells were harvested 48 hours after the medium exchange. The cells were lysed with lysis buffer (10mM Tris/HCl, pH=7.5; 100mM NaCl; 10mM EDTA; 0.5% Nonidet P-40; 0.05% Deoxycholic acid) for 10 minutes on ice, scraped off the plates and centrifuged for 10 minutes at 14000rpm at 4°C to remove cell debris. Cell lysates were analyzed for APP, LRP and β-actin by a 12% sodium dodecylsulfate polyacrylamide gelelectrophoresis (SDS-PAGE). The conditioned media were analyzed for sAPPβ by 6% SDS-PAGE and for Aβ by 4%-16.5% Tris-tricine gelelectrophoresis. After blotting onto a polyvinylidene difluoride (PVDF) membrane (GE Healthcare), unspecific binding sites were blocked with 20% horse serum in phosphate buffered saline containing 0.2% Tween 20 (PBS-T 0.2%), followed by incubation with antibodies in 10% horse serum in PBS-T 0.2%. Amyloid β and sAPPβ were detected using anti β-amyloid (1/5000, Rockland) and anti-sAPPβ-wt (1/100, IBL), respectively. APP was detected with anti-APP Y188 (1/5000, Abcam) and LRP was detected using IgG1-iS18 (1/10000, Affimed Therapeutics). As loading control for the cell cell lysates β-actin was detected with anti-β-actin (1/5000,

Sigma). Blots were developed using AceGlow (PeqLab). For vizualization the membranes were exposed on chemiluminescense films (RP New, CEA). Stripping of the membranes was performed using stripping buffer (100 mM glycine, 1% SDS, 0.1% Nonidet P40; pH 2.2).

1. Debatin, L. et al. Association between deposition of beta-amyloid and pathological prion protein in sporadic Creutzfeldt-Jakob disease. *Neurodegener Dis* **5**, 347-354 (2008).
2. Parkin, E.T. et al. Cellular prion protein regulates beta-secretase cleavage of the Alzheimer's amyloid precursor protein. *Proc Natl Acad Sci U S A* **104**, 11062-11067 (2007).
3. Gauczynski, S. et al. The 37-kDa/67-kDa laminin receptor acts as a receptor for infectious prions and is inhibited by polysulfated glycanes. *J Infect Dis* **194**, 702-709 (2006).
4. Gauczynski, S. et al. The 37-kDa/67-kDa laminin receptor acts as the cell-surface receptor for the cellular prion protein. *Embo J* **20**, 5863-5875 (2001).
5. Wenk, G.L. Neuropathologic changes in Alzheimer's disease. *J Clin Psychiatry* **64 Suppl 9**, 7-10 (2003).
6. De Strooper, B. & Annaert, W. Proteolytic processing and cell biological functions of the amyloid precursor protein. *J Cell Sci* **113 (Pt 11)**, 1857-1870 (2000).
7. Brion, J.P., Couck, A.M., Passareiro, E. & Flament-Durand, J. Neurofibrillary tangles of Alzheimer's disease: an immunohistochemical study. *J Submicrosc Cytol* **17**, 89-96 (1985).
8. Allinson, T.M., Parkin, E.T., Turner, A.J. & Hooper, N.M. ADAMs family members as amyloid precursor protein alpha-secretases. *J Neurosci Res* **74**, 342-352 (2003).
9. Kaether, C., Haass, C. & Steiner, H. Assembly, trafficking and function of gamma-secretase. *Neurodegener Dis* **3**, 275-283 (2006).
10. Haass, C. Take five--BACE and the gamma-secretase quartet conduct Alzheimer's amyloid beta-peptide generation. *Embo J* **23**, 483-488 (2004).
11. Lansbury, P.T., Jr. Structural neurology: are seeds at the root of neuronal degeneration? *Neuron* **19**, 1151-1154 (1997).
12. Rossjohn, J. et al. Crystal structure of the N-terminal, growth factor-like domain of Alzheimer amyloid precursor protein. *Nat Struct Biol* **6**, 327-331 (1999).
13. Perez, R.G., Zheng, H., Van der Ploeg, L.H. & Koo, E.H. The beta-amyloid precursor protein of Alzheimer's disease enhances neuron viability and modulates neuronal polarity. *J Neurosci* **17**, 9407-9414 (1997).
14. Sabo, S.L., Ikin, A.F., Buxbaum, J.D. & Greengard, P. The Alzheimer amyloid precursor protein (APP) and FE65, an APP-binding protein, regulate cell movement. *J Cell Biol* **153**, 1403-1414 (2001).
15. Zomosa-Signoret, V., Arnaud, J.D., Fontes, P., Alvarez-Martinez, M.T. & Liautard, J.P. Physiological role of the cellular prion protein. *Vet Res* **39**, 9 (2008).
16. Nikles, D. et al. Subcellular localization of prion proteins and the 37 kDa/67 kDa laminin receptor fused to fluorescent proteins. *Biochim Biophys Acta* **1782**, 335-340 (2008).
17. Morel, E. et al. Bovine prion is endocytosed by human enterocytes via the 37 kDa/67 kDa laminin receptor. *Am J Pathol* **167**, 1033-1042 (2005).
18. Ludewigs, H. et al. Therapeutic approaches for prion disorders. *Expert Rev Anti Infect Ther* **5**, 613-630 (2007).

19. Vana, K. et al. LRP/LR as an alternative promising target in therapy of prion diseases, Alzheimer's disease and cancer. *Infect Disord Drug Targets* **9**, 69-80 (2009).
20. Zuber, C., Ludewigs, H. & Weiss, S. Therapeutic approaches targeting the prion receptor LRP/LR. *Vet Microbiol* **123**, 387-393 (2007).
21. Auth, D. & Brawerman, G. A 33-kDa polypeptide with homology to the laminin receptor: component of translation machinery. *Proc Natl Acad Sci U S A* **89**, 4368-4372 (1992).
22. Ardini, E. et al. The 67-kDa laminin receptor originated from a ribosomal protein that acquired a dual function during evolution. *Mol Biol Evol* **15**, 1017-1025 (1998).
23. Sato, M. et al. Analysis of nuclear localization of laminin binding protein precursor p40 (LBP/p40). *Biochem Biophys Res Commun* **229**, 896-901 (1996).
24. Taraboletti, G., Belotti, D., Giavazzi, R., Sobel, M.E. & Castronovo, V. Enhancement of metastatic potential of murine and human melanoma cells by laminin receptor peptide G: attachment of cancer cells to subendothelial matrix as a pathway for hematogenous metastasis. *J Natl Cancer Inst* **85**, 235-240 (1993).
25. Zuber, C. et al. Invasion of tumorigenic HT1080 cells is impeded by blocking or downregulating the 37-kDa/67-kDa laminin receptor. *J Mol Biol* **378**, 530-539 (2008).
26. Xia, W., Zhang, J., Perez, R., Koo, E.H. & Selkoe, D.J. Interaction between amyloid precursor protein and presenilins in mammalian cells: implications for the pathogenesis of Alzheimer disease. *Proc Natl Acad Sci U S A* **94**, 8208-8213 (1997).
27. Powers, J.M. et al. Concomitant Creutzfeldt-Jakob and Alzheimer diseases. *Acta Neuropathol* **83**, 95-98 (1991).
28. Hainfellner, J.A. et al. Coexistence of Alzheimer-type neuropathology in Creutzfeldt-Jakob disease. *Acta Neuropathol* **96**, 116-122 (1998).
29. Schwarze-Eicker, K. et al. Prion protein (PrPc) promotes beta-amyloid plaque formation. *Neurobiol Aging* **26**, 1177-1182 (2005).
30. Lauren, J., Gimbel, D.A., Nygaard, H.B., Gilbert, J.W. & Strittmatter, S.M. Cellular prion protein mediates impairment of synaptic plasticity by amyloid-beta oligomers. *Nature* **457**, 1128-1132 (2009).

Acknowledgements The work was supported by the European Commission Grant NoE NeuroPrion

Author information Correspondence and requests for materials should be addressed to S.W. (weiss@lmb.uni-muenchen.de).

Figure legends

Figure 1| Analysis of Aβ, sAPPβ, APP and LRP levels secreted from and in HEK293FT cells treated with anti-LRP/LR antibody IgG1-iS18. Representative Western blot of HEK293FT cells, endogenously expressing APP, treated with 50µg/ml IgG1-iS18, IgG1-HD37 or no antibody for 48 hours. Cell lysates were analyzed for APP (**a**), LRP (**b**) and β-actin (**c**) by 12% SDS-PAGE. The conditioned media were analyzed for sAPPβ (**d**) by 6% SDS-PAGE and amyloid β (**e**) by 4-16,5% Tris-Tricine gelelectrophoresis. Analyses were performed twice, each in triplica. Molecular weight markers are indicated on the left.

Figure 2| Densitometric quantification of amyloid β levels released from HEK293FT cells after treatment with anti-LRP/LR antibody IgG1-iS18. Signals for amyloid β (Fig. 1e) were quantified using NIH ImageJ software. The amyloid β levels in the conditioned media of HEK293FT cells, treated with 50µg/ml IgG1-iS18, were significantly reduced by 99% ± 0.5% s.d. in comparison to amyloid β levels from non-treated cells (p-value<$5*10^{-8}$, two-tailed t-test). All error bars represent mean and s.d., n=6.

Figure 3| Densitometric quantification of LRP and APP levels in HEK293FT cells treated with anti-LRP/LR antibody IgG1-iS18. Western blot data of APP and LRP (Fig. 1a,b) were quantified using NIH ImageJ software. APP and LRP levels were not significantly altered upon antibody treatment with IgG1-iS18 or IgG1-HD37 in comparison to APP and LRP levels of untreated cells, respectively. All error bars represent mean and s.d., n=6.

Figure 4| Densitometric analysis of sAPPβ levels secreted from HEK293FT cells after treatment with anti-LRP/LR antibody IgG1-iS18. Signals for sAPPβ (Fig. 1d) were quantified using NIH ImageJ software. The sAPPβ levels in the conditioned media of HEK293FT cells, treated with IgG1-iS18, were significantly reduced by 99% ± 1% s.d. in comparison to sAPPβ levels released from non-treated cells (p-value<0.0005, two-tailed t-test). All error bars represent mean and s.d., n=6.

Figure 1

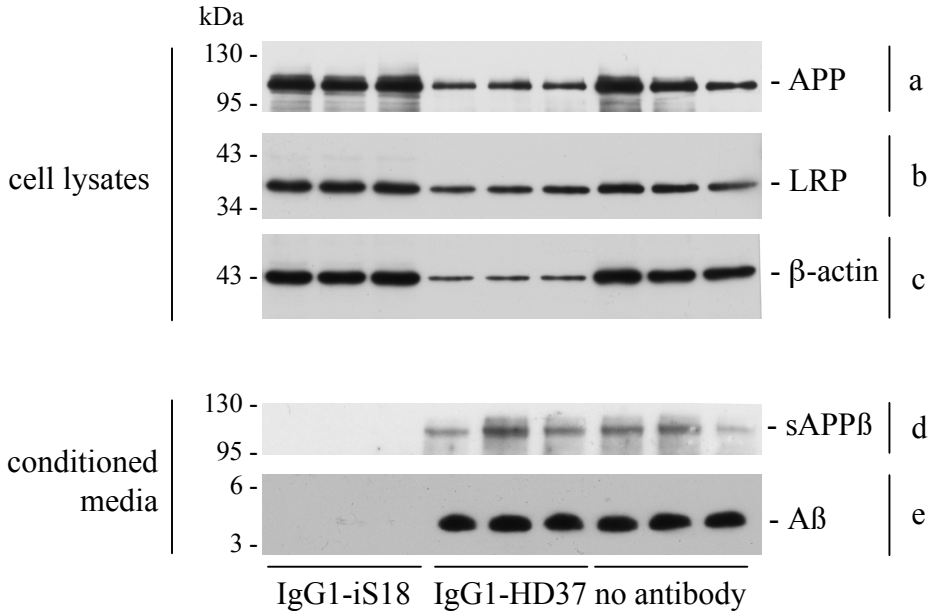

kDa
130 -
95 -
- APP
a
43 -
34 -
- LRP
b
cell lysates
43 -
- β-actin
c
130 -
95 -
- sAPPß
d
conditioned media
6 -
3 -
- Aß
e
IgG1-iS18
IgG1-HD37
no antibody

Figure 2

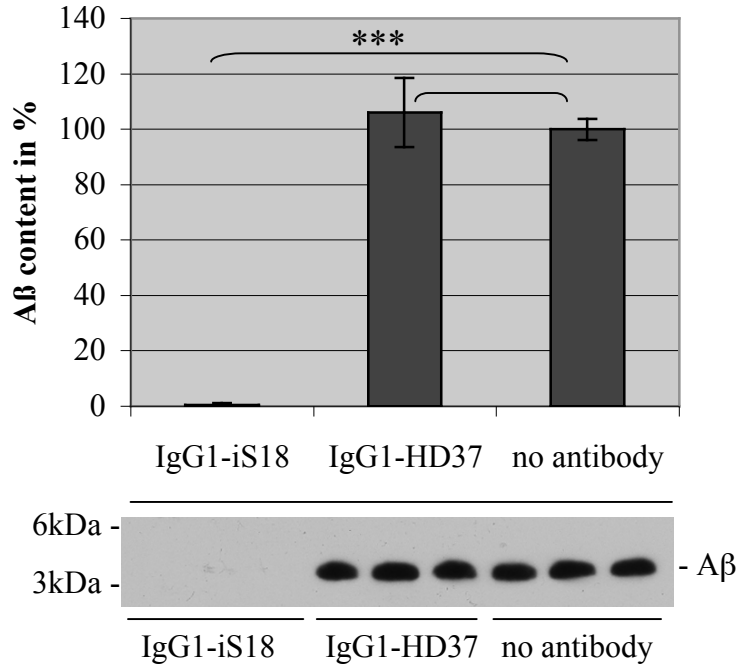

140
120
100
80
60
40
20
0
Aß content in %
***
IgG1-iS18
IgG1-HD37
no antibody
6kDa -
3kDa -
- Aβ
IgG1-iS18
IgG1-HD37
no antibody

Figure 3

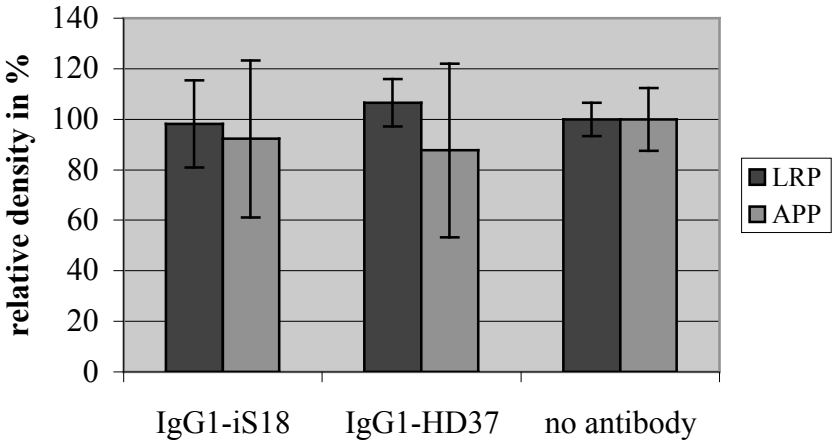
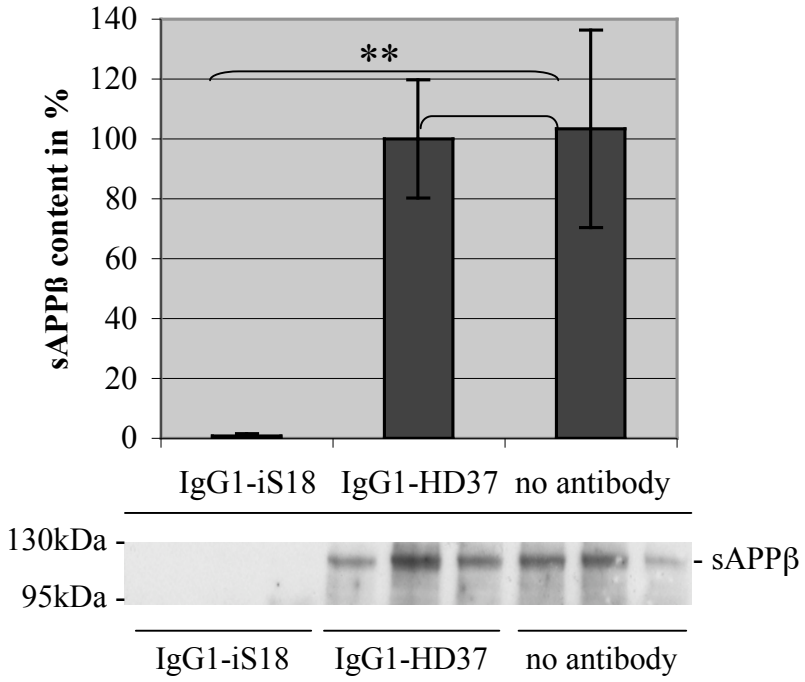

140
120
100
80
60
40
20
0
relative density in %
IgG1-iS18
IgG1-HD37
no antibody
LRP
APP

Figure 4


140
120
100
80
60
40
20
0
sAPPß content in %
**
IgG1-iS18
IgG1-HD37
no antibody
130kDa -
95kDa -
- sAPPβ
IgG1-iS18
IgG1-HD37
no antibody

Chapter VII

Subcellular localization of prion proteins and the 37kDa/67kDa laminin receptor fused to fluorescent proteins

published as:

Nikles, D., Vana, K., Gauczynski, S., Knetsch, H., Ludewigs, H. and Weiss, S., Subcellular localization of prion proteins and the 37kDa/67kDa laminin receptor fused to fluorescent proteins. *Biochim Biophys Acta* 2008. 5: 335-340.

Biochimica et Biophysica Acta 1782 (2008) 335–340

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Subcellular localization of prion proteins and the 37 kDa/67 kDa laminin receptor fused to fluorescent proteins

Daphne Nikles [1], Karen Vana [1], Sabine Gauczynski, Heike Knetsch, Heike Ludewigs, Stefan Weiss *

Laboratorium für Molekulare Biologie — Genzentrum-Institut für Biochemie der LMU München, Feodor-Lynen-Str. 25, 81377 München, Germany

ARTICLE INFO

Article history:
Received 23 January 2008
Received in revised form 12 February 2008
Accepted 12 February 2008
Available online 11 March 2008

Keywords:
Prion protein
PrP
Laminin receptor
LRP/LR
Subcellular localization
Cancer
Heterochromatin

ABSTRACT

The 37 kDa/67 kDa laminin receptor LRP/LR acts as a receptor for both PrP^{c} and PrP^{Sc} at the cell surface. Here, we further analyzed the subcellular localization of fluorescent labeled prion protein (PrP) and laminin receptor (LRP/LR) molecules. We show that EGFP-PrP is localized at the cell surface and in a perinuclear compartment, respectively. In contrast, a DsRed-ΔSP-PrP mutant lacking the signal peptide is almost exclusively found in the nucleus but does not colocalize with heterochromatin. Interestingly, LRP-DsRed efficiently colocalizes with EGFP-PrP in the perinuclear compartment and LRP-ECFP partly colocalizes with DsRed-ΔSP-PrP in the nucleus, respectively. We conclude that the interactions of PrP and LRP/LR are not restricted to the cell surface but occur also in intracellular compartments suggesting a putative role of LRP/LR in the trafficking of PrP molecules.

1. Introduction

The cellular prion protein PrP^{c} is one of the most studied proteins in eukaryotic cell biology, because it is not only the central player in prion diseases but it unraveled how an infectious agent could consist of protein only. The so-called protein-only hypothesis, which was formulated by Stanley B. Prusiner in 1982 [1] is nowadays well-grounded on recent studies [2,3]. However, it still remains an open task to determine the function of PrP^{c}, because *Prnp* knock-out mice showed no obvious phenotype [4]. There exist a tremendous number of postulated functions for PrP^{c} [5–11] including copper binding, signal transduction, neuroprotection and oxidative stress reactions.

Previously, a green fluorescent protein coupled PrP^{c} has been used in many studies for subcellular visualization of the protein [12–16]. In living cells, immunofluorescent studies showed an equal localization pattern compared to endogenous PrP [17]. Interestingly, a mutant version of PrP^{c} lacking its signal peptide translocates the protein from the endoplasmatic reticulum to the nucleus [18]. However, the finding cannot be explained by the presence of nuclear localization signals within the protein sequence [18]. Since PrP^{c} has RNA binding properties [19], the nuclear localization of truncated PrP^{c} may be of physiological relevance.

The 37 kDa/67 kDa laminin receptor (LRP/LR) plays a major role within the cellular functions of PrP^{c}. The protein is a multifunctional type II transmembrane protein with roles in ribosomal transcription, cell attachment, tumor metastasis formation and functions as a receptor for various viruses (for review: [20]). LRP/LR acts as a cell surface receptor for PrP^{c} [21] and for infectious prions [22,23]. Recently, several approaches for prion disease therapy targeting LRP/LR have been described (for review: [20,24,25]. These include (i) antibodies directed against the 37 kDa/67 kDa LRP/LR [26,27], (ii) siRNAs targeting the 37 kDa LRP mRNA [28] (Ludewigs et al., in preparation), (iii) transdominant negative LRP/LR mutants acting as a decoy [29] and (iv) polysulfated glycanes [22]. Blocking or down-regulation of LRP/LR by these molecular tools reduced invasion of tumorigenic fibrosarcoma cells (HT1080) recommending these molecules as alternative therapeutics for prevention of cancer metastasis and tumor progression [30]. In the present study, we used both fluorescence-coupled PrP^{c} and LRP/LR to visualize both proteins after transfection in eukaryotic cells. We generated cyan fluorescent LRP (LRP-ECFP), red fluorescent LRP (LRP-DsRed) and green fluorescent LRP (LRP-EGFP), respectively, as well as a red fluorescent prion protein mutant lacking its signal peptide (DsRed-ΔSP-PrP). In addition, we used full-length prion protein coupled to the green fluorescent protein (PrP-EGFP) [17]. Here, we describe the subcellular localization of these proteins after transient transfection in two different cell lines employing confocal immunofluorescence (IF) microscopy. We confirm that PrP-EGFP is localized in a perinuclear compartment and on the

* Corresponding author. Genzentrum-Institut für Biochemie der LMU München, Feodor-Lynen-Str. 25, D-81377 München. Tel.: +49 89 2180 76887; fax: +49 89 2180 76999.
E-mail address: weiss@lmb.uni-muenchen.de (S. Weiss).

Abbreviations: LRP/LR, laminin receptor precursor/laminin receptor; EGFP, enhanced green fluorescent protein; ECFP, enhanced cyan fluorescent protein; PrP, prion protein; PrP^{c}, cellular form of PrP; *Prnp*, PrP gene; ΔSP-PrP mutant, PrP mutant lacking its signal peptide; kDa, kilodalton; siRNA, small interfering RNA

[1] These authors contributed equally to this work.

0925-4439/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbadis.2008.02.003

cell surface. Moreover, we show that DsRed-ΔSP-PrP is translocated into the nucleus without colocalization with heterochromatin. Additionally, we observe a colocalization of LRP-DsRed and EGFP-PrP in the perinuclear compartment and a partly colocalization of LRP-ECFP and DsRed-ΔSP-PrP in the nucleus suggesting a potential role of PrP^c in gene regulation.

2. Material and methods

2.1. Plasmid constructions

For the generation of the plasmid pDsRed-ΔSP-PrP, the coding sequence for DsRed was amplified from the plasmid pDsRed-Express-N1 (Clonetech) using the oligonucleotides 5′-AgeI-DsRed (5′-GTCAAACCGGTCGCCACCATGGC-3′) and 3′-BglII-DsRed (5′-GTCAAAGATCTCA GGAACAGGTGGTGGCG-3′) and subsequently inserted into the target vector pEGFP-PrP [17] using the restriction sites AgeI and BglII. The plasmid pLRP-DsRed was cloned by inserting the murine laminin receptor coding sequence (amplified from pSFV1-moLRP::FLAG [22]) into the target vector pDsRed-Express-N1 (Clonetech) by use of BamHI- and SmaI-coding oligonucleotides 5′-BamHI-LRP and 3′-SmaI-LRP (5′-GTCAAGGATCCGCCACCCATGTCC-3′, 5′-GTCAACCCGGGGCCACCATGTCCGGAGCC-3′). The plasmid pLRP-EGFP was generated by insertion of the LRP cassette amplified from pSFV1-moLRP::FLAG using the oligonucleotides 5′-XmaI-LRP (5′-GTCAACCCGGGCCACCATGTCCGGAGCC-3′) and 3′-BamHI-moLRP (5′-GTCAAGGGATCCGGACCACTCAGTGGTGGC-3′) into the plasmid pEGFP-N3. The vector pLRP-ECFP was cloned in two steps. First, pECFP-N1 plasmid was generated via exchange of DsRed with the ECFP cassette amplified from pECFP-Endo vector (Clonetech) with the primers 5′-AgeI-ECFP (5′-GTCAAACCGGTCGCCACCATGGTG-3′) and 3′-NotI-ECFP (5′-GTCCAGCGGCCGCTACTTGTACAGCTCGTCCATG-3′) and subsequent insertion into pDsRed-Express-N1 vector. In the second step, the murine LRP sequence was inserted into the newly generated vector pECFP-N1 by restriction of the pLRP-DsRed plasmid using the restriction enzymes XmaI and AgeI. All generated plasmid sequences were verified by dideoxy sequencing.

2.2. Cell culture and transfections

Baby hamster kidney (BHK, ATCC CCL-10) and murine neuroblastoma cells (N2a, ATCC CCL-131) were cultivated in DMEM (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) and 1% non-essential amino acids. For calcium phosphate transfection, cells were plated at 30% confluency in 6 well plates prepared to contain one coverslip per well and were incubated overnight to enable cell attachment. In total, 8 μg of mixed plasmids plus 9 μl (2.5 M) calcium chloride completed to 90 μl with ultrapure water was prepared. Next, 90 μl of sterile filtered HBS buffer (sodium chloride 281 mM, HEPES 100 mM, sodium phosphate 1.5 mM, pH 7.12) was added while plasmids were vortexed for 1 min and precipitates were added onto the cells containing 25 μM chloroquin in DMEM. Media were changed once after 8 h and a second time after overnight incubation. Transfection experiments were carried out in three individual experiments.

2.3. Western blot analyses

48 h after transfection, cells were detached by trypsin (0.1% in PBS) lysed in 50 μl lysis buffer (10 mM Tris/HCl pH 7.5, 100 mM NaCl, 10 mM EDTA, 0,5% Nonidet P-40, 0,5% Desoxycholate) and incubated on ice for 30 min. Samples were centrifuged at 14,000 rpm in an Eppifuge (Heraeus) and supernatant was mixed with SDS sample buffer. Samples were boiled at 95 °C for 5 min and subjected to SDS polyacrylamide gel electrophoresis using a 12% separation gel. Samples were blotted on nitrocellulose membranes and blocked by horse serum (20% in PBS) followed by incubation with either anti-PrP antibody SAF 32 (1:5000) [31] or anti-LRP antibody W3 (1:5000) [21]. Blots were then incubated with the secondary anti-mouse and anti-rabbit HRP conjugated antibody (Sigma) (1:5000), respectively, following detection using Super-Signal detection kit (Pierce).

2.4. Immune fluorescence and confocal microscopy

24–48 h post transfection cells were fixed using 4% paraformaldehyde for 20 min at room temperature and were stained by 10 μg per ml DAPI for 10 min at room temperature. After threefold PBS wash cells were embedded using confocal matrix (MicroTechlab). The confocal analyses were carried out on a Leica DM IRE2 confocal microscope using the Leica confocal software TCS SP2 version 2.5.

3. Results

3.1. Cloning and expression of the fluorescence labeled prion protein and the 37 kDa/67 kDa laminin receptor

The fluorescent fusion proteins were generated on the basis of pEGFP-N1 and pDsRed-N1 as well as pECFP-C1 and pDsRed-C1 vector backbones, respectively. The insertion resulted in fluorescence-coupled variants of the mouse prion protein and the murine laminin receptor fusion proteins (Fig. 1A). The enhanced green fluorescent protein (EGFP) was placed in frame between the prion protein-derived signal peptide [9] to obtain the EGFP-PrP fusion protein. The variant lacking the signal peptide was fused to the C-terminus of the DsRed sequence (DsRed-ΔSP-PrP). In case of the laminin receptor (LRP/LR) fusion proteins, the fluorescent proteins were fused in frame to the C-terminal end of LRP/LR to obtain LRP-ECFP, LRP-DsRed and LRP-EGFP, respectively. The expression of the prion protein fusion protein variants EGFP-PrP and DsRed-ΔSP-PrP in BHK cells was confirmed by Western blotting showing that both proteins were present in cell lysates (Fig. 1B, left panel, lanes 1, 2). The EGFP-PrP was typically glycosylated showing a three band pattern

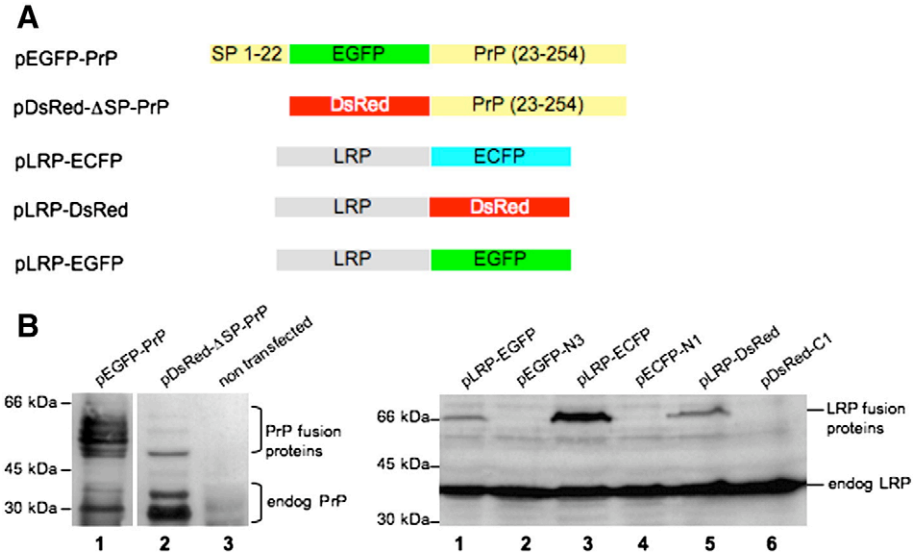


Fig. 1. Expression of the PrP fusion proteins and the 37 kDa/67 kDa laminin receptor in mammalian cells. (A) Fluorescent gene fusion constructs were generated and transiently transfected into BHK cells to investigate the subcellular localization of PrP and LRP/LR, respectively. The cDNA for the signal peptide (SP) of murine PrP^c was cloned N-terminally, the cDNA encoding amino acids 23–254 C-terminally to EGFP and DsRed. The sequence of murine LRP was cloned N-terminally to the ECFP, DsRed and EGFP proteins, respectively. (B) Expression of PrP derived fusion proteins was analyzed 48 h post transfection (left panel). Both EGFP-PrP (lane 1) and DsRed-DSP-PrP (lane 2) in comparison to non-transfected cells (lane 3) were detected using the PrP-specific mAb SAF 32. Expression of LRP derived fusion proteins was analyzed in BHK cells 48 h post transfection (right panel). LRP-ECFP derived cell lysate (lane 1) and LRP-DsRed derived cell lysate (lane 3) were compared to mock-transfected cells (lanes 2, 4, 6) using the polyclonal anti-LRP serum W3.

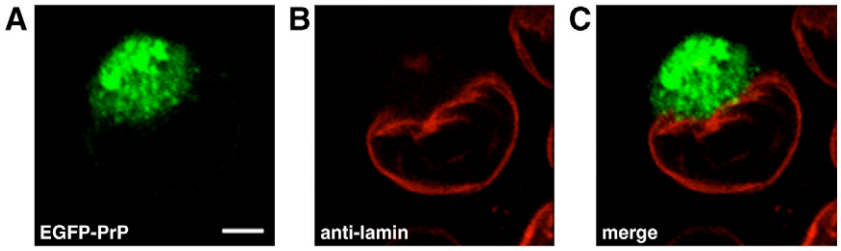


Fig. 2. Localization of green fluorescent protein coupled prion protein (EGFP-PrP) in a perinuclear compartment. (A) EGFP-PrP was examined 48 h post transfection in BHK cells using confocal microscopy. (B) Co-staining of the nucleic lamina with a lamin B-specific antibody followed by incubation with a secondary antibody conjugated with indocarbocyanine Cy3. (C) Merge of A and B. The bar represents 4 μm.

between 48 kDa and 60 kDa (Fig. 1B, left panel, lane 1), whereas the variant lacking the signal peptide was unglycosylated (Fig. 1B, left panel, lane 2). The increased molecular weight of the PrP proteins resulted from the EGFP fusion that has a molecular weight of approximately 35 kDa. Moreover, the specificity of the antibody stain was confirmed using non-transfected control cell lysate (Fig. 1B, left panel, lane 3). In addition, the expression of the LRP/LR fusion proteins LRP-ECFP (Fig. 1B, right panel, lane 1), LRP-DsRed (Fig. 1B, right panel, lane 5) and

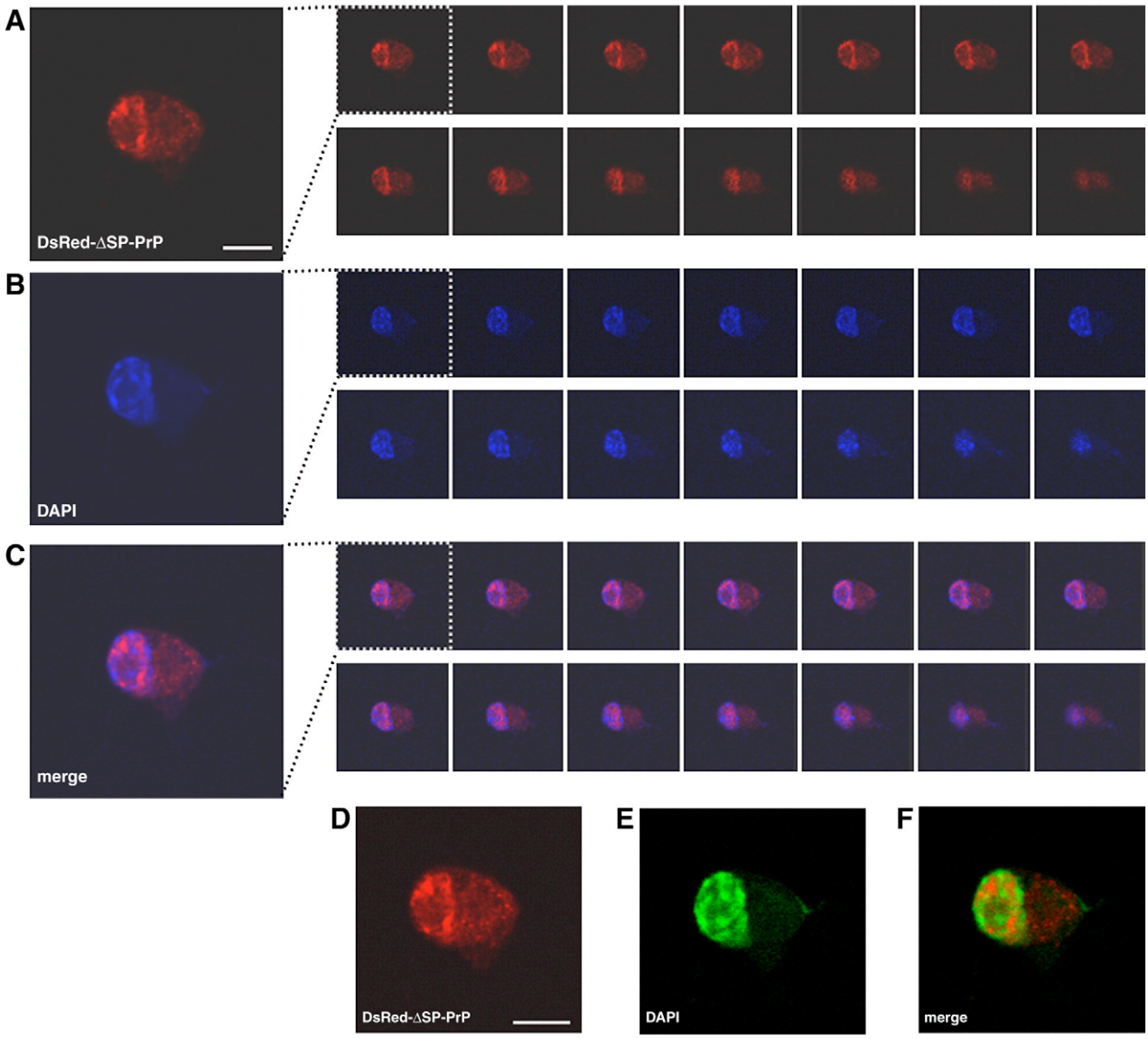


Fig. 3. The prion protein mutant DsRed-ΔΔSP-PrP does not colocalize with heterochromatin in the nucleus. (A) N2a cells were transiently transfected with DsRed-ΔSP-PrP and analyzed by confocal microscopy. *Z* sections through an individual cell were performed varying about 2.8 μm in the *z* plane in total (individual steps 0.2 μm). (B) The same cell was simultaneously stained by DAPI (10 μg/ml) and analyzed for heterochromatin/DNA staining, respectively. (C) Merge of A and B. The bar represents 6 μm. (D) N2a cells were transfected with DsRed-ΔSP-PrP and analyzed by confocal microscopy. (E) Simultaneously, the same cell was stained with DAPI (10 μg/ml) and analyzed for heterochromatin/DNA staining using the EGFP channel for better visualization. (F) Channels were merged. The bar represents 8 μm.

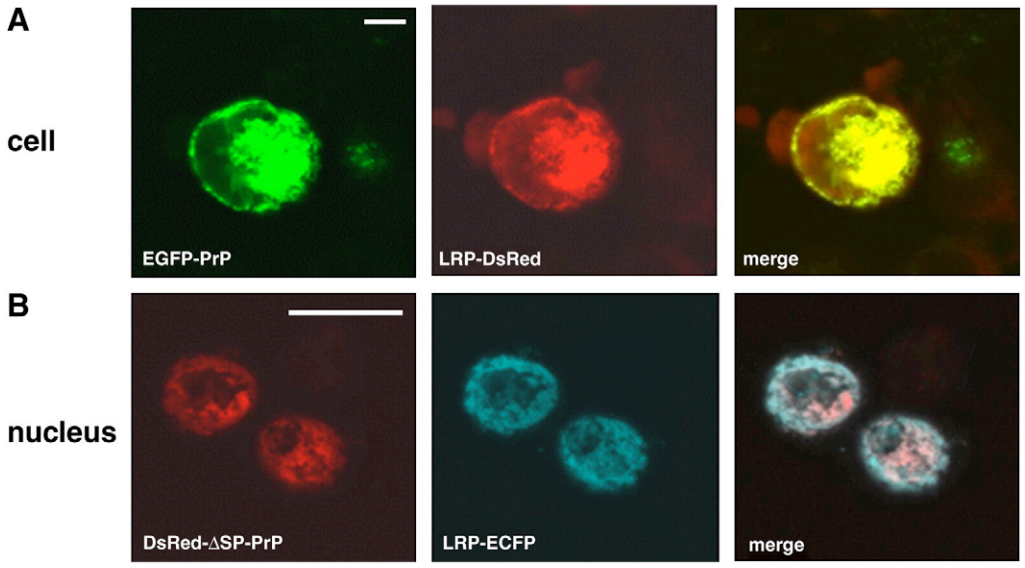


Fig. 4. Colocalization of the 37 kDa/67 kDa laminin receptor (LRP/LR) with green fluorescent protein coupled prion protein (EGFP-PrP) and in part-colocalization with the mutant prion protein DsRed-ΔSP-PrP. (A) BHK cells were transiently cotransfected with pEGFP-PrP and pLRP-DsRed. 48 h post transfection cells were fixed and the distribution of fluorescent proteins was analyzed by confocal microscopy. Green channel (left pannel), red channel (middle pannel) and merge (right pannel). (B) BHK cells were transiently cotransfected with pDsRed-ΔSP-PrP and pLRP-ECFP. 48 h post transfection cells were fixed and the distribution of fluorescent proteins was analyzed by confocal microscopy. Red channel (left pannel), blue channel (middle pannel) and merge (right pannel). The bar represents 20 μm.

LRP-EGFP (Fig. 1B, right panel, lane 3), respectively, has been verified employing the polyclonal LRP antibody W3 [25,32].

3.2. EGFP-PrP accumulates in the perinuclear compartment

EGFP-PrP was transfected into BHK cells and subcellular localization was examined 24 h post transfection by confocal fluorescence microscopy. In cells expressing high levels of the transgene an accumulation of EGFP-PrP in an endosomal compartment (Fig. 2A) which was localized in close proximity to the nucleus was observed (Fig. 2B, C). This finding is supported by previous publications demonstrating that EGFP-PrP is localized in either Golgi compartments or, after internalization of PrP^c, in endosomal compartments including an accumulation in the perinuclear compartment [16,17].

3.3. DsRed-ΔSP-PrP is localized in the nucleus and does not colocalize with heterochromatin

The mutant PrP DsRed-ΔSP-PrP lacking the signal peptide revealed a distinct localization pattern (Fig. 2B) in neuronal cells (N2a). The mutant PrP variant did not colocalize with EGFP-PrP (data not shown) and was localized in the nucleus and not in the cytoplasm (Fig. 3 A). The nucleic localization of PrP lacking its signal peptide has been reported previously in a study using antibody-based fluorescent-staining [18]. Here, we confirm this finding by transient transfection of DsRed-ΔSP-PrP in BHK cells (data not shown) in addition to N2a cells (Fig. 3). However, upon co-staining of the DNA with DAPI (Fig. 3B, E) no colocalization of red fluorescent DsRed-ΔSP-PrP and the heterochromatin (Fig. 3C) was detected. Due to the indistinct analysis of the merge of the blue DAPI stain and the red fluorescence of DsRed-ΔSP-PrP, the filter of the confocal microscope was changed from blue to green for the visualization of DAPI (Fig. 3E, F). Our finding implicates that the herein used DsRed-ΔSP-PrP does not colocalize with heterochromatin.

3.4. The 37 kDa/67 kDa laminin receptor colocalizes with EGFP-PrP and partly colocalizes with DsRed-ΔSP-PrP in the nucleus

Furthermore, we investigated the distribution of the 37 kDa/67 kDa laminin receptor (LRP/LR) upon cotransfection using the different PrP variants. Cotransfection of EGFP-PrP and LRP-DsRed resulted in a colocalization in the perinuclear compartment and on the cell surface (Fig. 4A). In earlier studies we showed that PrP^c and LRP/LR colocalize on the cell surface of neuroblastoma cells [21]. In addition, LRP-ECFP also localized in the nucleus of cotransfected DsRed-ΔSP-PrP cells. This finding argues that upon expression of the PrP mutant, LRP/LR was partly directed to or localized to the nucleus (Fig. 4B) and not localizing within the perinuclear compartment. Previously, it has been shown that LRP/LR is widely spread through all cellular compartments including the cytoplasm, the nucleus and the cell surface where it contributes to its multifunctional role in cell biology [33–35]. Although a typical colocalization pattern was not observed, we found that LRP-ECFP and DsRed-ΔSP-PrP were partly colocalized in the nucleus (Fig. 4B).

4. Discussion

4.1. Putative role of the prion protein mutant DsRed-ΔSP-PrP in the nucleus

The eukaryotic cell nucleus is organized within subnuclear compartments including the nucleic envelope, the eu- and heterochromatin as well as the nucleoli as sites of ribosome production and assembly. A variety of proteins are localized in the nucleus and are part of the ribosomal, replication or transcription machinery and the spliceosome, respectively. However, a putative role of a prion protein located in the nucleus, in case its signal peptide is genetically removed, is highly speculative. So far, there is no evidence that PrP lacking its signal peptide is present in the nucleus under physiological conditions. Nevertheless, the nuclear localization of DsRed-ΔSP-PrP is astonishing. As it was previously discussed, there is no evidence, that a nuclear localization signal is responsible for such action [18]. Until now it is speculative how PrP in the absence of its signal peptide targets the nucleus. Further analyses are required to unveil this mode of action.

4.2. The 37 kDa/67 kDa laminin receptor and the prion protein colocalize intracellularly

In the present report, we show for the first time the intracellular colocalization of LRP/LR with PrP. We hypothesize that the nucleic

localization of the mutant PrP might be based on the passive transport via an interaction with another protein. Such an interaction partner might be the 37 kDa/67 kDa laminin receptor LRP/LR which has been identified as the cell surface receptor for the cellular PrP and infectious prions [21–23]. To investigate this possibility, we generated fluorescent LRP variants, which were used for colocalization studies with both EGFP-PrP and DsRed-ΔSP-PrP. Our results are consistent with the wide cellular localization pattern of LRP-EGFP including the nucleus, the cytoplasm and the cell surface. Previously, it has been reported that LRP/LR, which is basically identical with the ribosomal protein p40 playing an important role in the translation process, is transported into the nucleus under specialized conditions [36]. It has further been shown that LRP/LR is located inside the nucleus, beside cytoplasmic and cell surface localization, where it binds to histones [34,35]. Further experiments are required to enlighten the unknown mechanism by which cytoplasmic PrP variants are targeted to the nucleus.

Since internal nuclear localization signals are not involved in nucleic import of the prion protein lacking the signal peptide [18], an alternative way of nuclear import may involve an adapter protein that contains a nuclear localization signal. It has been shown that the 37 kDa LRP is directed to the cellular nucleus after addition of midkine, a heparin binding growth factor [36,37]. According to the binding ability of PrP to LRP [21,38], we speculate that the nuclear localization of the PrP variant DsRed-ΔSP-PrP might be also dependent on midkine. This explanation, as well as the multifunctional role of LRP/LR in the cell, may account for our finding that depletion of LRP/LR does not totally impede nucleic transport of the mutant PrP. This issue shall be further addressed in cell biology studies to unravel the yet unidentified mode of action by which mutant PrP is transported into the nucleus.

4.3. Subcellular Localization of the mutant prion protein lacking the signal peptide

Previously, Crozet et al. described a chromatin association for the prion protein lacking its signal peptide [18]. However, the chromatin-removal method has some limitations which might not reveal reliable information on localization in vivo [39]. Here, we performed an additional colocalization experiment by the use of the DNA stain DAPI to confirm the chromatin association of mutant PrP. Astonishingly, we could barely find a colocalization of DAPI stained DNA with DsRed-ΔSP-PrP. This finding clearly argues against a tight chromatin binding of DsRed-ΔSP-PrP, because the distance of two molecules lacking colocalization clearly exceeds 200 nm in the *x*/*y* plane and even more than 400 nm in the *z* plane. The mutant protein is found in nucleic compartments containing a low DNA content, which may be nucleoli structures. Therefore, further experiments, in which subnuclear structures are specifically co-stained will be needed to investigate the subnuclear localization of DsRed-ΔSP-PrP.

In summary, we have confirmed, that EGFP-PrP is localized at the cell surface and in a perinuclear compartment within the cell and that mutant DsRed-ΔSP-PrP localized to the nucleus. Interestingly, we found that the latter PrP mutant failed to be colocalized with DAPI stained heterochromatin. Furthermore, in the present study we showed for the first time that (i) PrP-EGFP and LRP-DsRed colocalize in the perinuclear compartment in addition to a cell surface-colocalization and (ii) DsRed-ΔSP-PrP and LRP-ECFP partly colocalize in the nucleus, respectively. Thus, the 37 kDa/67 kDa LRP/LR and the prion protein reveal close proximity not only on the surface of eukaryotic cells but additionally in intracellular compartments. Investigations by immunoprecipitation or cellular fractionation will further unravel the role of PrP, PrP mutants and LRP/LR in the life cycle of prions.

Acknowledgements

We thank Dr. Kil Sun Lee from Brazil for the kind gift of the pEGFP-PrP plasmid. The work was supported by the Bundesministerium für Bildung und Forschung (grant 01-KO-0514), the European Commission (grant NoE-NeuroPrion FOOD-CT 2004-506579) and the Deutsche Forschungsgemeinschaft (grant WE 2664/2-1).

References

[1] S.B. Prusiner, Novel proteinaceous infectious particles cause scrapie, Science 216 (1982) 136–144.
[2] J. Castilla, P. Saa, C. Hetz, C. Soto, In vitro generation of infectious scrapie prions, Cell 121 (2005) 195–206.
[3] G. Legname, I.V. Baskakov, H.O. Nguyen, D. Riesner, F.E. Cohen, S.J. DeArmond, S.B. Prusiner, Synthetic mammalian prions, Science 305 (2004) 673–676.
[4] H. Bueler, M. Fischer, Y. Lang, H. Bluethmann, H.P. Lipp, S.J. DeArmond, S.B. Prusiner, M. Aguet, C. Weissmann, Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein, Nature 356 (1992) 577–582.
[5] B.T. Bragason, A. Palsdottir, Interaction of PrP with NRAGE, a protein involved in neuronal apoptosis, Mol. Cell. Neurosci. 29 (2005) 232–244.
[6] G.N. Hajj, M.H. Lopes, A.F. Mercadante, S.S. Veiga, R.B. da Silveira, T.G. Santos, K.C. Ribeiro, M.A. Juliano, S.G. Jacchieri, S.M. Zanata, V.R. Martins, Cellular prion protein interaction with vitronectin supports axonal growth and is compensated by integrins, J. Cell Sci. 120 (2007) 1915–1926.
[7] B. Krebs, A. Wiebelitz, B. Balitzki-Korte, N. Vassallo, S. Paluch, G. Mitteregger, T. Onodera, H.A. Kretzschmar, J. Herms, Cellular prion protein modulates the intracellular calcium response to hydrogen peroxide, J. Neurochem. 100 (2007) 358–367.
[8] A. Li, P. Piccardo, S.J. Barmada, B. Ghetti, D.A. Harris, Prion protein with an octapeptide insertion has impaired neuroprotective activity in transgenic mice, EMBO J. 26 (2007) 2777–2785.
[9] G. Mitteregger, M. Vosko, B. Krebs, W. Xiang, V. Kohlmannsperger, S. Nolting, G.F. Hamann, H.A. Kretzschmar, The role of the octarepeat region in neuroprotective function of the cellular prion protein, Brain Pathol. 17 (2007) 174–183.
[10] E. Morel, S. Fouquet, D. Chateau, L. Yvernault, Y. Frobert, M. Pincon-Raymond, J. Chambaz, T. Pillot, M. Rousset, The cellular prion protein PrPc is expressed in human enterocytes in cell–cell junctional domains, J. Biol. Chem. 279 (2004) 1499–1505.
[11] E. Paitel, C. Sunyach, C. Alves da Costa, J.C. Bourdon, B. Vincent, F. Checler, 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 (2004) 612–618.
[12] S. Barmada, P. Piccardo, K. Yamaguchi, B. Ghetti, D.A. Harris, GFP-tagged prion protein is correctly localized and functionally active in the brains of transgenic mice, Neurobiol. Dis. 16 (2004) 527–537.
[13] S.J. Barmada, D.A. Harris, Visualization of prion infection in transgenic mice expressing green fluorescent protein-tagged prion protein, J. Neurosci. 25 (2005) 5824–5832.
[14] Y. Gu, S. Verghese, R.S. Mishra, X. Xu, Y. Shi, N. Singh, Mutant prion protein-mediated aggregation of normal prion protein in the endoplasmic reticulum: implications for prion propagation and neurotoxicity, J. Neurochem. 84 (2003) 10–22.
[15] N.S. Hachiya, K. Watanabe, M. Yamada, Y. Sakasegawa, K. Kaneko, Anterograde and retrograde intracellular trafficking of fluorescent cellular prion protein, Biochem. Biophys. Res. Commun. 315 (2004) 802–807.
[16] A.C. Magalhaes, J.A. Silva, K.S. Lee, V.R. Martins, V.F. Prado, S.S. Ferguson, M.V. Gomez, R.R. Brentani, M.A. Prado, Endocytic intermediates involved with the intracellular trafficking of a fluorescent cellular prion protein, J. Biol. Chem. 277 (2002) 33311–33318.
[17] K.S. Lee, A.C. Magalhaes, S.M. Zanata, R.R. Brentani, V.R. Martins, M.A. Prado, Internalization of mammalian fluorescent cellular prion protein and N-terminal deletion mutants in living cells, J. Neurochem. 79 (2001) 79–87.
[18] C. Crozet, J. Vezilier, V. Delfieu, T. Nishimura, T. Onodera, D. Casanova, S. Lehmann, F. Beranger, The truncated 23–230 form of the prion protein localizes to the nuclei of inducible cell lines independently of its nuclear localization signals and is not cytotoxic, Mol. Cell. Neurosci. 32 (2006) 315–323.
[19] E. Derrington, C. Gabus, P. Leblanc, J. Chnaidermann, L. Grave, D. Dormont, W. Swietnicki, M. Morillas, D. Marck, P. Nandi, J.L. Darlix, PrPC has nucleic acid chaperoning properties similar to the nucleocapsid protein of HIV-1, C. R. Biol. 325 (2002) 17–23.
[20] K. Vana, C. Zuber, D. Nikles, S. Weiss, Novel aspects of prions, their receptor molecules, and innovative approaches for TSE therapy, Cell. Mol. Neurobiol. 27 (2007) 107–128.
[21] S. Gauczynski, J.M. Peyrin, S. Haik, C. Leucht, C. Hundt, R. Rieger, S. Krasemann, J.P. Deslys, D. Dormont, C.I. Lasmezas, S. Weiss, The 37-kDa/67-kDa laminin receptor acts as the cell-surface receptor for the cellular prion protein, EMBO J. 20 (2001) 5863–5875.
[22] S. Gauczynski, D. Nikles, S. El-Gogo, D. Papy-Garcia, C. Rey, S. Alban, D. Barritault, C.I. Lasmezas, S. Weiss, The 37-kDa/67-kDa laminin receptor acts as a receptor for infectious prions and is inhibited by polysulfated glycanes, J. Infect. Dis. 194 (2006) 702–709.
[23] E. Morel, T. Andrieu, F. Casagrande, S. Gauczynski, S. Weiss, J. Grassi, M. Rousset, D. Dormont, J. Chambaz, Bovine prion is endocytosed by human enterocytes via the 37 kDa/67 kDa laminin receptor, Am. J. Pathol. 167 (2005) 1033–1042.
[24] H. Ludewigs, C. Zuber, K. Vana, D. Nikles, I. Zerr, S. Weiss, Therapeutic approaches for prion disorders, Expert Rev. Anti Infect. Ther. 5 (2007) 613–630.
[25] C. Zuber, H. Ludewigs, S. Weiss, Therapeutic approaches targeting the prion receptor LRP/LR, Vet. Microbiol. 123 (2007) 387–393.
[26] C. Zuber, S. Knackmuss, C. Rey, U. Reusch, P. Rottgen, T. Frohlich, G.J. Arnold, C. Pace, G. Mitteregger, H.A. Kretzschmar, M. Little, S. Weiss, Single chain Fv antibodies

directed against the 37 kDa/67 kDa laminin receptor as therapeutic tools in prion diseases, Mol. Immunol. 45 (2008) 144–151.
[27] C. Zuber, G. Mitteregger, C. Pace, H.A. Kretzschmar, I. Zerr, S. Weiss, Anti-LRP/LR antibody W3 hampers peripheral PrPSc propagation in scrapie infected mice, Prion 1 (2007) 207–212.
[28] C. Leucht, S. Simoneau, C. Rey, K. Vana, R. Rieger, C.I. Lasmezas, S. Weiss, The 37 kDa/67 kDa laminin receptor is required for PrP(Sc) propagation in scrapie-infected neuronal cells, EMBO Rep. 4 (2003) 290–295.
[29] K. Vana, S. Weiss, A trans-dominant negative 37 kDa/67 kDa laminin receptor mutant impairs PrP(Sc) propagation in scrapie-infected neuronal cells, J. Mol. Biol. 358 (2006) 57–66.
[30] C. Zuber, S. Knackmuss, G. Zemora, U. Reusch, E. Vlasova, D. Diehl, V. Mick, K. Hofmann, D. Nikles, T. Fröhlich, G. Arnold, B. Brenig, E. Wolf, H. Lahm, M. Little, S. Weiss, Invasion of tumorigenic HT1080 cells is impeded by downregulating or blocking the 37 kDa/67 kDa laminin receptor, J. Mol. Biol. (in press). doi:10.1016/j.jmb.2008.02.004.
[31] M. Ito, M. Shinagawa, S. Doi, S. Sasaki, H. Isomura, K. Takahashi, H. Goto, G. Sato, Effects of the antiserum against a fraction enriched in scrapie-associated fibrils on the scrapie incubation period in mice, Microbiol. Immunol. 32 (1988) 749–753.
[32] R. Rieger, F. Edenhofer, C.I. Lasmezas, S. Weiss, The human 37-kDa laminin receptor precursor interacts with the prion protein in eukaryotic cells, Nat. Med. 3 (1997) 1383–1388.
[33] Y. Kaneda, K. Kinoshita, M. Sato, K. Tanaka, Y. Kaneda, The analysis of 40 kDa nuclear protein, p40, in interphase cells and mitotic cells, J. Cell Sci. 106 (Pt 3) (1993) 741–748.
[34] K. Kinoshita, Y. Kaneda, M. Sato, Y. Saeki, M. Wataya-Kaneda, A. Hoffmann, LBP-p40 binds DNA tightly through associations with histones H2A, H2B, and H4, Biochem. Biophys. Res. Commun. 253 (1998) 277–282.
[35] M. Sato, K. Kinoshita, Y. Kaneda, Y. Saeki, A. Iwamatsu, K. Tanaka, Analysis of nuclear localization of laminin binding protein precursor p40 (LBP/p40), Biochem. Biophys. Res. Commun. 229 (1996) 896–901.
[36] R.H. Salama, H. Muramatsu, K. Zou, T. Inui, T. Kimura, T. Muramatsu, Midkine binds to 37-kDa laminin binding protein precursor, leading to nuclear transport of the complex, Exp. Cell Res. 270 (2001) 13–20.
[37] Y. Shibata, T. Muramatsu, M. Hirai, T. Inui, T. Kimura, H. Saito, L.M. McCormick, G. Bu, K. Kadomatsu, Nuclear targeting by the growth factor midkine, Mol. Cell. Biol. 22 (2002) 6788–6796.
[38] C. Hundt, J.M. Peyrin, S. Haik, S. Gauczynski, C. Leucht, R. Rieger, M.L. Riley, J.P. Deslys, D. Dormont, C.I. Lasmezas, S. Weiss, Identification of interaction domains of the prion protein with its 37-kDa/67-kDa laminin receptor, EMBO J. 20 (2001) 5876–5886.
[39] K.M. Wan, J.A. Nickerson, G. Krockmalnic, S. Penman, The nuclear matrix prepared by amine modification, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 933–938.

Abbreviations

°C	degree Celsius
A	Adenin
AAV	adeno-associated virus
Aβ	Amyloid beta
AD	Alzheimer´s disease
ADAM	A dis-integrin and metalloprotease
ApoEε4	Apolipoprotein εE4
APP	Amyloid beta precursor protein
BACE	Beta-site-APP-cleaving-enzyme
BCA	Bicinchoninic acid
BHK	Baby hamster kidney
bp	Base pair
BSE	Bovine spongiform encephalopathy
C	Cytosin
cDNA	Copy deoxyribonucleic acid
CJD	Creutzfeldt-jakob disease
CNS	Central nervous system
CWD	Chronic wasting disease
ΔSP	Delta signal peptide
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco´s modified eagle medium
DNA	Deoxyribonucleic acid
DOC	Deoxycholic acid
DsRed	Discosoma sp red fluorescent protein
ECFP	Enhanced cyan flourescent protein
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
EGFP	Enhanced green flourescent protein
et al.	And others
FCS	Fetal calf serum
FFI	Fatal familial insomnia
Fig.	Figure
FT	Fast growing, SV40 large T-antigen

FVB	*Fv1*^{b} allele transgenic mouse
g	Gram
G	Guanin
GPI	Glycosylphosphatidylinositol
GSS	Gerstmann-Sträussler-Scheinker
h	Hours
HBS	HEPES buffered saline
HCl	Hydrogen chloride
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HRP	Horseraddish peroxidase
HT1080	Human fibrosarcoma cell line
i.c.	Intracerebral
IF	Immunofluorescence
IgG	Immuneglobuline G
kDa	Kilo Dalton
l	Litre
LR	Laminin receptor
LRP	Laminin receptor precursor
M	Molar
mAb	Monoclonal antibody
mg	Milligram
min	Minutes
ml	Millilitres
mM	Millimolar
mRNA	Messenger RNA
µg	Microgram
µl	Microlitres
µm	Micrometer
µM	Micromolar
N2a	Mouse neuroblastoma cell line
N2aSc+	Scrapie-infected Mouse neuroblastoma cell line
NaCl	Sodium chloride

NFT	Neurofibrillary tangles
nm	Nanometer
NSE	Neuron specific enolase
PAGE	Polyacrylamide gelectrophoresis
PBS	Phosphate based saline
PBS-T	Phosphate based saline, supplemented with Tween 20
PCR	Polymerase chain reaction
p.i.	Post inoculation
PK	Proteinase K
POD	Peroxidase
PrP	Prion protein
PrP^{c}	Cellular prion protein
PrPres	PK-resistent Prion protein
PrP^{Sc}	Prion protein scrapie
PVDF	Polyvinylidene Fluoride
RML	Rocky Mountain Laboratories
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
rpm	Rounds per minute
SAF	Scrapie associated fibrils
sAPPα	Soluble Amyloid beta precursor protein alpha
sAPPβ	Soluble Amyloid beta precursor protein beta
scFv	Single chain fragment variable
SD	Standart deviation
SDS	Sodiumdodecyl sulfate
shRNA	Short hairpin ribonucleic acid
siRNA	Small interfering ribonucleic acid
T	Thymidin
tg	Transgenic
TME	Transmissible mink encephalopathy
TSE	Transmissible spongiform encephalopathy
TU	Transducing units
VEE	Venezuelan equine encephalomyelitis virus
wt	Wild-type

Curriculum vitae

Heike Pflanz, geb. Ludewigs
Nordendstrasse 17
80799 München

---

fon: +49-89-35895033
mobil: +49-177-5015503
eMail: heike@pflanz.de

Geburtstag: 14.04.1977
Geburtsort: Lüneburg
Familienstand: verheiratet
Staatsangehörigkeit: deutsch

Berufs- und Forschungserfahrung:

o5.2006-03.2009	Doktorarbeit am Genzentrum, Institut für Biochemie der Ludwig-Maximilians-Universität München, Doktorvater: Professor Dr. Stefan Weiss
05.2005-11.2006	Teilzeitanstellung als Apothekerin in der Apotheke am Forum in München
04.2005-03.2006	Doktorarbeit an der Psychiatrischen Klinik der LMU, Institut für Molekulare und Klinische Neurobiologie,Doktorvater: Professor Dr. Benedikt Grothe, Institut für Neurobiologie der LMU
06.2004-03.2005	Vollzeitanstellung als Apothekerin in der Aeskulap-Apotheke in Rüsselsheim mit dem Schwerpunkt Zytostatikaherstellung
05.2004-06.2004	Teilzeitanstellung als Apothekerin in der Apotheke im Hauptbahnhof in Frankfurt am Main

Curriculum vitae

2003	zweiwöchiges Praktikum während des 7. Fachsemesters Pharmazie bei Prof. Dr. Steinhilber im Institut für Pharmazeutische Chemie der Goethe-Universität Frankfurt am Main
04.2000-03.2003	Teilzeitanstellung als PTA in der Apotheke im Hauptbahnhof in Frankfurt am Main

---

Ausbildung:

28.05.2004	Approbation zur Apothekerin
11.2003-04.2004	Pharmaziepraktikum in der Apotheke im Hauptbahnhof in Frankfurt am Main mit dem Schwerpunkt Methadon-Programm
05.2003-10.2003	Pharmaziepraktikum bei OctoPlus Technologies in Holland mit dem Schwerpunkt pharmazeutische Technologie
04.1999-04.2003	Pharmaziestudium an der Goethe-Universität Frankfurt am Main
08.1998-02.1999	Praktikum in der Dorf –Apotheke Tespe im Rahmen der Ausbildung
08.1996-07.1998	PTA-Schule an der Dr. von Morgenstern-Schule in Lüneburg
1996	Abitur

Fremdsprachen:

Englisch	fließend
Französisch	Grundkenntnisse
Spanisch	Grundkenntnisse

Curriculum vitae

Kongresse und Fortbildungen:

10.2008	NeuroPrion in Madrid, Spanien, mit Posterpräsentation
10.2008	NoE NeuroPrion in Madrid, Spanien, mit Vortrag
03.2008	NoE NeuroPrion in München, mit Vortrag
04.2007	ForPrion in München, mit Posterpräsentation
09.2007	NeuroPrion in Edinburgh, Schottland, mit Posterpräsentation
04.2006	ForPrion in München, mit Posterpräsentation
10.2006	NeuroPrion in Turin, Italien, mit Posterpräsentation
10.2005	Kongress 24. Symposium der Arbeitsgemeinschaft Neuropsychopharmakologie und Pharmakopsychiatrie, mit Poster-Präsentation
12.2005	Fortbildungsveranstaltung gemäß §15 GenTSV zum Erwerb der Sachkunde als Projektleiter oder Beauftragter für die biologische Sicherheit