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Single chain antibodies against the 37 kDa/67 kDa laminin receptor as tools for prion diseases therapy

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aus

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Erklärung

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Ehrenwörtlich Versicherung

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SUMMARY

Prions are unconventional pathogens that cause transmissible spongiform encephalopathies (TSEs). According to the "protein only" hypothesis, prions consist of an infectious protein that is capable of converting a normal host protein termed PrP^{c} into a protease resistant form termed PrP^{Sc}. PrP^{Sc} is poorly degraded by the host and accumulates in the CNS. Normal biological functions of PrP^{c} and mechanisms involved in neurodegeneration remain obscure. During the past two decades, considerable efforts have been made to elucidate prion diseases and in particular to identify PrP interactors for a better understanding in prion biology. A major break-through was the identification of the 37 kDa laminin receptor (LRP), which represents the precursor of the human 67 kDa high-affinity laminin receptor (LR), as the cell surface receptor for the cellular prion protein.

We investigated the role of LRP/LR in the propagation of PrP^{Sc} in chronically infected cells by different approaches. Three strategies resulted in downregulation or blocking of LRP and prevented PrP^{Sc} accumulation in different scrapie infected neuronal cell lines (i) transfection with an antisense LRP RNA expression plasmid (ii) transfection with small interfering (siRNAs) specific for the LRP mRNA and (iii) incubation with the polyclonal anti-LRP antibody, W3. We observed that the treatment with W3 abolished PrP^{Sc} deposition and reduced PrP^{c} levels after one week of incubation. PrP^{Sc} did not reappear in cells being cultured for 14 additional days without therapeutic antibody treatment. Taken together, these results indicate that LRP is not only required for PrP^{c} metabolism under non-pathological conditions but also has a pivotal role in prion propagation in a cell culture model. LRP/LR appears then to be a promising potential target for the development of therapeutics for the treatment of prion disease.

Due to these encouraging cell culture data, we decided to select single chain antibodies (scFv) encompassing a suitable format for therapy. ScFvs are composed of variable parts of heavy and light chains of an immunoglobulin that are connected by a

peptidic linker. The antibodies were screened on recombinant GST::LRP employing a phage display strategy. Two scFvs termed N3 and S18 were screened and selected by ELISA. Both antibodies were further characterized by western blotting and FACS analysis: both N3 and S18 specifically recognized mouseLRP and humanLRP overexpressed in mammalian cells under denaturating conditions (western blot) and under native conditions at the cell surface (FACS). Epitope mapping revealed that as expected both scFvs are directed against the extracellular part of LRP: S18 and N3 recognized amino acid residues 225-233 and 273-278, respectively. The ability of N3 and S18 to interfere with LRP/PrP interaction was tested by pull-down assays. In contrast to the control scFv C9 directed against the pre-S1 coat-protein of hepatitis B virus, both anti-LRP scFvs were able to block the specific LRP/PrP binding. In order to investigate a potential curing effect of scFv S18 in vivo, this scFv was tested in a scrapie mouse model by passive immunization. The application of S18 by intra-peritoneal injection was able to reduce PrP^{Sc} deposition in the spleen in comparison to mice injected with PBS or C9. However the survival times of S18 treated animals was not increased. Anti-LRP scFv S18 seems to contribute to block prion propagation in the periphery but it is likely that this effect was not enough strong to have an impact on the CNS invasion. Thus, we hypothesized that a strategy targeting directly the brain should be more effective. In this context, an approach based on the expression of single chain antibodies as secretory molecules in the brain via an adeno-associated virus (AAV) vector was initiated.

To assure secretion of the scFv expressed in mammalian cells, a signal sequence was fused to the scFvs. Tranfection experiments demonstrated that neuronal cells were able to express and secrete high quantities of both scFvs. Furthermore, the generated scFvs were still functional as shown by western blotting. To find the appropriate AAV serotype for scFv expression, neuronal cells were transduced with varying serotypes carrying a GFP. AAV serotype 2 was chosen due to (i) its good transduction performance in two neuronal cell lines and (ii) the possibility of its purification by affinity chromatography. The sequences encoding for the scFvs N3, S18 and C9 have been cloned in an AAV-based vector. The AAV system was also able to drive high expression of scFvs into the supernatant by transfection or transduction. rAAV-scFv particles were

produced and purifed for further stereotaxic injections into mice. Although the investigation of this therapeutic strategy is still in progress in a murine scrapie model, we already proved that a single injection of rAAV led to the expression of scFvs into the brain of mice 30 days post injection. This study represents the first gene therapeutic approach for the treatment of prion diseases.

CHAPTER I

Prion and prion diseases: an overview

1 Historical background and the prion hypothesis

- The first transmissible spongiform encephalopathy (TSE) was described in 1732 in sheep and called scrapie. The german neurologists Creutzfeldt and Jakob observed in the 1920s patients with brain lesions such as spongiosis, astrocytosis and gliosis. This disease was termed Creutzfeldt-Jakob disease (CJD). In the 1950s, an epidemy termed Kuru decimated an aborigine population in New Guinea with similar histological patterns to these previously observed in CJD patients (Gajdusek and Zigas, 1959). Although the presence of viral particles or nucleic acids coud not be convincingly demonstrated, this group of disorders was classified at that time in the group of slow viral diseases (Gajdusek, 1977). Nevertheless, the idea that the causative agent might be a protein began to emerge in 1967, and was in complete contradiction with the very foundations of molecular biology (Griffith, 1967). This suggestion was developed by Prusiner in the prion hypothesis that paved the way into a fascinating new biological phenomenon. In 1982, he coined the term Prion to describe the **pro**teinascous **in**fectious particles responsible for scrapie in sheeps and hamsters. Prusiner suggested that scrapie and some other close diseases, some inherited, some infectious and some sporadic were due to an unique mechanism : a misfoled protein that propagates and kills neuronal cells. The non-infectious cellular protein termed PrP^{c} converts to a misfolded infectious protein termed PrP^{Sc} (Prusiner, 1982; Prusiner, 1984). This theory was first greeted with great skepticism but is now favoured and accepted in the scientific community. It is now accepted that the causing agent of TSEs is the result of the conformational change or conversion of the cellular prion protein. The final proof of the so called "protein only" hypothesis was recently brought by Prusiner: infectious prions that have been created *in vitro* from a recombinant mouse prion protein allow transmission of the disease when inoculated in mice (Legname et al., 2004).

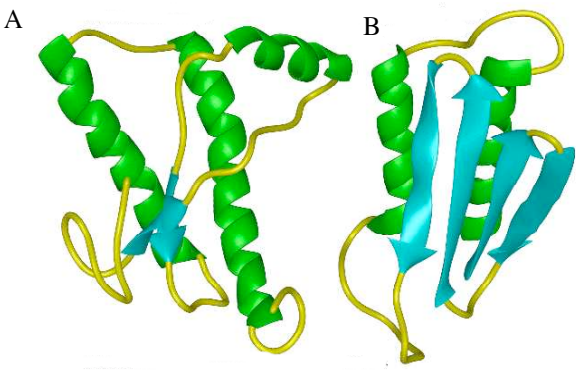

Figure 1. A NMR spectroscopy structure of human PrP^{c}. Note the presence of three α-helices and a ß-strand **B** Speculative conformation of PrP^{Sc} (no structural data is avalaible until now)

- The BSE crisis :

Although, scientific efforts to understand and fight TSEs began have been initiated in the middle of the 20th century, mass concern over TSEs raised with the occurence of bovine spongiform encephalopathy (BSE) also called mad cow disease is quite new. The first case of BSE was confirmed in 1987 and overall the UK about 180000 mad cows were identified (Figure 2). It is thought that prions were transmitted to cattle through meat and bone food prepared from sheep, cattle, pigs etc...processed for industrial use. Classical pathogens are eliminated by high temperature but prions need high temperatures plus high pressure or solvents such as sodium hydroxide for inactivation, so when in the animal food industry for economical reasons, the sterilization temperature was decreased from 130°C to 110°C, the agent was not inactivated anymore. Ingestion of BSE-contaminated tissue that had entered the food chain lead to a new form of Creutzfeldtjacob disease (vCJD) in humans. The ten first cases were detected in UK in 1996 (Bradley and Liberski, 2004) and meanwhile approximately 160 vCJD cases appeared worldwide.

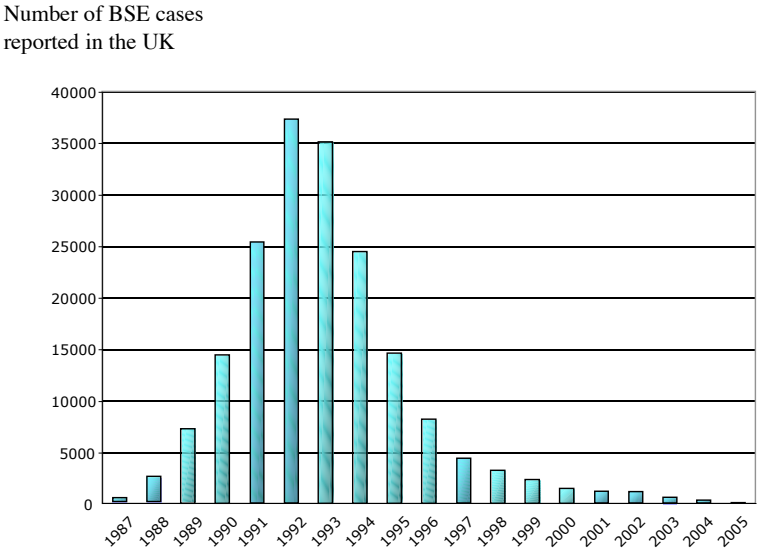

Figure 2. Number of BSE cases reported in the UK since 1987.

- Interestingly, one prion like element, het-s, could be found in a fungus (Coustou et al., 1997) and two in yeast. The non-Mendelian elements in *Saccharomyces cerevisiae* [PSI+] and [URE3] (representing respectively the protein Sup35p* and Ure2p*) are equivalent to mammalian prions (Wickner et al., 1995). Although the conversion of these proteins changes their function, these events are not harmful to the yeast.

2 One protein - 2 stories:

2.1 Prion protein terminology

All prion diseases are associated with accumulation of an abnormal isoform of a host coded protein (PrP), the cellular isoform of PrP is noted PrP^{c} and the disease related PrP isoform PrP^{Sc} (Sc for Scrapie).The truncated version of PrP^{Sc} lacking approx. 60 amino acids at the N-terminal part generated by proteolysis is termed PrP27-30 (the 27-30 refers to the estimated size of the protein bands when analyzed by gel electroporesis)

or PrP^{res} (Res for resistant) and polymerizes into amyloids. The term amyloid is used to describe various types of protein aggregation or deposit that shares specific characteristics. The core protein may be visualized by electron microscopy as scrapie-associated fibrils (SAF) also known as prions rods.

2.2 Structure and conversion mechanism

PrP is expressed as a precursor with a signal peptide (N-terminus) and a signal sequence (C-terminus). After processing, PrP^{c} is secreted and remains attached at the cell surface via its glycosyl-phosphatidylinositol (GPI) anchor (Stahl et al., 1987). Moreover, PrP^{c} can be glycosylated at positions 181 and 197. Subsequently 3 forms can be visualized by westernblot: the unglycosylated form, mono- and di-glycosylated forms. PrP also contains an octapeptide repeat motif which can bind copper.

PrP^{c} and PrP^{Sc} have the same primary structure but harbor conformational differences as shown by spectroscopic methods, PrP^{c} has a α-helix content a bit higher than PrP^{Sc}(42% versus 30%) and their ß-sheet content is drastically different: 3% for PrP^{c} and 43% for PrP^{Sc} (see Table 1, α-helix and ß-sheet contents are given for hamster PrP). The conversion is then thought to involve structural rearrangment of the polypeptide from α-helix to ß-sheet (Caughey et al., 1991; Pan et al., 1993).

	PrP^{c}	PrP^{Sc}
α-helices	42%	30%
β-strand	3%	43%
Protease resistance	NO	Partially resistant
Infectious properties	NO	YES

Table 1. Specific features of PrP^{c} and PrP^{Sc}.

Two models have been proposed for the replication process of prions: (i) the heterodimer model suggests that PrP^{Sc} forms an heterodimer with PrP^{c} followed by its conversion, the two states being separated by an activation energy barrier (Prusiner, 1991) (ii) the seeding model proposes that a small seed of PrP^{Sc} binds to PrP^{c} initiating a polymerization reaction (Jarrett and Lansbury, 1993) (Figure 3)

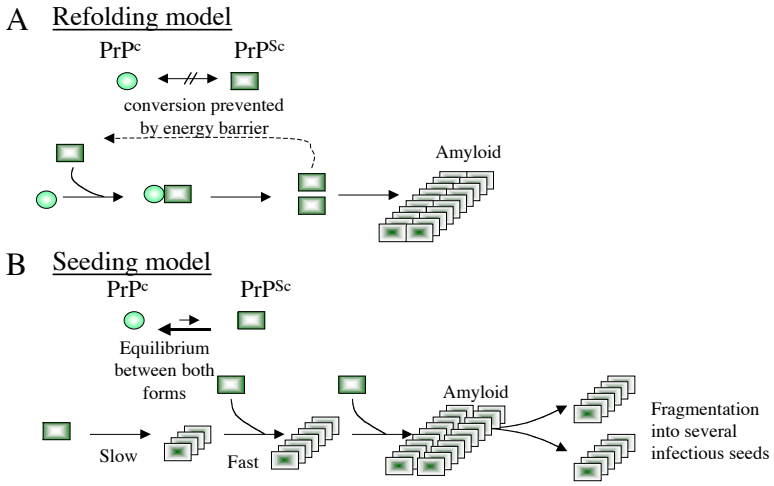

Figure 3. Model for the conformational conversion of PrP^{c} to PrP^{Sc} **A** Refolding model: the conformational change is kinetically controlled, a high activation energy barrier preventing spontaneous conversion. Interaction with PrP^{Sc} leads to PrP^{c} conversion **B** Seeding model: PrP^{c} and PrP^{Sc} or a PrP^{Sc}-like molecule are in equilibrium with PrP^{c} that is strongly favoured. PrP^{Sc} is only stabilized when it is integrated into a crystal-like structure seed or aggregates of PrP^{Sc}. Seed formation is rare; however, once a seed is formed, incorporation of monomer is rapid. Aggregates must fragment to generate increasing surfaces for aggregation.

The three-dimensional structure of PrP^{c} from different organisms has been solved by nuclear magnetic resonance (NMR) spectroscopy and reveals a highly conserved structure : mouse (Riek et al., 1996; Riek et al., 1997), syrian hamster (Donne et al., 1997), human (Zahn et al., 2000) (Figure 1), cattle (Lopez Garcia et al., 2000), chicken, turtle, frog and elk (Calzolai et al., 2005; Gossert et al., 2005). The core region is a globular domain containing three α-helices and a short anti-parallele ß-sheet, the N-terminal part is a disordered and flexible tail (Figure 1). The crystal structure of human

PrP^c reveals an oligomerisation mechanism resulting in a conformational swapping at the dimer interface. As suggested in the seeding model, such PrP dimers could accelerate the formation of a nucleus acting as a seed for the formation of PrP^{Sc} aggregates. The seeding model is supported in part by a very recent publication showing that non-fibrillar particles of 14-28 PrP are the most efficient initiator of TSE diseases (Silveira et al., 2005).

3 Cell biology of prion proteins

3.1 Physiological function of PrP^c

- *Knock-out models*

The physiological function of PrP^c is still enigmatic and poorly understood. The role of PrP^c has been investigated in knock-out mice leading to controversial results. Several lines of mice devoid of PrP^c have been generated by homologous recombination in embryonic stem cells. In some murine lines either no obvious phenotype (Bueler et al., 1992; Manson et al., 1994) or minor phenotypes such as altered circadian activity rythms and sleep pattern (Tobler et al., 1996) and anomalies in specific neuronal excitablility have been observed (Collinge et al., 1994; Manson et al., 1995). In contrast, other investigators reported symptoms of ataxia and extensive loss of Purkinje cells in another line (Moore et al., 1999; Sakaguchi et al., 1996). These contradictory results might be explained by (i) the different methods used: either disruption of the open reading frame or deletion of the open reading frame together with flanking regions (ii) the genetical background and (iii) the potential upregulation of the PrP^c homolog Doppel (Rossi et al., 2001; Silverman et al., 2000). The only clear function of the prion protein is its absolute necessity to propagate infectivity since PrP-knock-out mice generated are resistant to scrapie (Bueler et al., 1993).

- *Proposed role of PrP^c*

Synaptic function

PrP^c is found at the cell surface in neuronal cells in lipid rafts and has been detected in presynaptic nerve endings (Fournier et al., 1995; Herms et al., 1999). Though PrP^c is a GPI anchored protein, PrP^c seems to be also associated with synaptic vesicles. Therefore PrP^c is likely to cycle between these compartments. Interestingly, synaptin I which is associated with small synaptic vesicles is a PrP^c interacting protein and is located together with Grb2 in neuronal microsomal vesicles as well as PrP^c (Spielhaupter and Schatzl, 2001). This suggests a direct role in synaptic activity and/or in vesicles recycling.

Copper binding properties: for transport or anti-oxidative activity?

The PrP protein shows copper binding features and can bind four ions via the histidine-containing octarepeat region at the N-terminus of the protein (Brown et al., 1997; Viles et al., 1999) (Figure 4). The region is also implied in interaction of PrP^c to different ligands.Thus copper may be of structural importance and influence binding of PrP^c to other proteins. It has been reported that mice devoid of PrP^c show synaptosomal copper concentrations diminished by 50% as compared to normal mice (Kretzschmar et al., 2000). Therefore, PrP^c may serve as a copper buffer or may play a role in the re-uptake of copper into the presynaptic compartment. Based on *in vitro* experiments, an alternative role of PrP^c was proposed : a copper-dependent superoxide activity has been described for PrP^c suggesting a contribution against anti-oxidative stress (Brown et al., 1999).

Signalling properties

The activation of the tyrosine kinase Fyn might represent a pathway by which PrP^c influence synaptic function (Mouillet-Richard et al., 2000). Another interactor of PrP^c identified in signal transduction processes is Grb2 which is a connector between signals from extracellular receptors and intracellular proteins. Another contradictory point is the role of PrP^c in cell survival, indeed it has been referred to be involved either in apoptosis or neuroprotection. Nevertheless, more evidences have been accumulated that PrP^c has a neuroprotective function (Bounhar et al., 2001; Chiarini et al., 2002; Kuwahara et al., 1999). Concerning *in vivo* studies, PrP^c has been demonstrated to protect

the brain against Doppel-mediated cell death (Atarashi et al., 2003). Some other studies stated that PrP knock-out mice are more susceptible to brain injury and seizures compared to wild-type mice (Hoshino et al., 2003; Walz et al., 1999).

Long-term memory

A new notion emergedfrom studies on the sea urchin *Aplysia californa*: conformational replication of prions might provide a durable form of molecular memory. Sophisticated experiments were carried out on cytoplasmic polyadenylation element binding protein (CPEB) from the sea-slug *Aplysia californa*, which is a sequence specific RNA binding protein considered as a candidate for synaptic translational regulation (Richter, 2001). The authors demonstrated that this protein behaves as a prion in yeast and is required for cementing long-term memories in neurons from *Aplysia*. Their proposal, yet far from being proven, is that the memory storage is based on prion-like switches of CPEB (Si et al., 2003a; Si et al., 2003b). Although this protein has no homologies with PrP^c, other investigators examined long-term memory on humans with different PrP polymorphisms and carriers of either the 129(MM) or the 129(MV) (see paragraph 4.4) genotype recalled 17% more information than 129(VV) carriers (Papassotiropoulos et al., 2005). These data represent the first hint for a potential role for PrP^c in the formation of long-term memory in humans.

3.2 Trafficking of PrP^c

PrP^c is synthetized in the rough endoplasmic reticulum (ER) and after passing the secretory pathway, including the Golgi and secretory vesicles, reaches the cell surface where it is anchored via its GPI moiety. So far, the internalization pathway and the exact intracellular pathway taken by PrP^c arestill unclear. PrP^c is found in lipid-rafts that consist of specialized and organized sites at the plasma membrane rich in cholesterol and sphingolipids (Brown and London, 1998; Vey et al., 1996). Several informations have led to the suggestion that PrP^c/PrP^{Sc} might be internalized via caveolaelike domains which are organelles with features related to rafts : (i) PrP^c/PrP^{Sc} are found in these domains (Vey et al., 1996) and (ii) perturbation of cholesterol synthesis changes PrP^c trafficking

(Marella et al., 2002). However, the classical internalization pathway involving clathrin-pits may be impaired by cholesterol depletion (Rodal et al., 1999; Subtil et al., 1999). This second possible pathway is supported by numerous reports (Laine et al., 2001; Shyng et al., 1994; Sunyach et al., 2003), indicating that multiple internalization pathways for PrP^c cannot be excluded. A very recent publication proposes that following copper binding to the octapeptide repeats of PrP^c dissociates from lipid rafts, whereas the N-terminal region mediates its interaction with a transmembrane adaptor protein that engages the clathrin endocytic machinery (Taylor et al., 2005). This very interesting model might reconcile previous contradictory hypotheses. PrP^c has been demonstrated to be both in classical endosomes (Magalhaes et al., 2002) and in caveolin-containing endosomes (Peters et al., 2003). The need of an endocytic PrP^c receptor has been postulated years ago. Up to now, two molecules have been confirmed to mediate PrP^c and even PrP^{Sc} endocytosis: the 37 kDa/ 67 kDa laminin receptor (LRP/LR) and heparan sulfate proteoglycans (HSPGs).

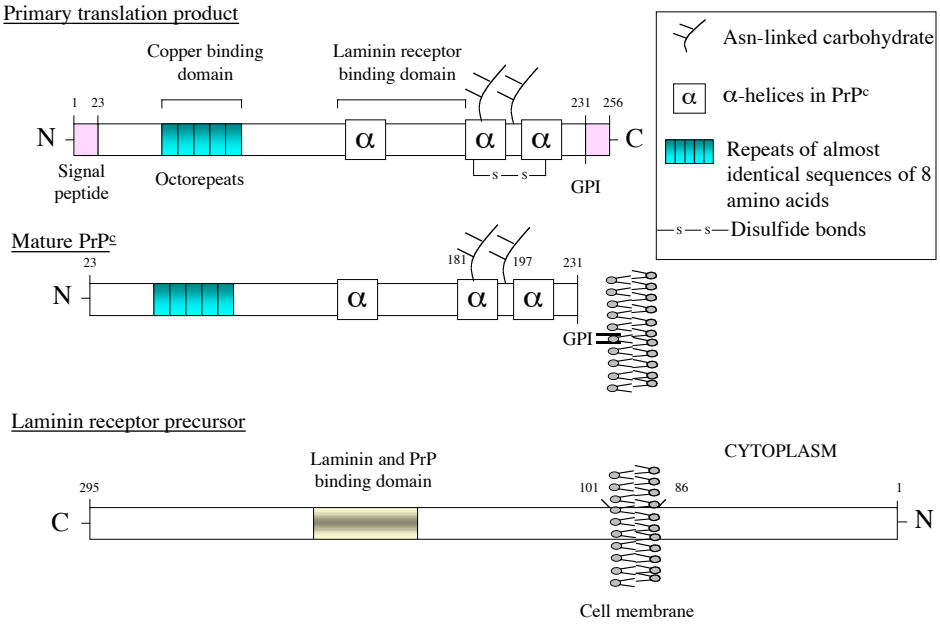

Figure 4. Organization of the human PrP^c and the laminin precursor protein (LRP). Maturation on the PrP precursor protein involves cleavage of the signal sequence, attachment of a GPI anchor and glycosylation at two sites (Asn 181 and Asn 197). The mature PrP^c is anchored at the outer surface of the plasma membrane

4 An overview of animal and human Transmissible Spongiform encephalopathies

4.1 Spread of PrP^{Sc}: the prion invasion

Although pathological damage in TSEs occurs in the central nervous system (CNS), the most likely natural port of entry of non experimental TSEs such as BSE and vCJD is via the gastro-intestinal tract (Terry et al., 2003). Supported by experimental data, a domino model for PrP^{Sc} propagation emerged : spread of PrP^{Sc} in the CNS occurs per continuitatem through conversion of PrP^{c} by adjacent PrP^{Sc} rather than a migration of PrP^{Sc} to the CNS. The spread is then based on a PrP^{c}-paved chain of cells that should not be interrupted by interposed cells lacking PrP^{c} for efficient propagation. The propagation consists of two aspects : lympho-invasion (Hilton et al., 2004; Jeffrey et al., 2000) and neuro-invasion. Elegant series of experiments performed by Aguzzi and colleagues have demonstrated the role of the immune system in scrapie propagation (Aguzzi and Heikenwalder, 2005). There are now strong evidences that PrP expressing hematopoietic cells (B and T cells, follicullar dendritic cells) facilitate prion transport from the entry site to secondary lymphoid organs (ganglions, spleen) where prions accumulate and replicate (Aucouturier et al., 2001; Huang et al., 2002; Klein et al., 1997; Montrasio et al., 2000). In a second step, PrP^{Sc} migrates from the periphery to the central nervous system. This transfer is thought to implicate peripheral nerves and be PrP^{c} dependent (Beekes et al., 1996; Beekes et al., 1998; Glatzel and Aguzzi, 2000). After gaining contact with the CNS, PrP^{Sc} propagates further in the brain leading to a progessive neurodegeneration. How PrP^{Sc} passes through the gastrointestinal tract remains speculative, it could be mediated by non specific endocytosis in lymphoid follicles present in the intestine (Peyer´s patches) thanks to M cells (Heppner et al., 2001a) or require a specific receptor and co-factors (Figure 5). A recent work performed on human enterocytes has shown that the internalization of BSE-prions is mediated by LRP/LR (Morel et al., 2005).

Barrier species and prions strains:

Passage of prions between species is characterized by prolonged incubation times, low rate of transmission or no transmission at all. Differences in *Prn-p* gene sequences have been demonstrated to be responsible for the species barrier (Race et al., 1995; Raymond et al., 2000).

One remarkable features of prion disease is the existence of distinct prions strains. Such strains harbor different biochemical properties such as susceptibility to proteinase K (PK) digestion or electrophoretic mobility after PK treatment (Bessen and Marsh, 1994). According to the "protein only" hypothesis, each strain is assumed to be associated with different PrP pathological conformation or isoform. Recently different phenotypes of CJD have been attributed to distinct human PrP^{Sc} types (Parchi et al., 1996). However, Parchi mentioned that prion strains or prion types could be artefacts, migration conditions and specially pH conditions of the samples may lead to different migration patterns (oral communication, international Prion Conference 2005, Düsseldorf).

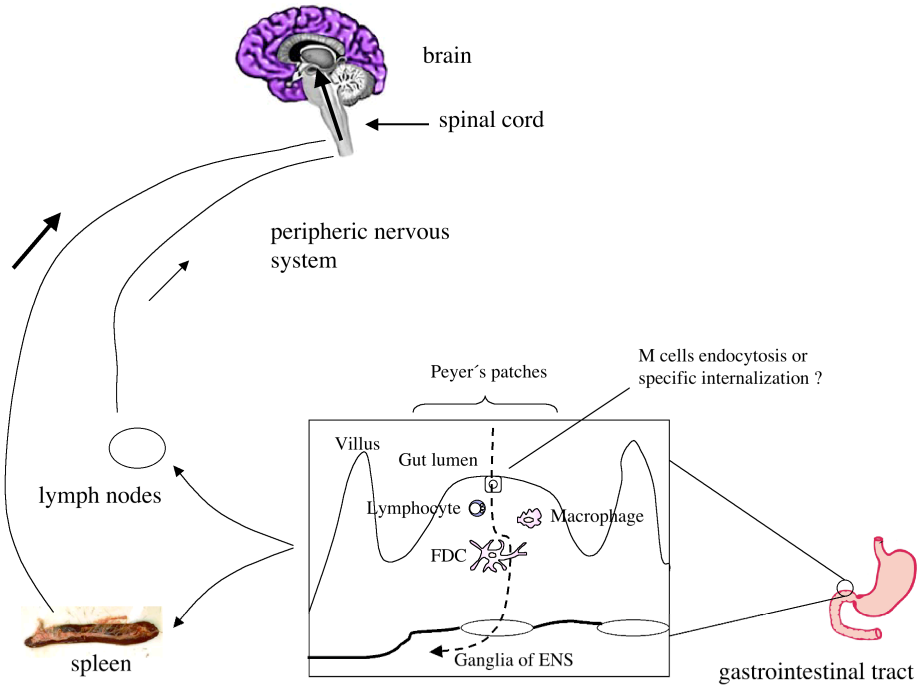

Figure 5. Model for the spread of prions in the organism following oral infection. The prion uptake might be mediated by M cell endocytosis and/or specific LRP/LR dependent internalisation by enterocytes.

4.2 Mechanism of neurotoxicity

How and why prion leads to neurodegeneration is one of the issues that are still obscure and controversial. Contrary to the dogma that PrP^{Sc} accumulation directly results in neuro-destruction, several lines of evidence suggest that PrP^{Sc} itself is innocuous. Although PrP^{Sc} is toxic to primary cultured neurons (Forloni et al., 1993; Muller et al., 1993), it has no effect on PrP^{c}-null neurons when inoculated in PrP^{c}-null mice (Bueler et al., 1993; Sailer et al., 1994). Furthermore neurotoxicity is restricted to PrP^{c}-expressing neurons grafted into the brain of $PrP^{0/0}$ mice despite accumulation and migration of PrP^{Sc} demonstrating that PrP^{c} is necessary for neurotoxicity (Brandner et al., 1996). Different hypothesis can be proposed:

- the adverse effects are due to PrP^{c} loss of function : distorting signalling events that PrP^{c} normally controls might lead to neuronal apopotosis. This hypothesis is conflicting because conditional PrP knock-out studies suggest that PrP^{c} depletion is not sufficient to mediate neurodegeneration.
- during prion invasion, aberrant forms of PrP with deleterious action are produced :
  In a transgenic model, Ma et al showed that forced cytosolic expression of PrP^{c} leads to severe neurodegeneration (Ma et al., 2002). This idea is illustrated by studies on naturally occuring prion diseases : a mutated form of PrP^{c} found in inherited forms have been demonstrated to be translocated to the cytoplasm to a greater degree than wild-type PrP^{c} (Ma and Lindquist, 2001; Ma and Lindquist, 2002). Moreover, mutations in a central hydrophobic region of PrP generating increased transmembrane forms of PrP cause neurodegeneration in some genetic diseases as well as in transgenic mice (Hegde et al., 1998; Hegde et al., 1999). This transmembrane form can also be subjected to degradation by cytoplasmic proteasomes involving access of PrP^{c} to the cytoplasm. In the last international prion conference (october 2005, Düsseldorf), different speakers reported that high infectious material in some cases is not associated with the presence of high quantity of PrP^{Res} but rather with low PK resistant PrP forms (Barron, Weissmann). Charles Weissmann proposed an updated protein only hypothesis which includes a protease sensitive form of PrP (PrP*) which

might be the pathogenic/replicative form rather than PrP^{Sc} (Figure 6).

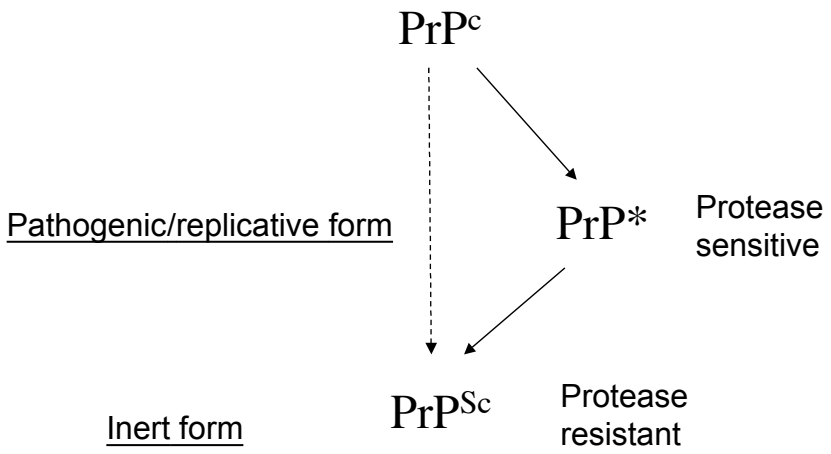

Figure 6. Modified protein-only hypothesis proposed by C. Weissmann (Prion conference, Düsseldorf, 2005)

4.3 Animal disease

- *Scrapie*

Scrapie is a naturally occuring disease in sheep and very rarely in goat. It has been present in Europe for more than 200 years. It owes its name to the phenomenon that the infected sheep animals scrape off their wool. Scrapie was the first TSE disease to be shown as experimentally transmissible (Cuille, 1938). Although there are no known case of genetic TSEs in animal comparable to human, allelic variations in the PrP sequence of sheep seem to influence the susceptibility to scrapie infection (Baylis and Goldmann, 2004). The allelic variants responsible for scrapie susceptibility are more efficiently converted to PrP^{Sc} by *in vitro* system (Sabuncu et al., 2003). However, the exact mechanism of pathogenesis is unknown.

- *Chronic Wasting disease (CWD)*

This form of TSEs affects cervids in North America such as mule-deer, white-taile deer. There are two distinct epidemics, one affecting free-ranging cervids, the other one

farmed cervids. The infection is believed to have occured by oral exposure under natural conditions via excreta or decomposed carcasses (Miller and Williams, 2003; Miller et al., 2004). It has been hypothetized that these epidemics have originally emerged from a sporadic form.

- *Transmissible Mink encephalopathy (TME)*

TME appears in farmed mink populations in different ranches in USA. The origin is not identified but it seems to be a food-borne disease (Marsh and Hadlow, 1992).

- *Bovine Spongiform encephalopathy (BSE or mad cow disease)*

After the confirmation of the first case of BSE in 1987, the epidemic raised its maximum with 3500 new infections per month in 1992/93. There are three hypotheses regarding the origin (i) "the sheep origin hypothesis" proposes that the causative agent of the disease was transmitted from sheep to cattle by feeding of meat and bone meal prepared from sheep infected with the scrapie agent (ii) "the bovine origin hypothesis" postulates that BSE emerged spontaneously in cattle (sporadic case) and was further incorporated into the food chain (iii) the very new still speculative "human origin hypothesis" assumes that human corpses found in indian rivers after funerals were incorporated into animal feed (Colchester and Colchester, 2005). In any case, it seems evident that recycling of infected bovine material within the cattle population amplified the epidemic.

- *Other diseases*

Some other cases of spongiform encephalopathies in captive and zoo animal have been detected including :Exotic ungulate encephalopathy (captive antelope,, bison etc...) and Feline spongiform encephalopathy (puma, tiger...) (Sigurdson and Miller, 2003).
No natural occuring transmissible spongiform encephalopathy has ever been identified in non-mammalian species, nevertheless prion-like elements exists in yeast, fungi and the sea urchin aplysia.

4.4 Human diseases

Human prion diseases occur in sporadic, transmitted and genetic (inherited) forms (Table 2). Polymorphism at codon 129 of the prion protein gene, where methionine or valine may be encoded (Goldfarb et al., 1994), is implicated both in susceptibility and phenotype of human prion diseases. *In vitro* experiments performed on both PrP variants revealed that the methionine 129 variant has a higher propensity to form ß-sheet-rich oligomers (Tahiri-Alaoui et al., 2004).

- *Sporadic form (sCJD)*

The most common disorder is the sporadic form of CJD which accounts for approx. 85% of all cases of human prion disease. This disorder occurs with an incidence rate of approx. 1 case/1 million/year with no epidemiological link with scrapie or other animal prion disease. The exact origin of the pathogenesis is unknown but it has been speculated that a random event may occur in the brain where PrP^{c} spontaneously converts to the abnormal isoform triggering the process in the absence of exogenous PrP^{Sc} (Prusiner et al., 1998) or alternatively that somatic mutations within the Prn-p gene may facilitate the conversion. No epidemical risk factor has been identified for sCJD. The disease affects mainly elderly individuals (peak onset at 60-69 years of age). Characteristic symptoms are rapidly progressive dementia, associated with disordered movement, visual disturbance and cerebellar ataxia. Most patients die within 6 months after onset of the disease.

The sporadic cases affect mainly patients homozygous for methionine with regards to a common PrP polymorphism at the amino acid at codon 129 (M/M) (Palmer et al., 1991)

- *Genetic forms*

Inherited or familial forms are autosomal dominant diseases and they are linked with pathogenic mutations or insertion in the octorepeat region of the prion protein gene (*Prn*-p) (Goldfarb et al., 1994; Goldfarb et al., 1992). These disorders have usually a specific clinical phenotype.

-familial CJD :genetic forms of CJD termed familial CJD (fCJD) represent 10-15% of CJD cases. The pathogenesis results of point mutations in the *Prn*-p gene and insertions/deletions in the octarepeat region.

-other familial forms of human prion disease:

Fatal familial insomnia (FFI) is mainly associated with a mutation at the codon 178(D→N) in conjonction with methionine homozygosity at codon 129 (Gambetti and Lugaresi, 1998). Patients show symptoms in form of progressive insomnia.

Gerstmann-Sträussler-Scheinker (GSS) syndrom occurs in five forms that are each related to a point mutation within the human *Prn-p* gene (Cervenakova et al., 1999; Doh-ura et al., 1989). The incidence rate is really low (1 case /10 million / year). In contrast to CJD, GSS is a slow progressive disease, ataxia is the dominant clinical sign and dementia is usually observed at a later stage.

- *Transmitted forms*

-Iatrogenic CJD: the larger number of iatrogenic cases have occured in the recipients of human growth hormons extracted from cadaveric glands. In rare cases, CJD has been transmitted by neurosurgical instruments, intracerebral electrodes and by dura mater graft.

-Kuru is a disorder among a tribe in Papua New Guinea, occuring chiefly in children and women, that has been postulated to be the results of cannibalism. Indeed children and women consumed mainly brains of deceased family members. Recent studies indicate that individuals homozygous for methionine at a polymorphic position 129 of the prion protein were preferentially affected during the kuru epidemic. The carriers of the alternative 129(M/V) and 129(V/V) genotypes had a longer incubation period and thus developed the disease at a later stage of the epidemic (Cervenakova et al., 1998).

-New variant form of CJD (nvCJD/vCJD): This new form emerged about 10 years after the first case of BSE (Deslys et al., 1997; Will et al., 1996). The early age of onset was unusual (average age of 26 years), as well as the duration of the disease (15 months vs 6 months for sCJD patients) and the histopathological features (presence of florid plaques in the brain). All patients tested so far for codon 129 polymorphism were

homozygous for methionine (Zeidler et al., 1997). There are now series of convincing findings suggesting that nvCJD is a new disease resulting from human exposure to BSE : (i) the isotype of PrP deposited in the brain of nvCJD has a similar glycosylation pattern to experimentally transmitted BSE (Collinge et al., 1996)(ii) florid plaques are present in the brain of macaque monkeys inoculated with BSE (Lasmezas et al., 1996) (iii) human PrP^{c} can be converted by bovine PrP^{Sc} into proteinase K resistant state (Raymond et al., 1997) (iv) transgenic mice expressing human PrP^{c} are infectable by the BSE agent (Hill et al., 1997). Interestingly, Asante et al published that two distinct disease patterns have been identified in mice challenged with BSE, one similar to nvCJD and the other one closely related to sporadic CJD (Asante et al., 2002). This finding might clarify the increasing number of sCJD cases reported in some countries, especially in Switzerland (Glatzel et al., 2002) that may be in fact caused by BSE.

	DISEASE	ORIGIN
sporadic forms	sporadic Creutzfeldt-Jakob disease	unknown : spontaneous conversion of PrP or somatic mutation in *Prn-p* gene ?
genetic forms	familial Creutzfeldt-Jakob disease	inherited mutation in *Prn-p* gene
	Fatal familial insomnia	inherited mutation in *Prn-p* gene
	Gerstmann-Sträussler-Scheinker syndrom	inherited mutation in *Prn-p* gene
acquired forms	Iatrogenic forms	cadaveric growth hormons treatments, electrodes, dura mater graft
	Kuru	consumption of brain during funeral feast
	new variant of Creutzfeldt-Jakob disease	consumption of meat from BSE infected cattle

Table 2. Classification of human transmissible spongiform encephalopathies

5 Role of a cellular prion protein receptor

To better understand TSEs, strong efforts have been done to identify molecules able to interact with PrP and so far a lots of interactors have been found. David Harris proposed the existence of a cellular receptor for prions, his idea was that a prion receptor should participate in the propagation of prions through its intenalization. The endocytosis of the GPI anchored PrP^c has been predicted to be mediated by a transmembrane protein which might connect PrP^c to clathrin. Two membrane proteins have been proposed to act as a receptor for PrP^c (1) the murine stress inducible protein I (STI1) (2) the 37kDa laminin receptor (LRP). Moreover, several groups established that HSPGs (heparan sulfate proteoglycans) present at the cell surface can also bind to PrP^c and act as a receptor for PrP^{Sc}.

5.1 The murine stress inducible protein I

A brazilian group identified a 66kDa membrane protein as a putative prion receptor by complementary hydropathy (Martins et al., 1997). Several years later, they determined that this PrP^c ligand was mSTI1, a heat shock protein, first described in a macromolecular complex with Hsp70 and Hsp90 chaperone family members. They could demonstrate a specific binding *in vitro* by pull-down assays and at the cellular level and observed that STI1 induce neuroprotective signals that rescue cells from apoptosis (Zanata et al., 2002). More recently, another group assigned that STI1 is involved in PrP^c dependent SOD activation (Sakudo et al., 2005). Nevertheless, though the authors detected a small fraction of STI1 at the cell surface (6%) of non-neuronal cells, so far no transmembrane domain or signal peptide have been recorded for this protein. Furthermore, since chaperonins oftenly bind all manner of probe (at least ten other chaperonins have been reported to interact with PrP^c: Hsp70, Hsp 90, GroEL...), this findings need further confirmation.

5.2 The 37/67kDa laminin receptor

LRP was first identified as a PrP^c interactor in a yeast-two-hybrid screen by our group (Rieger et al., 1997). This interaction was confirmed by co-infection and co-transfection assays in insect and mammalian cells. It has been further demonstrated that LRP is present in higher amounts in several organs and tissues of scrapie-infected mice and hamsters such as brain, spleen and pancreas compared to uninfected animals suggesting a good correlation between LRP levels and prion propagation. These findings were further strenghtened by a series of experiment: (1) LRP is located at the cell surface (2) PrP and LRP colocalize in neuronal and non-neuronal cells (3) PrP internalization is LRP-dependent (Gauczynski et al., 2001b). A more recent work in our laboratory postulates that LRP is not only the receptor for PrP^c but also for PrP^{Sc} (Gauczynski et al., submitted). This finding was confirmed by a french research group demonstrating that human enterocytes internalize BSE prions in a LRP/LR dependent manner (Morel et al., 2005).

The observation that laminin binds to the cancer cell surface led several groups to attempt to purify the putative laminin receptor by affinity chromatography. In 1983, independent laboratories reported the characterization of an apparently unique 67kDa protein isolated from membrane fraction of cancer and muscle cells (Malinoff and Wicha, 1983; Rao et al., 1983). This molecule was called high affinity 67kDa laminin receptor (LR). Nevertheless the cDNA isolated only contains a coding sequence for a smaller polypeptide with a molecular weight of 37kDa acting as a precursor of the LR termed laminin receptor precursor (LRP, Figure 4) (Rao et al., 1989). The maturation mechanism is still controversial and different scenarios have been postulated to explain this 37kDa/67kDa polymorphism: homodimerization, heterodimerization, fatty acid interaction (Buto et al., 1998; Landowski et al., 1995)...but the relationship between LRP and LR remains still a mystery. LRP/LR is a multifunction protein and might be involved in three cellular functions:

-cell-matrix interaction due to its laminin binding capacity: increased level of LR correlates with the progression of solid tumors since adhesion to the basement membrane and invasion are critical steps in the metastatic cascade (Cioce et al., 1991; Sanjuan et al., 1996). Thus this protein is a useful prognostic marker in different cancers (de Manzoni et al., 1998; Waltregny et al., 2001).

-translational function in association with ribosomes: LRP is identical to the p40 ribosomal protein and is thought to be a component of the translational machinery, being specifically associated with the 40S ribosomal subunit (Auth and Brawerman, 1992; Demianova et al., 1996).

-DNA-binding protein suggested to be involved in maintenance of nuclear structures: this function is less documented and awaits further elucidation but LRP was shown to localize in the nucleus and be tightly associated with nuclear structures (Kinoshita et al., 1998; Salama et al., 2001; Sato et al., 1996). Salama et al speculate that LRP might be a shuttle protein which might clarify its localization in different compartments.

Regarding evolutionary studies, LR is considered to originate from a ribosomal protein that progressively acquires new roles (Ardini et al., 1998). The LRP sequence shows a high degree of homology among mammalian species and the gene has been identified in different species*: Saccharomyces cerevisiae* (Davis et al., 1992; Demianova et al., 1996), *Arabidopsis thaliana*(Garcia-Hernandez et al., 1994), *Drosophila melanogaster*(Melnick et al., 1993), the archaebacterium *Haloarcula marismortui* (Ouzonis et al., 1995).

In additon to these functions, LRP/LR have been charaterized as the receptor for various pathogens: prions, Venezuelan equine encephalitis virus (Ludwig et al., 1996), Sindbis virus (Wang et al., 1992), Dengue virus (Thepparit and Smith, 2004; Tio et al., 2005), bacterial toxin CNF1 (Chung et al., 2003). On the other hand the presence of laminin-specific receptor on the surface of micro-organisms may influence their pathogenicity, and laminin binding protein related to LRP have been observed on the surface of *Candida albicans* (Lopez-Ribot et al., 1996) and the parasite *Leishmania donovani* (Bandyopadhyay et al., 2003).

5.3 Heparan sulfate proteoglycans (HSPGs)

Glycoaminoglycans (GAGs) such as heparan sulfate (HS) are long unbranched side chains of proteoglycans that are found in several cellular compartments including the cell surface. HS are directly connected to prion pathogenesis since they accumulate in cerebral prion amyloid plaques as it does in Alzheimer´s disease (Fukuchi et al., 1998;

Snow et al., 1989). Furthermore, a work conducted in our laboratory suggested that beside the direct interaction site between PrP and LRP, there is an indirect binding site implicating HSPGs (Hundt et al., 2001). Since HSPGs are involved in PrP binding, we suggested that they act as co-factors or co-receptors for PrP. HSPGs are initial attachment receptors for a series of virus such as AAV, HIV-1 and many more (Coombe and Kett, 2005). The group of Taraboulos recently published that heparan sulfate are binding and uptake receptors for prions in agrement with another work from other investigators (Ben-Zaken et al., 2003; Hijazi et al., 2005).

5.4 Neural cell adhesion molecule (NCAM)

NCAM has been identified as a PrP interactor at the surface of neuronal cells although this receptor has neither a role in PrP^{Sc} propagation in animals nor in PrP internalization (Schmitt-Ulms et al., 2001). NCAM is reported to enhance neurite outgrowth via activation of p59fyn (Beggs et al., 1997; Beggs et al., 1994), PrP^{c} contributes to this mechanism by recruiting NCAM to lipid rafts, which is essential for promotion of neurite outgrowth (Santuccione et al., 2005).

CHAPTER II

Therapeutic approaches for the treatment of prion diseases

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Abstract

Prion diseases are a group of lethal neurodegenerative disorders associated with misfolding of a host protein, the prion protein PrP^{c}. But despite increasing knowledges concerning this class of disease, up to now no therapeutic approach has been found to cure the disease or stabilize the pathogenesis in humans. A variety of potential therapeutic compounds have been tested in experimental models and some of them have been proven to prevent prion infection in rodents. However, none of them was successfull to cure the disease at a late stage. Nevertheless since the BSE crisis that might have infected a number of humans by dietary exposure, increased efforts have been done in the scientific community both in experimental therapy and basic research.

1 Introduction

Transmissible spongiform encephalopathies (TSEs) or prion diseases are neurodegenerative lethal disorders. This class of illnesses includes Creutzfeldt-Jakob disease (CJD) in humans and bovine spongiform encephalopathy (BSE) in animals. Prions are remarkable pathogens since they represent a new class of agents, infectious proteins. The disease-associated prion protein termed PrP^{Sc} results from the confomational change of the host cellular prion protein PrP^{c}. Due to its protease resistance PrP^{Sc} is not degraded by the host proteolysis systems (i.e the proteasomal or the lysosomal degradation pathways) and accumulates in the central nervous system (CNS). In humans, these diseases occur in infectious, sporadic and genetic forms. The attempts to develop an effective means of treatment began after the UK BSE epidemic, initially amplified by feeding cattle with meat and bone meal that was suspected to be responsible for the emergence of the new variant form of CJD (vCJD) in humans. Since many aspects in prion biology remain unclear, prion diseases are fascinating biological enigma. The physiological function of the prion protein and the mechanisms leading to neuronal damage are still poorly understood.

2 Curing of TSEs: a challenge?

Only a few drugs have been tested in patients suffering from CJD and up to now, no efficient therapy is available and TSEs are invariably lethal. The disease is rapidly progressive and when the first symptoms appear, there are already irreversible brain damages, the absence of an early diagnosis is then the first limitation. Moreover clinical studies are rendered really difficult due to the rarity of the disease and the variablity of the pattern of the disease and as these diseases are lethal, ethically no placebo can be accepted. Because of unusual properties of TSEs, standard approaches (antiviral or antibacterial substances) are useless. Hence, it is necessary to develop new strategies for the treatment of prion diseases.

When the infection source is peripheric, the scrapie agent rises to lymphoid organs before to transfer to the CNS (Jeffrey et al., 2000), then a post-exposure prophylaxis may be feasible preventing neuro-invasion. After the neuro-invasion began, the compounds used should cross the non-permeable blood brain barrier or be administered directly into the brain.

The challenge to cure TSEs or improve the survival time is dual: it will require both improvement in diagnostics and identification of efficient compounds that are non-toxic and able to cross the blood brain barrier. So far more than 20 compounds able to enhance prion clearance in cultured cells have been identified and several of them have been shown to extend the incubation times of mice or hamsters but only when administered around the time point of prion inoculation. Nervertheless, regarding increasing investigations and knowledge concerning TSE, prion therapy seems to be feasible. Multi-therapy might also be required for therapy of prion diseases as done in the treatment of HIV.

3 Models for therapeutic investigation

3.1 *In vitro* models

The development of a cell-free conversion assay, binding or polymerisation assays allow the evaluation of putative inhibitory effect of compounds interacting either with PrP^{c} or PrP^{Sc}. Since protein misfolding cyclic amplification (PMCA) reactions performed on brain homogenates gives higher yields of misfolded PrP than *in vitro* conversion (Supattapone, 2004), it might be adapted to high-throughput screening.

3.2 Cellular models

Cell culture systems represent relevant experimental models for TSEs: the scrapie agent can chronically infect some neuronal cell lines such as murine neuroblastoma cell lines (e.g. N2a) which are frequently used as well as hypothalamic neural cell lines (i.e. GT1) (Solassol et al., 2003). These cells have contributed to the screening and the study of potential therapeutic compounds. The cells are infected with a scrapie brain inoculum from the same species. Nevertheless, these models are limited: (i) the cell lines employed are mainly from murine origin and might not be transposable to humans, (ii) the infectivity is not always stable and can be lost after several passages lacking obvious reasons, (iii) they constitute a simplified model compared to *in vivo* propagation (role of immune system, nervous system) and as a consequence not all compounds with *in vitro* activity are effective *in vivo*.

3.3 Animal models

TSE agent can be transmitted to laboratory animals such as mice and hamsters by using rodent adapted strains. These models are most relevant for evaluation of drugs although (i) the incubation times are relatively long (several months), (ii) the strains are not natural and (iii) the physiology is different from humans. The incubation times depend on the inoculation route: mice die approx. 150 and 200 days, respectively, after intracerebral and intraperitoneal administration of the scrapie agent. The spleen, the main location of peripheral PrP^{Sc} propagation can be analyzed approx. 90 days after PrP^{Sc} inoculation: here early signs of a prion infection reflected by the presence of PrP^{res}, can be monitored. The primate system, however, is probably the closest model to the human

situation, since these animals can be inoculated with CJD. However, the primate model require huge cost regarding animal care facilities and the incubation times are in the range of several years.

4 Targets and Strategies for the treatment of TSEs

Different ways for the intervention in prion diseases are possible and many are under investigation. Many investigators tried to target PrP^{c} or PrP^{Sc} itself but it is also possible to interfere with lympho-invasion that implicates the immune system or to target PrP specific receptors or co-receptors (Table 1).

Targets/Compounds	Effect in scrapie infected cells	Effect in rodent models		Remarks
		prophylactic	therapeutic	
PrP^{c} ◆ anti-PrP antibodies	Yes	Yes	No	delivery by passive immunization
◆ anti-PrP scFv antibodies	Yes	Yes	?	transgenic expression
◆ immunization against PrP		Yes	?	
PrP^{Sc} ◆ binding compounds				
↪IDX		Yes		drug is preincubated with scrapie inoculum
↪Tetracycline		Yes		
↪Congo Red	Yes	Yes	?	
◆ ß sheet breakers		Yes	?	peptides are added to scrapie inoculum
◆ increasing clearance (branched polyamines)	Yes	?	?	
Raft domains				Effect restricted to certain scrapie strains
◆ polyene antibiotics				
↪amphotericin B	Yes	Yes	Yes	
↪MS-8209	?	Yes	Yes	less toxic than amphotericin B
Immune system				
◆ FDC maturation inhibition (soluble lymphotoxin ß receptor)		Yes	?	
◆ Complement inhibition		Yes	?	
Heparan mimetics (DS500, pentosan polysulfate)	Yes	Yes	?	DS500 is toxic
LRP ◆ anti LRP antibodies	Yes	?	?	
Quinacrine	Yes	No	No	Controversial results in human trials
Chlorpromazine	Yes	?	?	

Table 1. Therapeutic strategies for the treatment of prion diseases investigated in scrapie infected cells and experimental models.

4.1 PrP as a target

- *Molecules that act on PrP^c*

As shown in PrP-knock-out mice, depletion of PrP^c prevents scrapie infection (Bueler et al., 1993). Hence, PrP^c is of great interest as a target for intervention in TSEs. As a proof of principle it was elegantly demonstrated that transgenic mice expressing anti-PrP antibodies are protected against peripheral prion infection (Heppner et al., 2001b). Moreover, antibodies against PrP inhibit propagation of PrP^{Sc} in cell culture (Enari et al., 2001; Peretz et al., 2001 ; Perrier et al., 2004). Other investigators have reported that passive immunization with anti-PrP monoclonal antibodies reduces the infectivity in the spleen of mice peripherally infected with prions (White et al., 2003). However, another group pointed out that the injection of large amounts of anti-PrP antibodies caused massive neuronal apoptosis and the authors speculate that this might be due to PrP cross-linking via the antibodies (Solforosi et al., 2004). A very recent study proposes to use miniantibodies (single chain antibodies) as an alternative approach (Donofrio et al., 2005). Animals with TSEs do no elicit immune response against PrP^{Sc} but active immunization or vaccination have been actively investigated. This approach implicates to break immune tolerance to PrP that might be achieved by different strategies (e.g. PrP dimerization, PrP cross-linking to a bacterial protein) (Gilch et al., 2003; Koller et al., 2002; Rosset et al., 2004). Different groups reported low protective effects on infected mice (Polymenidou et al., 2004; Schwarz et al., 2003; Sigurdsson et al., 2003). However, innovative strategies are emerging such as expression of PrP at the surface of viral particles (Nikles et al., 2005) and these might contribute to increase the levels of auto-antibodies which might act as the limiting factor.

- *Molecules that interact with PrP^{Sc}*

A key feature for prion diseases is the conversion of PrP^c to PrP^{Sc}. Chemicals able to bind to PrP^{Sc} may sequester and enable it from serving as a template for replication. Several molecules which are able to interact specifically with PrP^{Sc} or amyloids have been tested: iodoxorubicin (Tagliavini et al., 1997), tetracycline (Forloni et al., 2002;

Tagliavini et al., 2000) and Congo Red (Caughey and Race, 1992; Ingrosso et al., 1995) might act by sequestering the template for conversion.

Another group of anti-TSE compounds consists of specifically designed synthetic PrP peptides termed ß-sheet breakers. These peptides with sequence homologies to PrP^{c} and an increase in proline content are able to interact with PrP^{Sc} and change its secondary structure (Chabry et al., 1998). As a consequence, they lead to a decrease in the protease resistance of PrP^{Sc} allowing a better clearance of PrP^{Sc}. The ß-sheet breakers indeed mediate prolongation of the life span of PrP^{Sc}-infected mice when the scrapie inoculum is mixed with such ß-sheet breakers (Soto et al., 2000).

- *Enhancement of PrP^{Sc} clearance*

PrP^{Sc} is partially resistant to proteolytic degradation as assayed by proteinase K *in vitro* but there are obviously natural mechanisms to destroy it. Indeed, PrP knock-out mice inoculated with high-dosis of prions, revealed clearance of PrP^{Sc} within 2 weeks. The half-life of PrP^{Sc} is evaluated at 24h in scrapie-infected cells (Ertmer et al., 2004).
To increase PrP^{Sc} clearance by the organism represents a reasonable strategy. Branched polyamines that are thought to stimulate PrP^{Sc} clearance in endolysosomes result in curing of chronically infected cells (Supattapone et al., 1999; Supattapone et al., 2001). An inhibitor of the tyrosine kinase c-abl also activates PrP^{Sc} lysosomal degradation (Ertmer et al., 2004).

- *Indirect effect : disturbance of raft biology*

Some polyene antibiotics such as amphotericin B and MS 8209 normally used as anti-fungal agents are cholesterol interactors, which might interfere with raft integrity. They are believed to impair PrP^{c} or PrP^{c}/PrP^{Sc} endocytosis and thus slow-down PrP^{Sc} accumulation as proved in chronically infected cells (Mange et al., 2000). MS 8209 is particularly efficient in infected hamsters (Adjou et al., 1999; Adjou et al., 2000; Demaimay et al., 1997).

4.2 Drugs able to cross the blood brain barrier

Drugs licensed for humans for some other indications have been tested: that is the case of chlorpromazine and quinacrine which are efficient in infected cells (Korth et al.,

2001) but failed to prolong survival times in rodent models (Barret et al., 2003; Collins et al., 2002). Quinacrine has been evaluated in different small clinical trials for CJD patients but no significant effect has been observed although some studies reported a transient improvement in visual stimulation (Benito-Leon, 2004; Furukawa et al., 2002; Kobayashi et al., 2003; Nakajima et al., 2004). Furthermore quinacrine treatments led to liver dysfunctions.

4.3 Immune system

Follicular dendritic cells (FDC) play an important role in peripheral infection since they are responsible for PrP^{Sc} accumulation in the germinal centres of secondary lymphoid organs. They may be directly involved in the connection between lympho-invasion and neuro-invasion since they are closely located to sympathetic nerve endings. The differentiation and maturation of FDCs that are mediated via a molecular dialogue with B-cells represent two steps required for splenic PrP^{Sc} accumulation. Both lymphotoxin ß and TNF are implicated in this process, suggesting that they are potential targets (Aguzzi and Heikenwalder, 2005; Brown et al., 2000). The maturation mechanisms can be impaired by neutralization of the lymphotoxin ß receptor using a soluble form of it (Mabbott et al., 2003; Mohan et al., 2005). When applied to i.p. infected mice, a single injection is efficient to prevent early PrP^{Sc} accumulation within the spleen but not after oral challenging.

4.4 Neuronal damage rescue

- *Neurons grafting*

Therapeutic approaches involving replacement or rescue of damaged neurons may help to delay clinical symptoms of disease. Brown et al. reported that grafting of embryonic cell graft devoid of PrP in hippocampus protects against scrapie neuronal loss. Despite no prolongation in the incubation period of the disease it has been observed that the treated area retains 50% more neurons than controls in mice grafted at a relatively late

stage of the disease (Brown et al., 2001). Transplantation of cells to multiple sites in the brain might further extend the incubation times.

- *Neuroprotective molecules*

Since the main feature of TSEs is neuronal death, any drug with the ability to impair the cascade associated with this event would be of interest.

4.5 PrP receptors

- *Heparan Sulfate Proteoglycans (HSPGs) / Glycoaminoglycans (GAGs)*

HSPGs consist of a protein core covalently linked to glycoaminoglycans. These molecules that are either secreted or inserted into the plasma membrane are important components of the extracellullar matrix and many of them act as co-receptors. Viruses such as HIV-1 (Saphire et al., 2001), Dengue virus (Hilgard and Stockert, 2000) , Adeno-associated virus (AAV) (Summerford and Samulski, 1998) and Sindbis Virus (Ryman et al., 2004) use HSPGs or GAGs as initial attachment receptors.

HSPGs have a binding affinity to PrP and have been shown to play an active role in PrP^{c}/PrP^{Sc} internalization (Hijazi et al., 2005; Horonchik et al., 2005). For this reason heparan sulfate mimetics (HMs) may be good candidates for therapeutic interventions (Caughey and Raymond, 1993). However their *in vivo* use was first limited by toxicity (e.g. Dextran sulfate 500 (Farquhar and Dickinson, 1986) and pentosan sodium polysulfate (Diringer and Ehlers, 1991)). New compounds optimized by group substitutions emerged and have been shown to hamper PrP^{Sc} formation both in cell culture and in animals (Adjou et al., 2003).

- *The 37 kDa/67 kDa laminin receptor (LRP/LR) as a target*

We identified the laminin receptor (LRP/LR) as the cell surface receptor for PrP^{c} (Gauczynski et al., 2001b; Rieger et al., 1997). According to our working model (Figure 1), we postulate that interfering in LRP-PrP^{c}/PrP^{Sc} interaction might first decrease prion entry in the intestinal tract, but also hamper PrP^{c} internalization leading to a reduction of PrP^{Sc} formation in the endocytic pathway. We hypothesize that LRP may also promote cell to cell propagation of PrP^{Sc}.

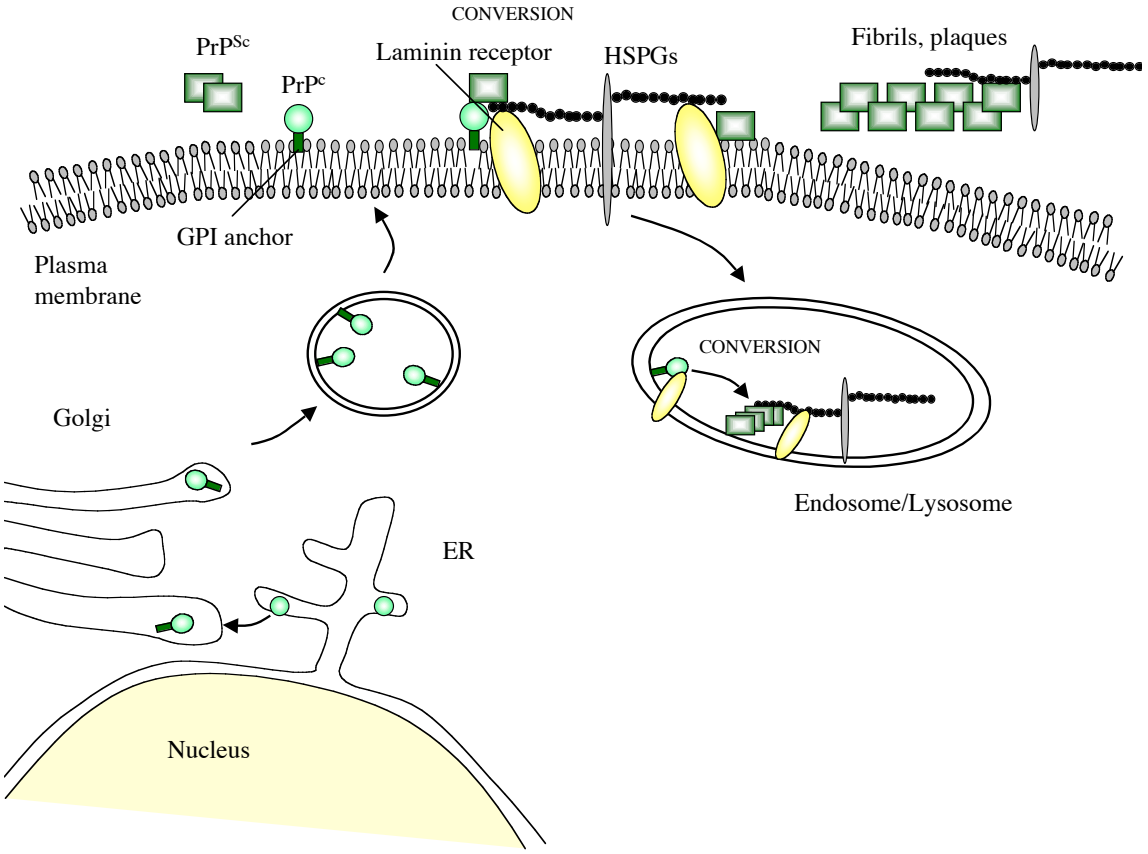

Figure 1. Role of the laminin receptor in the biogenesis of prions. The cellular isoform of the prion protein PrP^{c} is synthetized and glycosylated in the ER where the GPI anchor is added. PrP^{c} is transported to the plasma membrane where it interacts with the 37 kDa/67 kDa laminin receptor. During prion infection, the abnormal prion isoform PrP^{Sc} binds to PrP^{c} that is converted to PrP^{Sc} by a conformational change. This process occurs on the cell surface or in endocytic vesicles such as endosomes, lysosomes or endolysosomes. The prion propagation is thought to be affected by factors such as the laminin receptor and heparan sulfate proteoglycans (HSPGs). LRP/LR and HSPGs act as co-factors/co-receptors for PrP^{c} and for the infectious prion protein PrP^{Sc}. PrP^{Sc} is thought to accumulate in lysosomes and in extracellular deposits rather than in the plasma membrane.

LRP is necessary for PrP^{Sc} propagation in scrapie infected cells as reported previously. Thus, the knock-down of LRP by antisense LRP RNAs or by siRNA technology eradicates PrP^{Sc} in chronically infected cells. This can also been achieved by polyclonal antibodies directed against LRP leading to the possibility of development of new experimental therapies based on antibodies (Leucht et al., 2003). Recently, it was shown that the polyclonal antibody W3 directed against LRP blocked the internalization of the bovine infectious prion protein by human enterocytes, demonstrating that LRP/LR acts as a receptor for infectious prions (Morel et al., 2005). Because polyclonal antibodies

are not applicable for therapeutic purposes, an alternative approach is to take advantages of a new technology: single chain antibody fragments (scFvs) (Bird et al., 1988) (Figure 2). The development of a TSEs therapy based on scFvs directed against LRP/LR is a promising alternative or a complementary approach to anti-PrP antibodies.

5 Single chain antibodies as therapeutical tools

Antibody-based therapeutics are beginning to realize the promise enclosed in their earlier denomination "magic bullets".

Monoclonal antibodies have found applications in diagnosis and in treatment of various diseases, including cancer. Approx. 20 of them have the approval for therapeutic use in humans such as Rituxan® for lymphoma and Herceptin® for breast cancer. However, to improve this technology smaller antibodies have been engineered which exhibit better tissue penetration and enable binding specificity encoded by a single polypeptide gene. Among these novel antibodies, single chain antibodies are the smallest one with sizes of approx. 30kDa versus 150kDa for entire immunoglobulins. A scFv comprises the variable domain of the heavy and light chains (V_H and V_L) of a monoclonal antibody joined by a linker peptide (Figure 2). The advantages of scFvs compared to Igs make them interesting tools for therapy of neurodegenerative diseases: (i) they can be easily selected and expressed in bacterial systems, (ii) they should better penetrate brain tissues, (iii) they can be delivered by gene therapy, and (iv) they do not induce immune responses due to the lack of the Fc part. ScFvs already proved great potential in several publications and clinical trials predominantly in the cancer field. Nevertheless, new scFvs are emerging for neurodegenerative diseases. Lui et al developed scFvs directed against ß amyloids as an alternative to monoclonal antibodies that provoke dangerous side effects in patients. These scFvs can eliminate toxic effect of aggregated Aß-peptides causing Alzheimer's disease in cells (Liu et al., 2004). ScFvs directed against huntingtin have been engineered and might be helpful to treat Huntington disease (Khoshnan et al., 2002). Transgenic animals overproducing scFvs against PrP^c are protected against TSEs. Very

recently, it was shown that anti-PrP scFvs produced by genetically modified cells contribute to block prion propagation in chronically infected cells in co-culture (paracrine inhibition) (Donofrio et al., 2005).

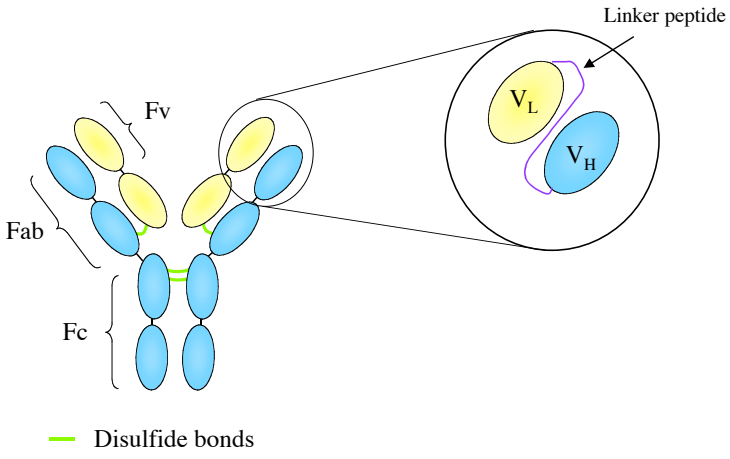

Figure 2. Schematic representation of an entire IgG molecule and a single chain antibody.
Ig are composed of two similar light chains (L) and 2 similar heavy chains (H). Light chains contain one constant domain and one variable domain (V_L), whereas heavy chains consist of three constant domains and one variable domain (V_H). A heterodimer of V_H and V_L linked by a peptide is called single chain Fv fragment (for fragment variable) and is still capable to bind the antigen. Single chain antibodies directed against PrP or LRP might be powerful tools in therapy of prion diseases.

6 Delivery of antibodies for TSEs

6.1 Passive immunotransfer

The easiest way to deliver the antibody is by intraperitoneal or intravenous injections. However, huge amounts of recombinant antibodies are required and due to the blood brain barrier, it is likely that only a small quantity of them might act in the brain where most of PrP^{Sc} accumulates. It has been previously estimated that 0,1% of injected Immunglobulins (Igs) might enter the brain (Bard et al., 2000), but no data concerning scFv have been reported so far. Therefore this approach will be favoured for prophylactic treatment or at early stage of the disease to delay the peripheral invasion. Nevertheless, intracerebral infusion might be an alternative route for delivery. A single injection of antibody in the third ventricule of mouse brain led to diffusion throughout the entire brain within 24h (Chauhan et al., 2001). This strategy has been tested for the administration of

the anti-prion drug pentosan polysulfate by implanting a continuous intraventricular drug infusion device (Doh-ura et al., 2004).

6.2 Gene transfer via viral vectors

Vector	Inflammatory potential	Limitations	Advantages	Use in experimental model of neurodegenerative diseases		
				Parkinson	Alzheimer	Huntington
lentivirus	low	integration may induce oncogenesis	persistent gene transfer	GDNF[1]	ApoE[2]	GDNF[3] CNTF
herpesvirus	high	inflammatory response	strong tropism for neurons	GDNF[4]	-	-
adeno-associated virus	low	inflammatory response	persistent gene transfer, non inflammatory, non pathogenic	GDNF[5]	ApoE[6]	GDNF[7]
adenovirus	high	small packaging capacity 5kb		GDNF[8]	-	BDNF[9]

Table 2. Overview of advantages and disadvantages of viral vectors suitable for brain targeting. The experimental delivery of therapeutic molecules via viral vectors for three neurodegenative diseases are mentioned in this table. Viral vectors are oftenly used to express neurotrophic factors in the CNS such as BDNF (brain derived neurotrophic factor), GDNF (glial cell line-derived neurotrophic factor), CNTF (ciliary neurotrophic factor) and ApoE (apolipoprotein E).
[1](Azzouz et al., 2004), [2] (Dodart et al., 2005), [3] (Regulier et al., 2003), [4] (Fink et al., 2003), [5](Wang et al., 2002), [6](Feng et al., 2004), [7](Kells et al., 2004), [8](Chen et al., 2003), [9](Bemelmans et al., 1999)

The use of scFvs for TSEs is limited by large scale production and biodistribution. In order to circumvent these limitations antibody based gene therapy can be used for instance via *in vivo* gene transfer using viral vectors. Vectors carrying a therapeutical gene can be packaged into a viral coat allowing efficient gene transfer in the absence of viral gene expression in target cells. Several viral vector system are suitable to infect cells

of the nervous system: adeno-associated viruses, lentiviruses, herpesviruses and adenoviruses (Table 2). A single micro-injection of a recombinant virus into the brain might support continuous and sustained transgene expression.

6.3 Engineered cells producing antibodies

Genetically engineered cells might be the source of sustained concentrations of soluble antibody fragments, capable of achieving long-term expression. The cells can be encapsulated in immunoprotective devices to avoid rejection. The use of hybridoma cells producing monoclonal antibodies is an obvious option. Nevertheless, this approach is limited by the short life span of hyridoma cells. Muscle cells are good candidates since they are long-living cells and are capable to sustain secretion of monoclonal antibodies for several months *in vivo* (Noel et al., 1997).

Although we are far away to cure TSEs in humans, interventions in the progression of TSEs in animal models, particularly with the development of immunotherapy, is a source of optimism. It is likely that increasing experimental data on prion biology will contribute to the emergence of novel therapeutic approaches in the future.

CHAPTER III

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

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Abstract

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

Introduction

Transmissible spongiform encephalopathies (TSEs) are a group of neurodegenerative disorders that include Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle, and scrapie in sheep (Aguzzi and Weissmann, 1998; Lasmézas and Weiss, 2000; Lasmézas, 2000; Prusiner et al., 1998; Weissmann, 1999). The main pathogenic event in the development of TSEs is the conversion of PrP^{c}, the normal cellular form of the prion protein, to PrP^{Sc}. An important feature of PrP^{Sc} is its partial resistance to proteases, which makes it biochemically distinguishable from PrP^{c} (Caughey and Raymond, 1991). Recently, we identified the laminin receptor (LRP/LR) as the cell-surface receptor for PrP^{c} (Gauczynski et al., 2001b). Heparan sulfate proteoglycans (HSPGs) have been shown to function as cofactors or co-receptors for the

binding of PrP^c to the LRP/LR (Hundt et al., 2001). The LRP/LR has been shown to interact directly with PrP^c in the yeast two-hybrid system (Rieger et al., 1997). This interaction was confirmed by pull-down assays in cotransfected COS-7 cells and co-infected insect cells (Rieger et al., 1997). Furthermore, increased levels of the LRP were found in the brain, spleen and pancreas of scrapie-infected mice and hamsters, as well as in scrapie-infected neuroblastoma cells, which are a well characterized *in vitro* model for scrapie infection (Rieger et al., 1997). These data suggest a link between the LRP/LR and prion propagation.

The non-integrin LRP/LR is a multifunctional protein that is required for cell differentiation, movement and growth (for review see (Gauczynski et al., 2001a)). The LRP cDNA encodes a 37-kDa precursor protein (LRP), also known as p40, and has been cloned from different species by several groups. This protein has been reported to be ribosome-associated, to bind to the histones H2A, H2B and H4 and to be the precursor of the metastasis-associated 67 kDa mature high-affinity laminin receptor (LR) (for review (Gauczynski et al., 2001a; Leucht, 2002)). The 67-kDa LR is consistently upregulated in aggressive carcinomas, suggesting a role in cell homeostasis and cohesion. The amino-acid sequence of the receptor is highly conserved through-out evolution, with at least 98.3% homology between the mouse, human and bovine sequences and 99% homology between the rat and human sequences (for review (Gauczynski et al., 2001a; Leucht, 2002)). Published data suggest the existence of at least six LR genes in the mouse genome; one of these is localized on chromosome nine and at least two copies are thought to be functional (Douville and Carbonetto, 1992). Using TRIBE-MCL, an algorithm for the detection of protein families (Enright et al., 2002), five LR genes were identified when the program was used to search the latest mouse draft genome sequence (Mouse Genome Sequencing Consortium, 2003, available at http://www.ensembl.org). The LRP gene on chromosome nine has seven exons and six introns but, in contrast with earlier results (Douville and Carbonetto, 1992), no LRP/LR gene on chromosome six has been identified. Interestingly, genes that affect susceptibility to prions have been identified on mouse chromosome nine (Stephenson et al., 2000).

PrP-specific antibodies have successfully been used in preventing prion propagation *in vitro* and *in vivo* as follows: first, the accumulation of PrP^{Sc} in scrapie-infected

neuroblastoma cells was inhibited by PrP-specific antibodies (Peretz et al., 2001a), second scrapie infection was abolished by transgenic expression of PrP-specific antibodies in mice (Heppner et al., 2001b). The epitope recognized by the antibody that has the most potent effect on PrP^{Sc}, D18, consists of amino-acid residues 132-156 of PrP, which includes helix A (residues 145-155). Because PrP residues 144-179 have been shown to constitute a binding site for the LRP/LR, we investigated whether antibodies directed against the LRP/LR, the cellular receptor of PrP^{c}, can also be used to interfere with the metabolism of PrP^{Sc}. To ablate LRP/LR expression from all putative LRP/LR-encoding genes we used an antisense RNA and a small interfering RNA (siRNA) approach. We investigated whether these strategies had an effect on prion propagation in several scrapie-infected cells systems.

Results and Discussion

Antisense LRP RNA prevents PrP^{Sc} propagation

To produce LRP antisense messenger RNA, we cloned a region of LRP complementary DNA from nucleotide position -65-901 into the expression plasmid pCI-neo in the antisense orientation to produce the pCI-neo-asLRP plasmid. After transient transfection of pCI-neo-asLRP into ScMNB cells we confirmed antisense LRP RNA expression in these cells (Fig. 1A). The level of the LRP mRNA was greatly reduced 48 h after transfection (Fig. 1B). Using phosphoimaging, this reduction was quantified and LRP mRNA levels were found to be 80-85% of normal LRP/LR mRNA expression levels. A similar reduction in target mRNA has been shown in other studies that have used the antisense RNA method to downregulate the expression of myelin basic protein (Katsuki et al., 1988) (80% reduction), and *Wnt-1* (Erickson et al., 1993) (up to 98% reduction). At the level of protein expression, no LRP protein was detected by western blotting 48 h after transfection (Fig. 1C). Analysis of cells 72 h after transfection showed an absence of the PrP^{Sc} propagation (Fig.1D) in cells with reduced LRP levels (Fig.1C).

Levels of PrP^{Sc} were unaffected in cells transfected with pCI-neo as compared with untransfected cells (Fig 1D). In ScMNB cells we were able to detect only the detect only the diglycosylated form of PrP using the SAF70 antibody, whereas in ScN2a cells and ScGT1 cells we observed the classic three-band pattern. We observed a reduction in PrP^{c} level after antisense LRP RNA transfection (Fig. 1D), which might be caused by an altered PrP^{c} metabolism. Previous studies have indicated that PrP^{c} internalization is highly dependent on the presence of the LRP/LR at the cell surface (Gauczynski et al., 2001b), where the LRP/LR binds PrP^{c} through two distinct domains: the octapeptide region and the region encompassing amino acids 144-179 of PrP^{c} (Hundt et al., 2001). This is consistent with a very recent study, in which it was found that the octarepeat region is essential for internalization of PrP^{c} (Nunziante et al., 2002). Hence, the altered PrP^{c} levels seen in this study are likely to be due to perturbed metabolism of the protein.

LRP-specific siRNAs prevent PrP^{Sc} propagation

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

We also tested the efficiency of the reduction of LRP expression using siRNAs in ScGT1 cells, which show a robust PrP^{Sc} phenotype (that is, these cells propagate PrP^{Sc}

over a long period of time). The results were consistent with those obtained using ScN2a cells, with a strong reduction of PrP^{Sc} correlated with LRP downregulation (Fig. 2C).

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

LRP/LR-specific antibodies have been used successfully to compete with recombinant prion proteins for binding to the LRP/LR in different mammalian cell types (Gauczynski et al., 2001b), showing that the LRP/LR has a crucial role in the metabolism of PrP^{c}. Using the LRP/LR-specific antibody, W3 (Rieger et al., 1997), in ScN2a cells we observed a reduction of PrP^{Sc} to undetectable levels (Fig. 3A, B). The antibody was used at concentrations of 6-64 μg ml^{-1}. At a concentration of 12 μg ml^{-1} a reduction in PrP^{Sc} levels was observed. At a higher concentration (64 μg ml^{-1}), PrP^{Sc} accumulation was completely abolished after incubation for three days, indicating a dose-dependent effect (Fig. 3A). In a time course experiment, we found a complete clearance of PrP^{Sc} after incubation for one week, using an antibody concentration of 32 μg ml^{-1} (Fig. 3B). These results are consistent with a previous study, in which different anti-PrP antibodies were used to reduce PrP^{Sc} levels in cultured cells (Peretz et al., 2001)(Table I). In that study, PrP antibody concentrations of 1.2-10.0 μg ml^{-1} were sufficient to clear PrP^{Sc} from ScN2a cells after one week of incubation (Table 1).

We also incubated ScN2a cells in which PrP^{Sc} had been previously cleared by W3 for a further two weeks without any antibody, and showed that no PrP^{Sc} reappeared (Fig. 3B). PrP^{c} levels in W3-treated cells were reduced after seven days of incubation with W3, but were completely restored after a further two-weeks incubation in the absence of the antibody (Fig. 3B).

Table 1 | Efficacy of anti-PrP and anti-LRP/LR antibodies in clearance of PrP^{Sc} from ScN2a cells

Antibody	Anti-LRP/LR	Anti-PrP[1]			
Incubation time	1 week	1 week			
Antibody	W3	D18	D13	R1	R2
Effective Concentration ($\mu g\ ml^{-1}$)	32	1.2	2.5	10	10

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

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

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

METHODS

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

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

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

Inhibition studies using LRP antisense RNA. ScMNB cells were grown in a six-well plates to 60% confluence. Cells were transfected with pCI-neo-asLRP and pCI-neo (control plasmid) using Lipofectamine (Invitrogen) in accordance with the manufacturer´s instructions. Transfection efficiencies were determined using a chloramphenicol acetyltransferase construct, and were estinated to be approximatively 80% on average (data not shown). Cells were harvested 72 h after transfection, lysed and analysed by western blotting.

Inhibition studies using small interfering RNAs. Four pairs of complementary 21-nucleotide RNA corresponding to regions of the LRP cDNA were made (Ambion). As a control, the lamin A/C RNA duplex was used (Elbashir et al., 2001b). The single-stranded complementary RNAs (Ambion) were annealed in annealing buffer (provided by the manufacturer) for 1 min at 90 °C, followed by incubation for 1 h at 37 °C. The RNA duplexes were transfected into ScN2a cells (cultured in Opti-MEM medium, Invitrogen) using Oligofectamine (Invitrogen) in accordance with the manufacturer´s instructions. ScGt1-7 cells were seeded in 60-mm Petri dishes ($5x10^5$ cells per dish) and transfected the following day with 10 μg of each of the 21-nucleotide RNA pairs using Exgen 500 (Fermentas) in accordance with the manufacturer´s instructions.

Ribonuclease protection assays. Total RNA was purified from transfected ScMNB cells and used in a ribonuclease protection assay using the RPA III kit (Ambion). An antisense riboprobe was made by *in vitro* transcription from pCI-neo-asLRP, following linearization of the plasmid with *Eco*RI, in the presence of [α-^{32}P]UTP. The antisense riboprobe was combined with the total RNA and the mixture was then precipitated. The precipitates were dissolved in hybridization buffer, denatured and hybridized with the total RNA. This was followed by incubation with Rnase for 30 min at 37°C,followed by inactivation of the Rnase and ethanol precipitation of the RNA. Protected RNA fragments were separated on an 5% acrylamid/urea gel and visualized using a Storm 860 phosphorimager equipped with ImageQuant software.

Reverse-transcriptase-PCR

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

Western blot analysis. Cytoplasmic lysates were made using a buffer containing 10 mM Tris/HCl pH 7.5, 100 mM NaCl, 10 mM EDTA, 0.5 % Triton X-100, 0.5 % sodium desoxycholate. After centrifugation, the total protein content of the lysates was measured (BCA-Protein Assay, Pierce)and equal amounts of protein were analysed. For PrP^{Sc} detection,. cell lysates were digested with proteinase K (20 μg ml^{-1}) for 1 h at 37°C . The reaction was stopped by the addition of Pefabloc (1mM) and the proteins were denatured with 6 M guanidine hydrochloride. Samples were boiled in SDS sample buffer and analysed on an SDS-polyacryamide gel containing 12.5% acrylamide. For PrPc and PrPSc detection (from ScN2a cells), 10% Bis-Tris gels with MES running buffer (NuPAGE, Invitrogen) were used. Proteins were blotted on a polyvinyllidene difluoride membrane, blocked and incubated overnight with the monoclonal antibody SAF70, SAF 32 or SAF84 (diluted 1:5000 in blocking solution) or A7 (diluted 1:2.500 in blocking solution) for PrP detection. The polyclonal anti LRP/LR antibody W3 (Rieger et al., 1997) (1:2000), or the monoclonal antibody 43512 (1 μg ml^{-1}) were used for LRP/LR detection and anti-ß Actin antibody (chemicon) (1:5000) for ß-actin detection.. After washing with TBS/0.05% Tween 20 the blot was incubated for 1 hwith a peroxidase-conjugated secondary antibody (Sigma) (1:2500). Detection was carried out by enhanced chemiluminescence (Western Lightning, NEN).

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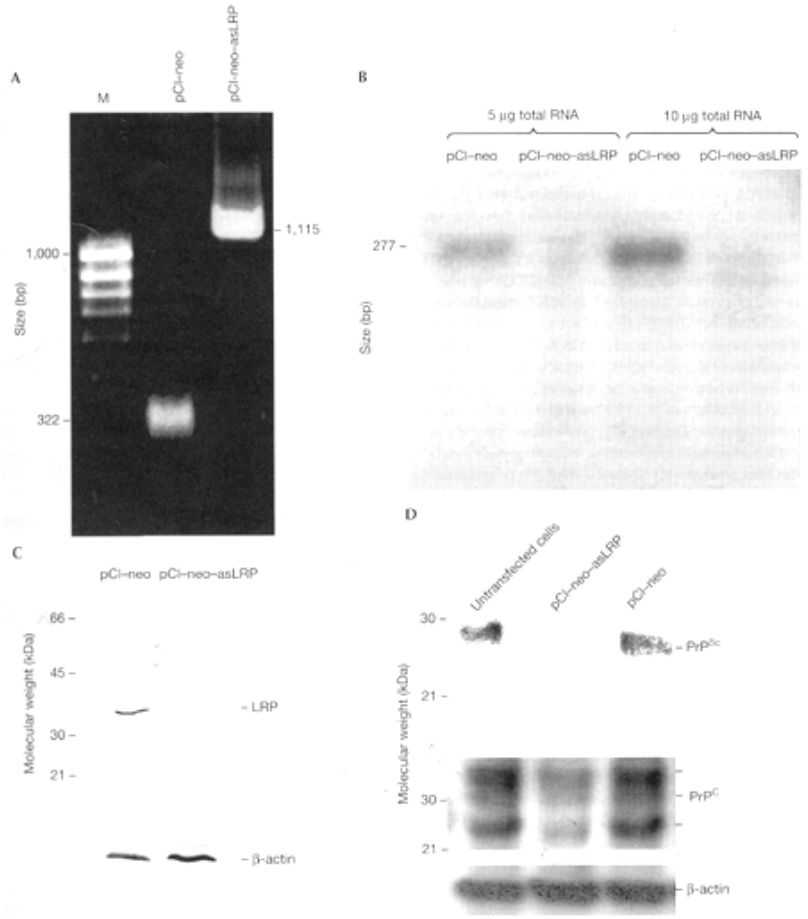

Figure 1. Abolition of PrP^{Sc} propagation using laminin receptor precursor (LRP) antisense RNA. **(A)** Analysis by PCR with reverse transcription of total RNA extracts from transfected ScMNB cells. Oligodesoxythymidine-primed complementary DNA was amplified by PCR using specific primers for the plasmid pCI-neo plasmid. This gave a 322-bp cDNA fragment for the pCI-neo transfected cells and a 1115-bp cDNA fragment for the pCI-neo-asLRP transfected cells. **(B)** A ribonuclease protection assay was carried out on total RNA from cells transfected either with pCI-neo or pCI-neo-asLRP; the RNA was then separated using a 5% acrylamide/urea gel. 5 μg or 10 μg of total RNA was used, and in both cases the level of LRP messenger RNA was reduced by 80-85% in cell transfected with pCI-neo-asLRP (quantified by posphorimaging). **(C)** Western blot analysis of cell lysates from pCI-neo and pCI-neo-asLRP-transfected ScMNB cells assayed 48 hours after transfection. LRP was detected using the polyclonal anti-LRP/LR antibody, W3. β-actin was detected using an anti-β-actin antibody as loading control. **(D)** ScMNB cells were transfected with pCI-neo and pCI-neo-asLRP. The PrP^{Sc} content of ScMNB cells was determined 72 h after transfection. The monoclonal anti-PrP antibody SAF70 was used for PrP^{Sc} detection and the SAF32 was used for detection of PrP^{c}.

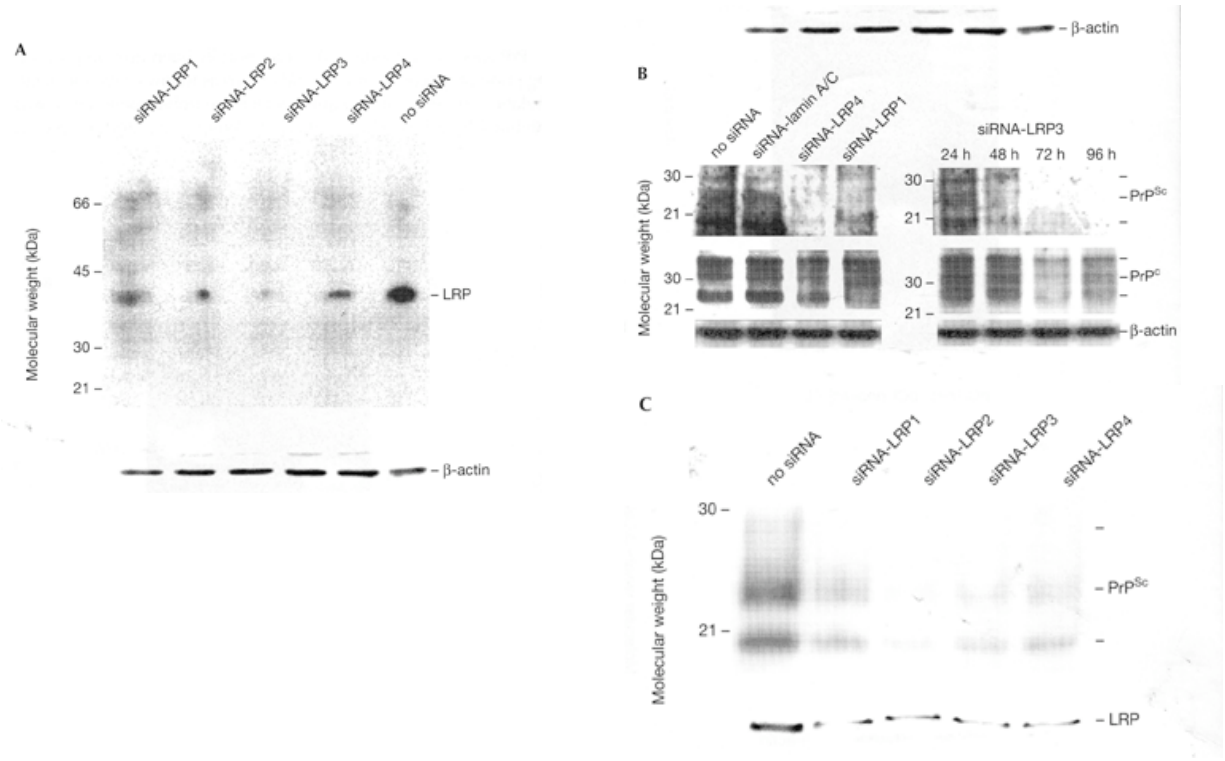

Figure 2. Inhibition of PrP^{Sc} propagation using small interfering RNAs. **(A)** Western blot analysis of ScN2a cells transfected with small interfering RNAs (siRNA). cells were analysed 72h after transfection using the polyclonal anti-laminin receptor (LRP/LR) antibody W3. **(B)** The effect of siRNAs on PrP^{Sc} propagation was assayed 72h after transfection (left panel). The time-dependent effect of siRNA-LRP3 on PrP^{Sc} propagation (right panel)was analysed using the SAF70 antibody; PrP^{c} was detected using the SAF32 antibody. β-actin was detected using an anti-β-actin antibody as loading control.

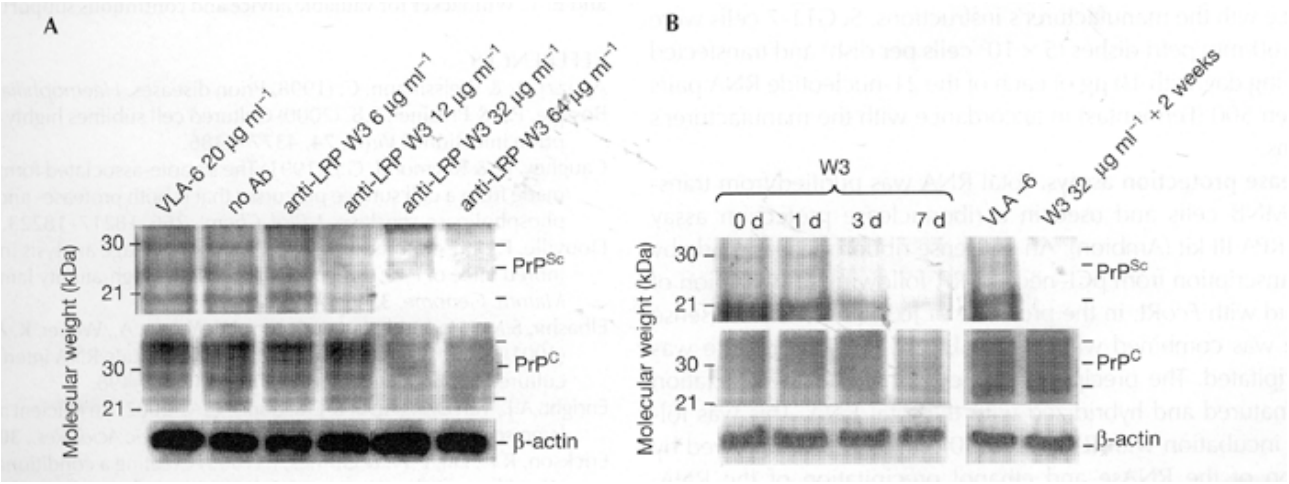

Figure 3. The effect of the W3 anti-laminin receptor (LRP/LR) antibody on PrP^{Sc} propagation. **(A)** ScN2a cells were incubated with W3 at varying concentrations. The PrP^{Sc} content was determined after a 72h incubation with W3. An anti VLA-6 (integrin-type laminin receptor) antibody was used as control. PrP^{Sc} was detected with the A7 polyconal antibody, PrP^{c} was detected with the SAF32 antibody. **(B)** ScN2a cells were incubated with W3 at 32μg ml^{-1} for varying durations. The last lane shows W3-treated ScN2a cells after an additional 2-week incubation without any antibody. PrP^{Sc} was detected with the SAF 70 antibody, PrP^{c} was detected with the SAF32 antibody. β-actin was detected using an anti-β-actin antibody as a loading control.

CHAPTER IV

Single chain Fv antibodies directed against the 37kDa/ 67kDa laminin receptor reduce peripheral PrP^{Sc} propagation

Submitted as:

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Single chain Fv antibodies directed against the 37kDa/67kDa laminin receptor reduce peripheral PrP^{Sc} propagation ***J. Virol****. (2005)*

Abstract

Transmissible spongiform encephalopathies are a group of disorder associated with the deposition of PrP^{Sc}, an abnormal form of the cellular prion protein PrP^{c}. The 37 kDa/67 kDa laminin receptor (LRP/LR) has been identified as the prion protein receptor and several lines of evidence strongly suggest that this protein plays a role during prion pathogenesis. A recent *in vitro* study indicates that anti-LRP antibodies are able to abolish PrP^{Sc} propagation in chronically infected cells. We therefore developed recombinant single chain antibodies (scFvs) directed against LRP suitable for therapeutic use. The specific LRP/LR recognition of two of the selected scFvs S18 and N3 was confirmed by Western blotting and FACS analysis. Both scFvs were capable to abrogate PrP/LRP interactions *in vitro*. After passive immunotransfer of the scFv S18 antibody via intraperitoneal injection into C57BL6 mice one day prior to intraperitoneal RML prion inoculation, reduced PrP^{Sc} levels were observed in the spleen 90 days post scFv injection. Although intraperitoneal injection of scFv S18 did not prolong the incubation times in RML inoculated mice, the *in vivo* data concerning the peripheral PrP^{Sc} propagation are encouraging and illustrate that immunotherapeutic approaches targeting LRP are worth pursuing

Introduction

Prion diseases are slow, invariably fatal neurodegenerative diseases with no known therapy. This group of infectious disorders includes Creutzfeldt-Jakob disease in humans, scrapie in sheep, and bovine spongiform encephalopathy (BSE) in cattle. This class of disease is remarkable since the causative agent is not a classical pathogen such as bacteria or viruses but an infectious protein. Prions are an abnormally folded form of the benign cellular prion protein (PrP^{c}). A key event in the disease pathology is the conversion of PrP^{c} into the infectious isoform referred to as PrP^{Sc} that accumulates in the brain. PrP^{Sc} has distinct biochemical properties including insolubility and partial resistance to proteolytic digestion (Caughey and Raymond, 1991). It is not yet clear

where the conversion process takes place but it is thought to occur either at the plasma membrane (Kaneko et al., 1997) where PrP^c is anchored via GPI or after internalization (Arnold et al., 1995). The molecules leading to PrP binding and internalization are thus a prerequesite for prion replication.

We identified the non-integrin 37 kDa/67 kDa laminin receptor (LRP/LR) as the cell-surface receptor for PrP^c together with heparan sulfate proteoglycans (HSPG) as cofactors (Gauczynski et al., 2001b; Hundt et al., 2001) and showed that LRP/LR is required for PrP^{Sc} propagation in scrapie infected cells (Leucht et al., 2003). LRP/LR is expressed in the human small intestinal mucosa (Shmakov et al., 2000), suggesting that LRP might be directly implicated in prion invasion. Very recently, we proved that bovine prions are endocytosed by human enterocytes via 37 kDa/67 kDa LRP/LR (Morel et al., 2005). These data were confirmed by the fact that moPrP27-30 binds LRP/LR dependent to mammalian cells (Gauczynski et al., submitted). A polyclonal anti-LRP specific antibody (Rieger et al., 1997) is able to interfere with (i) PrP^{BSE} internalization, (ii) PrP27-30 cell binding (Gauczynski et al., submitted) and (iii) with PrP^{Sc} propagation in cultured neuronal cells, the classical model to investigate the therapeutic potential of compounds in prion diseases (Leucht et al., 2003). These data strongly suggest that LRP might act as a promising target in the prophylaxis and/or therapy of prion diseases with anti-LRP/LR specific antibodies as powerful therapeutic/prophylactic tools. Since polyclonal antibodies are inappropriate for therapeutic applications, we developed monoclonal single chain antibodies (scFv) (Bird et al., 1988) directed against LRP. ScFvs, which consist of immunoglobulin heavy and light chain variable domains connected by a peptide linker, are a commonly used antibody format. ScFvs are attractive therapeutic agents in this particular case due to their small size, their high specificity and low immunogenicity and the absence of the Fc part that is responsible for activation of the complement cascade (Raag and Whitlow, 1995). Another major advance is the rapid selection process by phage display technology bypassing hybridoma technology and immunization (McCafferty et al., 1990).

In the present report, we developed tools for antibody based therapy of prion diseases using LRP, the prion receptor as a target. ScFvs directed against LRP have been selected by phage display using a naive and a synthetic library (Clackson et al., 1991).

Two scFvs have been selected and further characterized by western blotting and FACS analysis. The feasibility of this approach was confirmed by *in vitro* experiments proving that the selected scFvs were able to prevent interaction between LRP and PrP. Furthermore, treatment with S18 is able to reduce PrP^{Sc} peripheral accumulation in mice infected with prions.

Materials and Methods

Phage display selection and enzyme-linked immunosorbent assay (ELISA) screening. Three rounds of selection were performed on a GST::LRP fusion protein expressed in Baculovirus infected Sf9 cells (Rieger et al., 1997). Approximately 10^{12} phages from each library resuspended in PBS, 0.1% tween, 2 % milk were incubated with polystyrene immobilized GST::LRP. Phages that did not specifically bind were removed by ten washing steps with PBS, 0.1% tween. Bound entities were eluted by using Glycine-HCl, pH 2.2, and after neutralisation with 2 M Tris/HCl, pH 8, the eluate was used for infection of freshly grown E. coli XL1 Blue cells. Cells successfully transduced with phagemids encoding the human scFvs were selected for ampicillin resistance and were subsequently infected with M13K07 helper phage to generate phage progeny displaying scFv for the following *in vitro* selection. After the third round of selection individual colonies were grown in LB medium containing 100 µg/mL ampicillin and 20 µg/mL tetracycline at 30 °C. Cells were harvested by centrifugation and resuspended in 200 mM Tris-HCl, pH 7.5, 20% Sucrose, 1 mM EDTA. During incubation on ice the outer membrane is destroyed so that soluble periplasmic proteins including the scFv are released into the liquid. After elimination of cellular debris by centrifugation, the crude extracts were tested in ELISA for scFv antibody fragments binding the GST::LRP fusion protein.

Detection of scFv bound to immobilized GST::LRP (200ng/well) was carried out by using an anti His HRP-conjugate (Qiagen, 1μg/mL). The signal was developed with the tetramethyl benzidine solution (TMB) (KPL) and detected at 450 nm after termination of the reaction with 0.5 M H_2SO_4.

ScFvs expression and purification. The clones S18 and N3 were subcloned as *NcoI-NotI* restriction fragments into the vector pSKK2 (Le Gall et al., 2004) resulting in the plasmids pSKK2-S18 and pSKK2-N3, respectively. The clone C9 encoding for a scFv antibody directed against preS1 a hepatitis B coat protein (Persing et al., 1987) was subcloned into pSKK2 resulting in pSKK2-C9. The constructs were transformed into the *E. coli* RV308 and plated onto 2 YT agar containing 100ug/mL ampicillin and 50mM glucose. For expression, bacteria were cultured at 26°C in 2 YT medium supplemented with ampicillin and glucose until a cell density between 0.6 and 0.8 at an optical density of 600nm was achieved. After centrifugation bacterial pellets were resuspended in fresh YTBS medium supplemented with 1M D-sorbitol and 2,5mM betaine with 0,2mM IPTG and grown at 21°C overnight. The cells were resuspended in 50mM phosphate buffer pH 8, 300mM NaCl containing 20mM imidazole, ß-mercaptoethanol and protease inhibitors (PMSF, aprotinin, leupeptin). After snap-freezing in liquid nitrogen, the lysate was digested 1h with 1mg/mL lysozyme and centrifuged at 4°C 14500rpm for 1h. The supernatant was incubated with equilibrated Probond Nickel-chelating resin (Invitrogen). Beads were washed with the buffer described and finally eluted with 50mM phosphate buffer pH 8, 300mM NaCl and 250mM imidazole. For their application in animal experiments, scFvs were further purified by size exclusion chromatography using a sephadex S200 column and filter sterilized.

Expression of mouse LRP::FLAG and human LRP::FLAG in the Semliki-Forest-Virus (SFV) System. Expression of mouse and human LRP::FLAG was described previously (Gauczynski et al., 2001b). Briefly, SFV based vectors carrying mouse LRP::FLAG or human LRP::FLAG were used. Recombinant SFV-1 RNAs were generated by *in vitro* transcription and transfected into BHK cells by electroporation.

FACS analysis (flow cytometry). Single-cell suspensions were prepared in PBS, 2% fetal calf serum, 20 mM EDTA, 0.01% sodium azide (FACS buffer). For flow cytometry, cells were incubated with the primary antibody at concentrations of ~1 μg/10^6 cells for 15 minutes at room temperature. Cells were washed in FACS buffer before incubation with

FITC-conjugated anti-myc or anti-rabbit antibodies for 15 minutes at room temperature. After washing in FACS buffer, data acquisition and analysis were performed with an EPICS XL-MCL (Coulter) flow cytometer. A polyclonal anti-gal-3 antibody (Gauczynski et al., 2001b) and the scFvs N3 and S18 were used as primary antibodies.

Western blotting. Cells were lysed in 10mM Tris-HCl pH 7,5, 10mM NaCl, 10mM EDTA, 0,5% TritonX-100 and 0,5% sodium Deoxycholate. After centrifugation, equal amounts of protein were resuspended in SDS sample buffer and heated to 90°C for 10 minutes. Beads were eluted directly in SDS sample buffer. Samples were analyzed on a 12% SDS-polyacrylamide gel and blotted onto a polyvinylidene difluoride membrane, blocked with 5% milk and incubated with the primary antibodies N3, S18 (diluted 1:1000) or anti-LRP 43512 (diluted 1:5000). The scFvs were detected with an anti-c-myc antibody (Santa Cruz 1:1000), followed by a peroxidase conjugated anti-mouse antibody (Santa Cruz 1:5000). Detection was performed by enhanced chemiluminescence (Perkin Elmer Life Sciences).

Epitope mapping. The entire sequence of human LRP was covered by 92 different synthetic peptides of 15 amino acids length. The N-terminus of each peptide was shifted with respect to the previous peptide by three amino acids, leading to an overlap of 12 amino acids. Synthesis was performed on a cellulose membrane (AIMS, Braunschweig) using Fmoc chemistry (PyBop/NMM activation, Trt/tBu/Pbf/Boc side chain protection) according to the SPOT-synthesis method of Frank (Frank, 1992) using a spotting robot (Syro, MultiSynTech GmbH, Witten, Germany). Detection was performed as described for western blotting.

Generation of recombinant proteins. pGEX-4T (Amersham) was used for GST expression and pGEX-2T-huPrP23-230 for GST::huPrP23-230 expression. HuPrP23-230 was cloned into pGEX2T as described for the construction of pGEX2T-haPrP23-231 (Weiss et al., 1995). The cDNA fragment encoding huLRP (aa 1-295) was amplified by PCR and subcloned via *BamHI* and *EcoRI* into expression vector pGEX-4T. GST, GST::huPrP23-230 and GST::huLRP were produced in *E.coli* BL21 cells as described for

GST and GST::haPrP (Weiss et al., 1995). These proteins were purified by glutathione-sepharose affinity chromatography (Amersham Biosciences). GST::LRP was digested with 5 units biotinylated thrombin (Thrombin kit, Novagen) in a final volume of 500µL. The supernatant was incubated with streptavidin agarose beads to remove the recombinant thrombin according to the manufacturer´s instructions.

Pull-down assay. 4 µg LRP were pre-incubated with 100µg scFv. GST::PrP beads, GST beads and unloaded beads were first saturated with bovine serum albumine for 1h. 25µL beads were then added to the reaction in a final volume of 350µL binding buffer (50mM TrisHCl pH 8, 300mM NaCl, 0,025% NP40) for 1 hour at room temperature. The beads were washed 4 times in the same buffer. Bound proteins were eluted directly in SDS loading buffer for SDS-PAGE analysis.

Animal Experiments. *Analysis of peripheral PrP^{Sc} accumulation (spleen analysis) of mice injected with scFvs.* 3 groups of 6 C57BL/6 female mice were injected intraperitoneally with 100 µL of a 10% RML brain homogenate prepared from the brains of terminally sick mice. The mice were treated intraperitoneally once a week with 1mg of antibodies (S18 or C9) diluted in PBS for a total period of 8 weeks, the first treatment being given one day prior to RML prion inoculation. Control mice were treated with PBS solution. 90 days after prion inoculation, mice were sacrificed and spleens were homogenized in PBS to 10% w/v. The homogenates were adjusted to 5mg/ml and digested with 50 µg/ml of proteinase K (1h, 37°C). 150 µg of total protein were analyzed by SDS-PAGE followed by Western Blotting using the antibody SAF83. Undigested lysates were used as a loading control. The density bands obtained were analyzed using NIH software.

Analysis of the incubation times of mice treated with scFvs. 3 groups of C57BL6 female mice were intraperitoneally injected with S18, C9 and PBS followed by inoculation with RML prions. The same conditions were applied as for the analysis of the peripheral PrP^{Sc} accumulation described above. Mice were sacrificed when two of the TSE-specific symtoms described by Sethi et al. appeared (Sethi et al., 2002).

Investigation of side effects of the scFvs. To investigate side effects of scFv, 2 groups of 6 C57BL/6 mice were injected with PBS or S18 during 8 weeks. The animals were

sacrificed 4 and 8 weeks or 120 days after beginning of treatment, blood was collected in EDTA and analysed for immune cell content (Vet-Med-Labor, Munich). Different organs (liver, brain, kidney, spleen) were collected and analysed.

RESULTS

Selection of scFv against LRP by phage display

Specific phage display fragments binding to GST::LRP were selected from a naïve and a synthetic phage scFv library (Schwarz et al., 2004). After 3 rounds of affinity selection on GST::LRP, 47 individual clones from each library were selected to test their ability to recognize GST::LRP by ELISA. 66% of the selected clones from the naïve library and 53% from the synthetic library showed a positive signal (Fig.1 a, b, d).
The specific LRP recognition of the selected antibodies was further confirmed by Western Blot Analysis. The selected antibodies clearly recognized GST::LRP but not GST (Fig. 1c), demonstrating that the antibodies specifically recognized the LRP part of the fusion protein. A *Bst*NI fingerprinting of the DNAs of the 13 clones selected from the naïve library showed that 10 clones were identical. One clone was identified twice and another one revealed an individual restriction pattern (Fig. 1d). Due to their strong antigenic recognition, we selected the scFv clones S18 and N3 for further characterization.

Specific recognition of native and denatured LRP by scFvs S18 and N3 expressed in *E.coli*

The scFvs S18 and N3 were expressed in *E.coli* and purified by IMAC. Fig. 2 a shows the purified antibodies with molecular weights of approx. 35 kDa analyzed on a polyacrylamide gel.

To investigate the ability of the scFvs S18 and N3 expressed in *E. coli* to recognize denaturated LRP, we used them to probe Western Blots of cell lysate from BHK cells overexpressing human LRP::FLAG and murine LRP::FLAG, respectively. Purified S18 and N3 specifically recognized mouseLRP::FLAG and humanLRP::FLAG overexpressed in BHK cells with the Semliki Forest Virus sytem as well as the endogenous LRP (Fig 2 b). In contrast to S18, N3 also recognized the 67 kDa LR form (Fig. 2b). In order to use the scFvs S18 and N3 for therapeutic purposes, they should be able to recognize LRP/LR on the cell surface under native conditions. To assess if the antibodies were able to recognize the native LRP/LR on the cell surface, a FACS analysis was carried out on non permeabilized LRP transfected BHK cells. The FACS profiles obtained with both scFvs illustrate a specific staining of the cell surface for LRP/LR whereas no staining could be detected with a control antibody directed against gal 3 (ß-galactoside lectin galectin-3) (Fig.2 b). These data were confirmed by immunofluorescense techniques with the same cells (data not shown). Taken together, these results indicate that N3 and S18 specifically recognize the denatured and the native form of LRP on the cell surface.

Epitope Mapping of scFvs S18 and N3

For therapeutic applications of the scFv antibodies, S18 and N3 should be directed against the extracellular part of the receptor. Here the antibodies should directly compete with the binding of PrP^{c} and PrP^{Sc} by saturating the receptor at the cell surface. In order to identify the epitopes on LRP for S18 and N3 an epitope mapping was performed. 92 synthetic 15 mer peptides covering the LRP sequence and overlapping by 12 amino acid residues each were synthesized on a cellulose membrane. Under Western blot conditions, the membrane was incubated with the scFv N3, S18 or C9. The scFv C9 is directed against preS1, a hepatitis B coat protein and was used as a control (Persing et al., 1987). Four intense dots were visualized for N3 and three for S18 (Fig. 3a). The control scFv C9 did not recognize any dots (data not shown). Comparing the signal position with the corresponding peptide sequence, we identified the epitope $EEFQGEWTA_{225\text{-}233}$ for S18 and $TEDWSA_{273\text{-}278}$ for N3. Both epitopes are located in the extracellular part of LRP

(Fig. 3b). However, these epitopes are not located in the direct PrP binding site stretching from aa 161 to 179 (Hundt et al., 2001). Nevertheless, an indirect binding site via HSPGs has been described and is thought to be located between amino acids 180-285 of LRP (Hundt et al., 2001).

ScFvs S18 and N3 interfere with PrP/LRP interaction

We next studied the ability of these scFvs to interfere with the PrP/LRP interaction. GST::huPrP23-230 interacts with huLRP *in vitro* (Fig. 4) in a pull down assay as previously shown for FLAG::huPrP23-230 and GST::huLRP (Fig. 4 M in (Hundt et al., 2001)). In contrast to the control antibody C9, both scFv antibodies S18 and N3 after pre-incubation with huLRP were able to block the GST::huPrP23-230/huLRP interaction (Fig. 4). These data suggest that the selected scFvs might be promising tools for the treatment of prion diseases.

The scFv S18 reduced PrP^{Sc} accumulation in the spleen in a murine scrapie model by passive immunization

We first examined whether passive immunization with anti-LRP scFv exhibit side effect. Mice were injected with 1 mg of S18 per week or PBS during a period of eight weeks. Animals were killed at different time points after injection and blood was analysed. No significant differences could be observed between PBS and S18 treated mice leading to the conclusion that scFv S18 revealed no side effects and was suitable for passive immunization. Mice were intraperitoneally injected with 1mg of the scFvs S18 and C9, respectively, once a week for a period of 8 weeks. These mice were intraperitoneally challenged with Rocky Mountain Laboratory (RML) scrapie brain homogenate one day after beginning passive immunization. We determined the PrP^{Sc} levels in the spleen, an organ known to be an early site of PrP^{Sc} deposition, 90 days after challenging, which corresponds to the plateau phase of PrP^{Sc} accumulation. Compared to mice injected with PBS or with the control scFv antibody C9, two (out of six) mice injected with S18 exhibit a strong reduction of the PrP^{Sc} level in the spleen (Fig. 5). However, no significant differences could be observed in the survival time between the

control groups (PBS and C9) and the group treated with the anti-LRP scFv S18 (Fig. 6 Table 1). A Student-test proved that the survival time obtained are statistically equal The differences observed in the average are likely to be due to the small size of S18 and C9 groups.

DISCUSSION

In order to develop new anti-prion therapies, we selected and characterized scFvs directed against LRP by phage display. Two scFvs N3 and S18 were selected from a naïve and a synthetic scFv library using recombinant human GST-LRP as a target molecule. The capacity of the selected scFvs to block the PrP/LRP interaction in vitro (i) and the anti-prion effect on the peripheral PrP^{Sc} accumulation in a murine model (ii), recommend scFvs directed against LRP as potentially efficient tools in TSEs therapy.
Since a new variant form of CJD resulting from the consumption of BSE contaminated material has been identified in the UK and several other countries (Ironside et al., 1996; Will et al., 1996), therapeutic and prophylactic strategies have emerged with the aim to combat TSEs.

Among the numerous molecules harboring an anti-prion activity (for review: (Gauczynski et al., 2001a)) antibodies might be promising alternative tools for the therapy of prion diseases. Antibodies against PrP inhibit PrP^{Sc} propagation in cell culture (Enari et al., 2001; Peretz et al., 2001 ; Perrier et al., 2004) and also in mice (Heppner et al., 2001b; White et al., 2003). Since these antibodies have no or only low affinities for PrP^{Sc}, they might work by saturating PrP^{c} at the cell surface thereby reducing the availability of PrP^{c} for conversion. Recently, however, anti-TSE strategies focusing on PrP as the main target have been contested, since PrP antibodies injected into the brain of mice led to rapid neuronal apoptosis (Solforosi et al., 2004). The observed toxicity might be due to PrP^{c} crosslinking by the anti-PrP antibodies. Thus, LRP/LR seems to be a realistic alternative candidate to avoid secondary effects caused by an anti-PrP based therapy.

The selected anti-LRP scFv antibodies S18 and N3 are directed against an epitope located within the indirect PrP/LRP binding site

We used GST::LRP as target to screen two phage-display libraries. Two of these scFvs, S18 selected form the synthetic scFv library and N3 selected from the naïve library, were further characterized and their specific LRP recognition was demonstrated. The epitopes for S18 and N3 on LRP have been mapped and are located between aa225 and aa233 and aa273 and aa278, respectively. Both epitopes are within the extracellular domain of LRP and might target an indirect HSPG dependent PrP/LRP binding site, which is thought to stretch from aa180 to aa285 of LRP (Hundt et al., 2001). It is likely that both scFv might interfere with PrP binding to LRP via steric hindrance.

The selected anti-LRP scFvs S18 and N3 prevent PrP^{c}/LRP interaction *in vitro* and S18 reduces peripheral PrP^{Sc} accumulation *in vivo*

LRP is the cellular receptor for PrP^{c} and has been recently proven to be required for PrP^{BSE} internalization by human enterocytes (Morel et al., 2005) and moPrP27-30 binding to eukaryotic cells (Gauczynski et al., submitted) suggesting that LRP/LR acts as a receptor for PrP^{Sc}. Therefore, it is likely that this protein required for PrP^{c} and PrP^{Sc} internalization might promote cell-to-cell propagation of infectivity. ScFvs directed against LRP represent attractive tools in prion disease therapy. We therefore investigated whether these anti-LRP antibodies show inhibitory effects on prion replication *in vivo* by passive immunization. S18 exhibits a clear tendency to reduce PrP^{Sc} levels in the spleen even if this effect is not pronounced. However, the treatment did not prolong the incubation time in mice inoculated RML prions. This partial effect can be explained by the fact that the reduction of PrP^{Sc} observed in the spleen was not sufficient to delay the neuroinvasion. The inhibitory potential of antibodies on PrP^{Sc} propagation resulting in prolonged incubation times might depend on the long term delivery of high antibody levels as observed in case of monoclonal anti-PrP antibodies. Treatment of mice with these kind of antibodies resulted in a delay of the onset of the prion disease and strongly

reduced PrP^{Sc} levels in the spleen (White et al., 2003). However, a high antibody amount of 2 mg had to be injected twice a week during the entire life span of the mice. Due to the limited production yield of scFv S18 and N3 in our *E.coli* system, which cannot achieve the high amounts of the hybridoma technology (Kohler and Milstein, 1975), we treated C57BL6 mice with 1 mg of antibody per week for a period of only 8 weeks, which might explain a weaker reduction of PrP^{Sc} levels in the spleen and no significant prolongation of the incubation times. Furthermore, scFvs have generally a shorter half life than immunoglobulins (Maack et al., 1979). These technical limits might be overcome by taking advantages of scFv *in vivo* expression by gene transfer, e.g. employing viral vector systems. The scFvs affinity might also be improved by mutagenesis techniques which might be concomitant with an increase in antibody stability (Adams and Schier, 1999; Kobayashi et al., 1999; Natarajan et al., 2005; Schier et al., 1996).

Advantages of scFvs compared to classical monoclonal antibodies for therapy of TSEs

Monoclonal antibodies consist of a light and a heavy chain encoded by 2 individual genes, whereas single chain miniantibodies consist only of the antigen recognition site of the light and heavy chain connected by a peptide linker. Therefore gene transfer is facilitated by scFvs. The delivery of scFvs is also not limited by their size and any transfer system such as a viral vector system might be applied (Afanasieva et al., 2003; Arafat et al., 2002). *In vivo* transduction might contribute to continuous and sustained scFv expression at target sites where PrP^{Sc} accumulates such as the central nervous system or lymphoid organs. Full-size immunoglobulins can be delivered by passive transfer, but it is unlikely that significant amounts will reach the brain due to their large size. In contrast, scFvs exhibit better tissue penetration and can therefore rapidly reach the clinical study phase e.g. for anti-tumoral investigations (Azemar et al., 2003; Mayer et al., 2000). A very recent work reported that anti-PrP single chain antibodies expressed in mammalian cells exert a paracrine anti-prion activity (Donofrio et al., 2005). Therefore it might be interesting to consider a bi-therapy associating anti-LRP and anti-PrP scFvs to improve the anti-prion treatment.

In conclusion, production and selection of scFvs against the prion receptor is the first step towards targeting therapeutic antibodies into the brain via gene therapy. The ability of the anti-LRP scFv antibodies to abrogate LRP/PrP binding and to decrease peripheral PrP^{Sc} accumulation makes them promising candidates for further development of protecting molecules for prion diseases.

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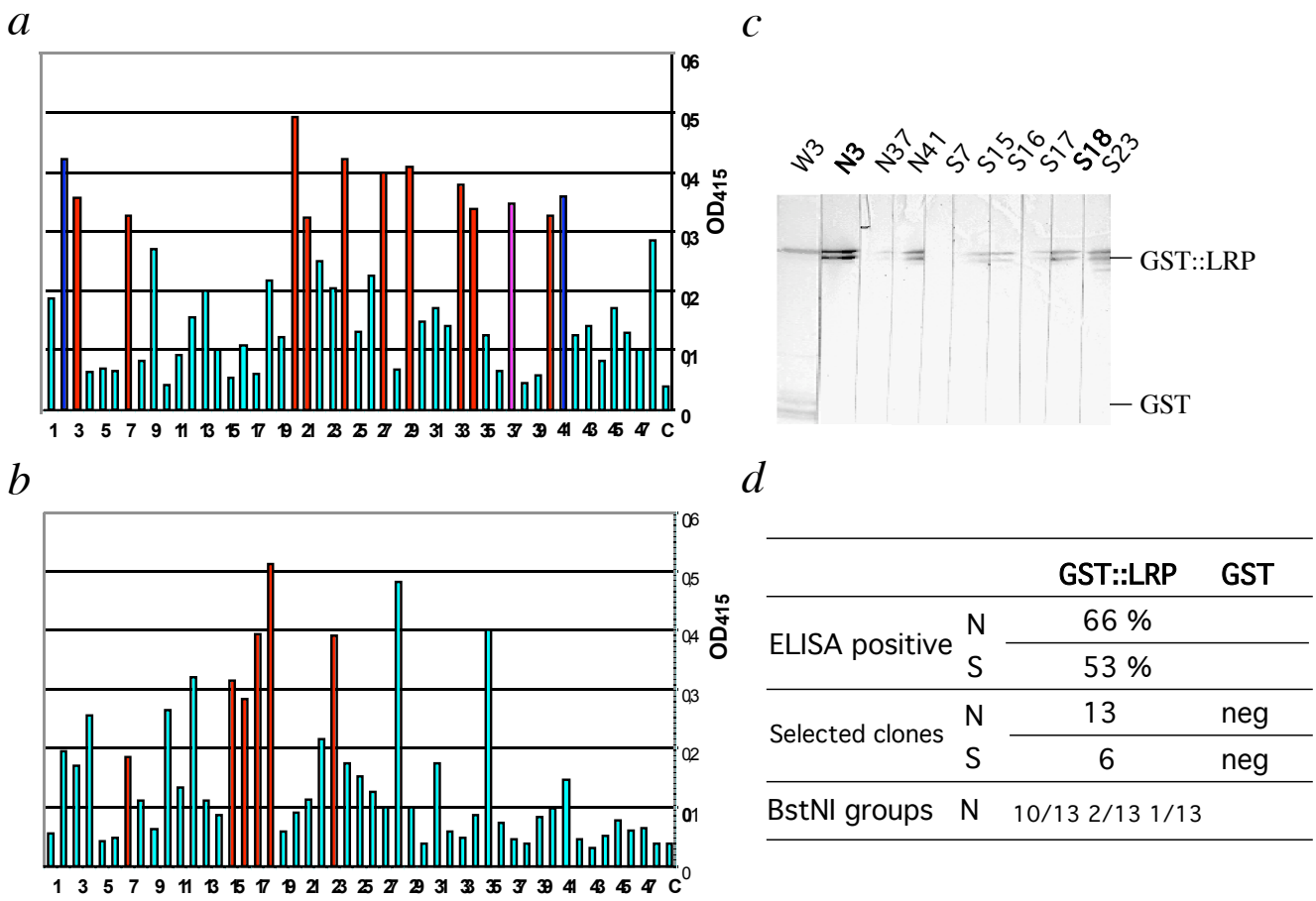

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		GST::LRP	GST
ELISA positive	N	66 %	
	S	53 %	
Selected clones	N	13	neg
	S	6	neg
BstNI groups	N	10/13 2/13 1/13	

Figure 1. Selection of scFvs by phage display. Clones of each library (a: naive library b: synthetic library) were analyzed for their ability to bind GST::LRP by ELISA. c: Unique clones were used for the detection of recombinant GST and GST::LRP by western blotting d: Selection summary: A *Bst*NI fingerprinting of the DNAs of the 13 clones selected from the naïve library revealed 10 identical clones. One clone was identified twice and another one revealed an individual restriction pattern. All clones tested recognized specifically GST::LRP. Due to their strong antigenic recognition, scFv clones S18 and N3 were selected for further characterization. N: Naive library, S: Synthetic library.

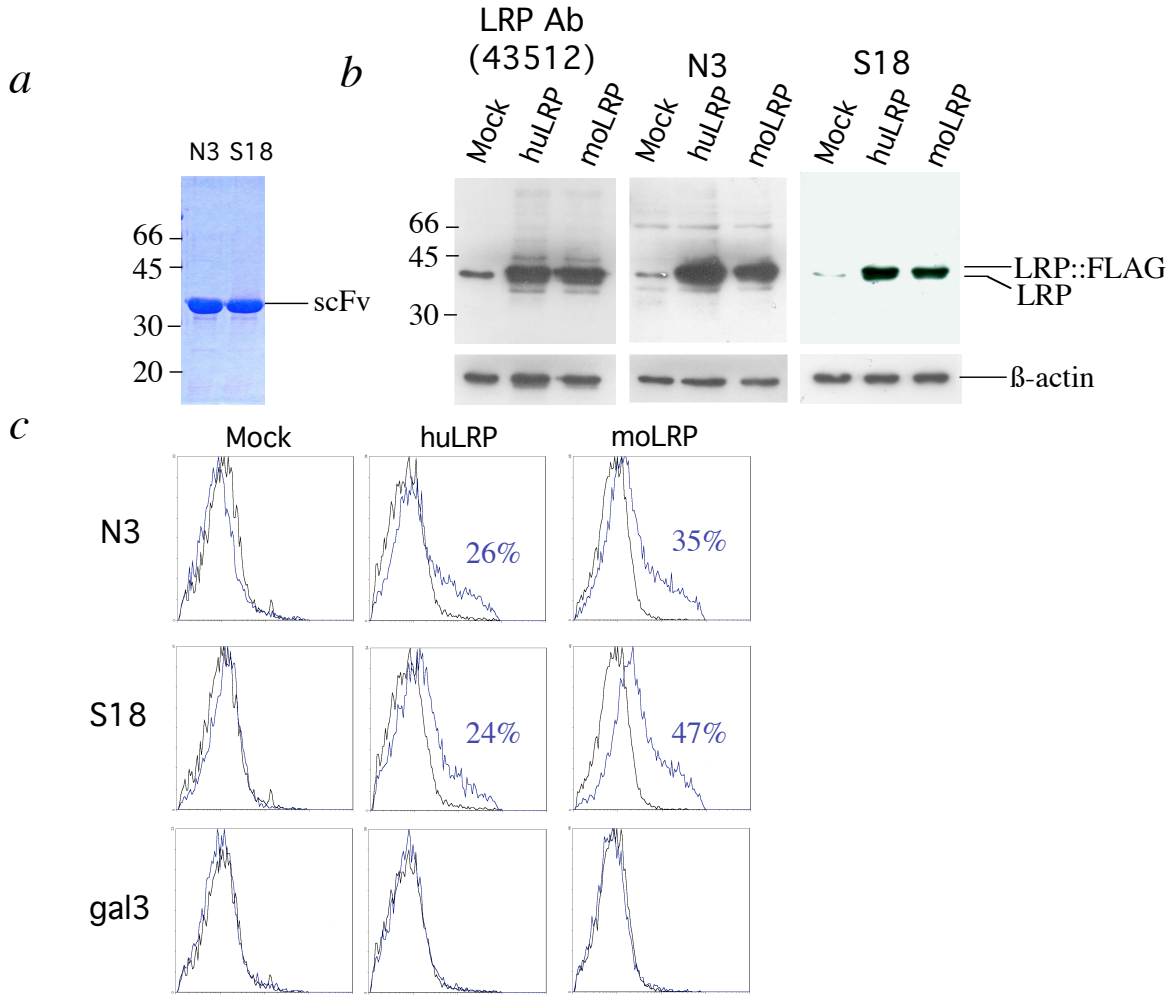

Figure 2. scFvs N3 and S18 specifically detect denatured and native LRP. a : SDS-PAGE analysis of purified scFvs expressed in *E. coli*. Proteins were visualized by Coomassie brillant blue staining. b: BHK cells were transfected with SFV RNA, SFV huLRP::FLAG RNA , SFV huLRP::FLAG RNA. Cell lysates were analysed by western blotting, using N3, S18 or the monoclonal anti-LRP antibody 43512 as control. c: Non-permeabilized cells were analysed by FACS using sc Fvs N3, S18 or the anti-gal-3 antibody.

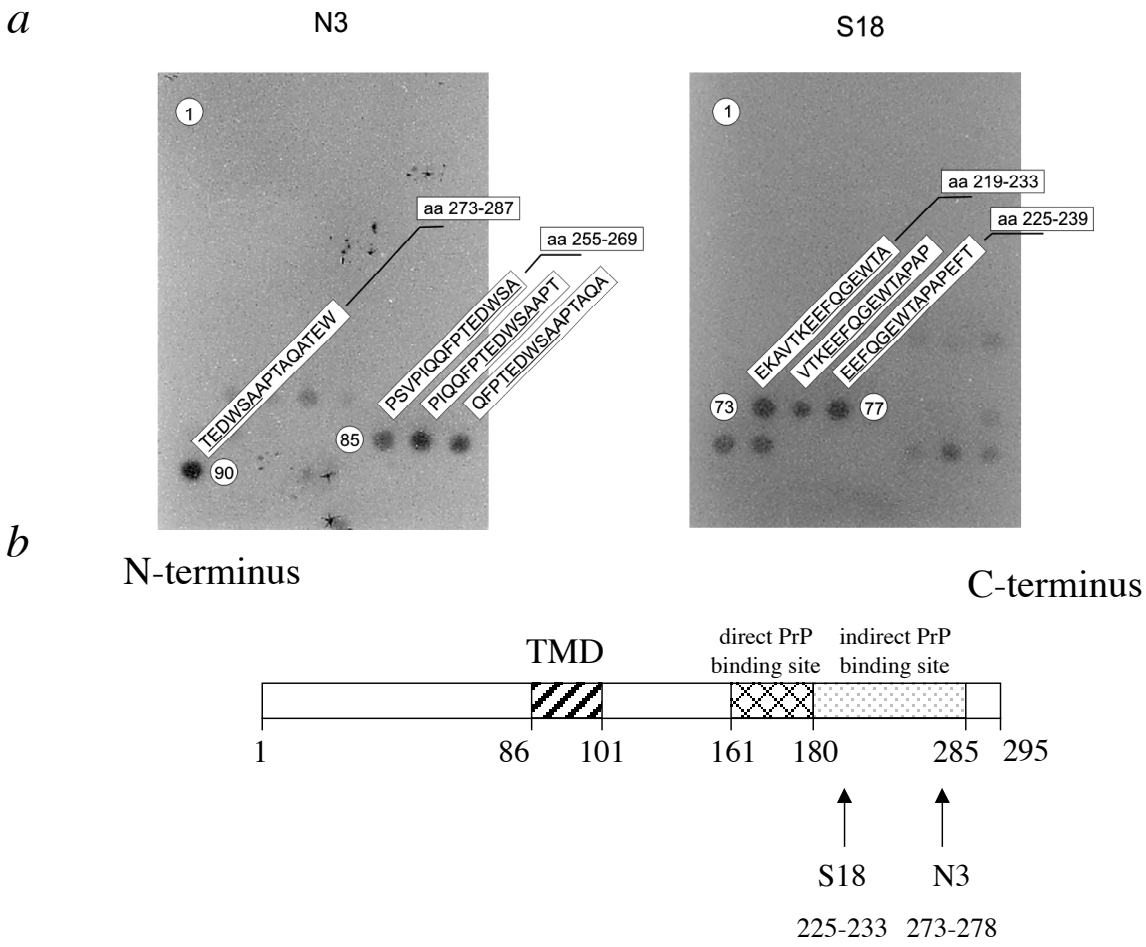

Figure 3. Epitope mapping of scFv S18 and N3. a: Membranes covering huLRP sequence were hybridized with N3 and S18. The sequence of the peptides detected is indicated as well as the aa number. b: Schematic representation of LRP, its PrP binding sites, and epitopes. Binding sites are shown : the direct binding site to PrP between aa 161-179, the suggested indirect binding domain between aa180-285, N3 epitope aa273-278, S18 epitope aa225-233.

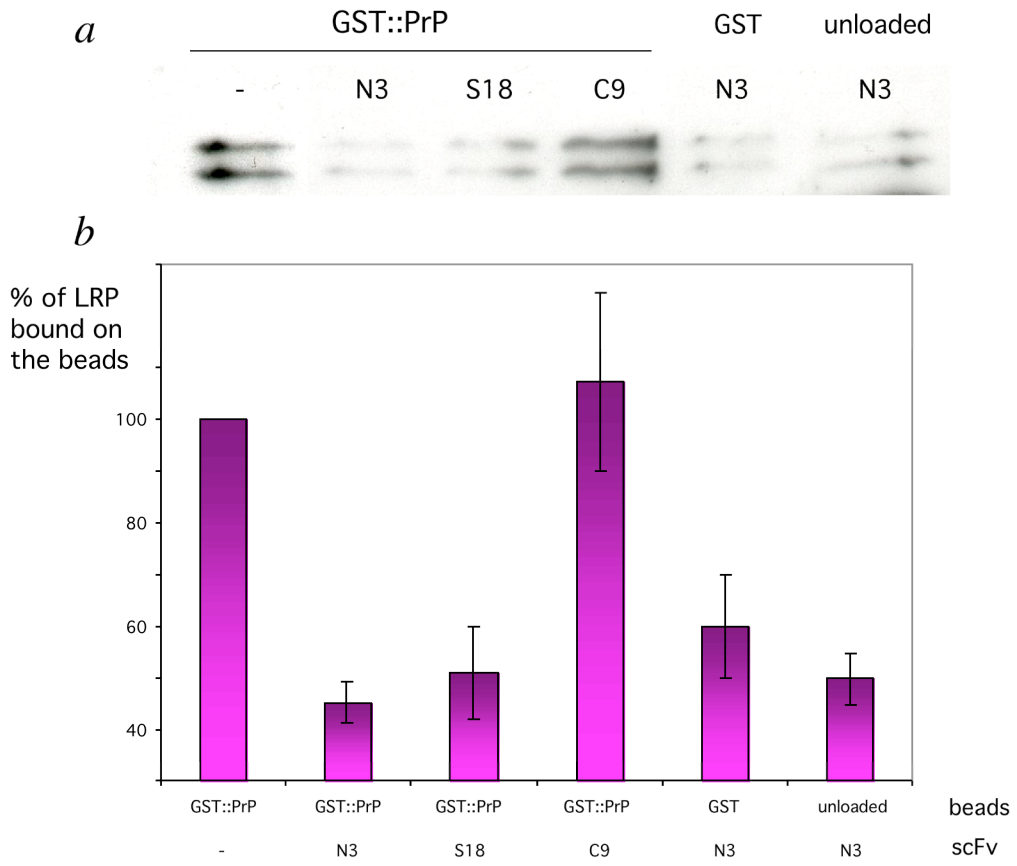

Figure 4. S18 and N3 are able to prevent the PrP/LRP interaction in vitro. a: GST::huPrP immobilized on sepharose glutathione beads was incubated with recombinant LRP in presence or absence of scFvs as indicated. GST immobilized on beads and unloaded beads were employed as negative controls. After elution, LRP bound on beads was analysed by western blotting using N3 for detection. b: Quantitative analysis of western blots performed by densitometric evaluation of the LRP level in 3 independent experiments.

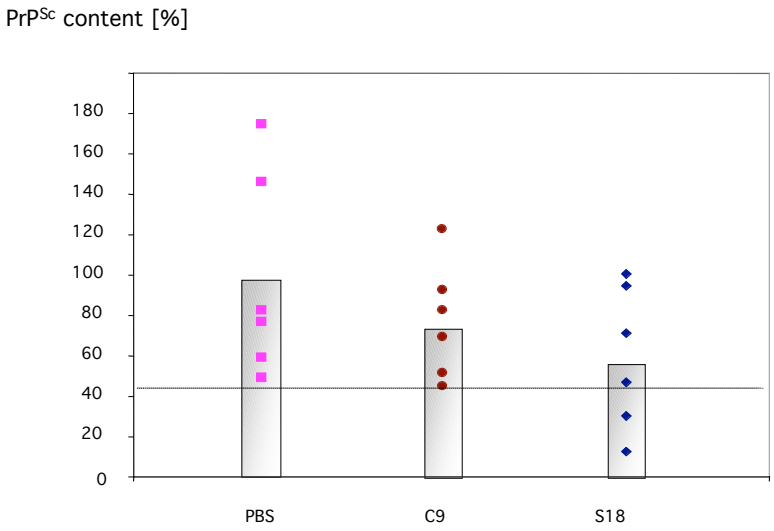

Figure 5. Spleen analysis of mice inoculated with prions after treatment with PBS, C9 or S18. Each group consists of 6 animals. Spleens have been analysed 90 days after scrapie inoculation by western blotting after PK digestion. The density of the bands has been measured with the NIH software and the values have been used to produce the graph presented. The average of the PrP^{Sc} content of mice injected with PBS was set to 100%. Bars represent the average of PrP^{Sc} levels and each individual value is also shown. Two (out of six) mice injected with S18 exhibit a strong reduction of the PrP^{Sc} level (below dashed line).

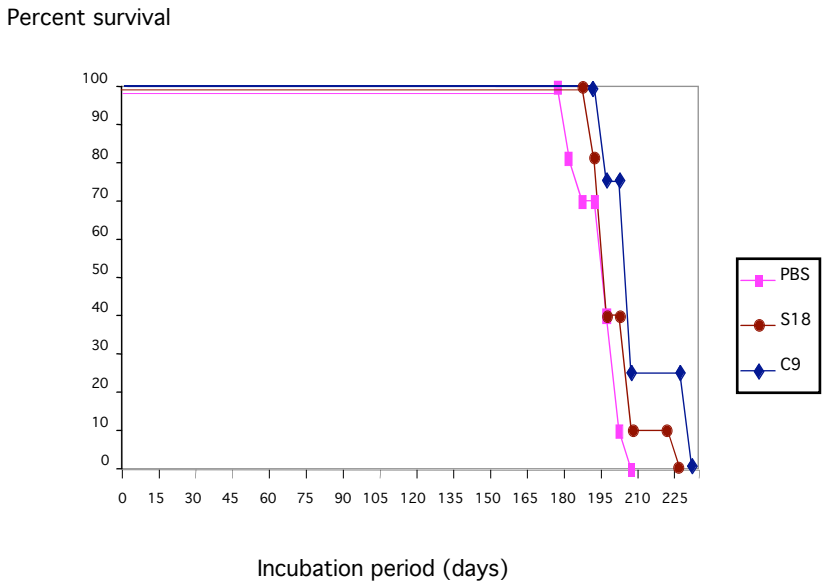

Figure 6. Survival time of mice inoculated with RML prions after intraperitoneal treatment with PBS, C9 or S18. The S18 group and the C9 group consist of 5 and 4 animals, respectively, and the PBS group of 10 animals. There was no significant difference in survival.

Experimental group	Incubation period, days (mean+/- SD)	Number of mice (affected/inoculated)
PBS	191 +/- 9	10/10
S18	200 +/- 12	5/5
C9	207 +/- 12	4/4

SD = standard deviation

Table 1. Summary of survival times of C57BL6 mice infected with the single chain antibodies S18 and C9

CHAPTER V

Delivery of anti-laminin receptor single chain antibodies into the brain via AAV vectors for prion disease Gene therapy

Submitted as:

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Delivery of anti-laminin receptor single chain antibodies into the brain via AAV vectors for prion disease gene therapy ***J. Virol.*** (2005)

Abstract

Prion diseases are untreatable neurodegenerative disorders, associated with the deposition of a disease-related form of prion protein (PrP). Experimental evidence suggest that hampering the interaction between PrP and its receptor, the non integrin laminin receptor (LRP/LR) may provide a novel therapeutic strategy to inhibit PrP^{Sc} propagation. Based on this consideration, we previously screened anti-LRP single chain antibodies (scFv) and proved that one of these contributes to reduce PrP^{Sc} deposition in peripheric organs. In the present work, we have focused on the development of an *in vivo* expression system of scFvs based on adeno-associated virus (AAV) vectors. As a proof of principle, we show that neuronal cells were able to secrete high levels of functional scFvs by transient transfection. Furthermore, our data demonstrate that rAAV serotype 2 vectors carrying scFv enable transgene expression in their secretory form. These vectors were administered in mice by stereotaxic intracerebral injection and the expression of scFvs was confirmed one month post injection.

Introduction

Prion diseases are invariably lethal neurodegenerative illnesses affecting humans and animals. None of the affected individuals can be treated or cured effectively. Brain from affected individuals contains the abnormal form of the prion protein, PrP^{Sc}, which propagates mainly in the brain and in the lymphoreticular system. PrP^{Sc} is distinct from the host protein PrP^{c} by its biochemical properties such as proteinase K sensitivity and insolubility but harbors the same amino acid sequence. It is now accepted that the generation of PrP^{Sc} from PrP^{c} involves conformational changes accompanied by modifications in the secondary structure of the protein. Prion diseases represent a unique class of disorders caused by an infectious protein as the infectious agent.

Different studies have pointed out the pivotal role of the 37kDa/67kDa laminin receptor (LRP) in prion infection. LRP has been shown to act as a PrP^{c} receptor (Gauczynski et

al., 2001b) and is responsible for bovine PrP^{Sc} internalization by human enterocytes (Morel et al., 2005). The fact that LRP is overexpressed in tissues and organs of infected animals strongly suggests that this protein is not only implicated in prion entrance after oral infection but also in other aspects of pathogenesis (Rieger et al., 1997). We proposed that the implication of LRP in PrP life cycle supports PrP^{Sc} replication. Consequently this protein attracts particular attention as a target for prion diseases therapy. Multiple strategies on LRP inactivation have been shown to be successfull by inhibiting PrP^{Sc} propagation *in vitro*: (1) down regulation of LPR via antisense or siRNA strategies totally blocks PrP^{Sc} propagation and (2) saturation of LRP at the cell surface with anti-LRP antibodies also abrogates PrP^{Sc} accumulation (Leucht et al., 2003). Monoclonal antibodies are attractive therapeutic agents and almost 20 of them obtained FDA approval for therapeutic use in patients. Nevertheless immunotherapy is limited by the immunogenicity of murine derived antibodies and the restricted tissue penetration. Alternative systems have been developed by engineering e.g. single chain antibodies (scFv). In contrast to entire immunoglobulins, scFv are much smaller in size that allows them to penetrate into tissue and they do not provoke an immune response (for review (Sanz et al., 2005)). Recently, we selected two scFv (S18 and N3) directed against LRP from a human antibody phage-display library (Rey et al., submitted). These antibodies are able to abolish the interaction between PrP and LRP. Furthermore one of them (S18) exhibits an anti-prion effect on PrP^{Sc} peripheral accumulation in a murine model. Despite advantages offered by scFv, due to their short half-life, passive immunization implicitates at least weekly injections of antibodies at high doses. An alternative to obtain sustained therapeutic concentration is to take advantage of gene transfer by viral vectors. For this purpose, we chose to express the single chain antibodies S18 and N3 as secretory molecules *in situ* via adeno-associated virus (AAV) vectors. Originally, the first AAV has been found as a contaminant in adenovirus stocks (Atchison et al., 1965). AAV is a member of the parvovirus family. For a productive infection AAV depends on co-infection of an unrelated helper virus as adenovirus or herpesvirus and is therefore classified as a Dependovirus. Up to now 11 serotypes have been identified named AAV type 1 to AAV type 11 (Gao et al., 2002; Mori et al., 2004). Although the other seroptypes have recently attracted increased attention, AAV type 2 is the most prominent

and best characterized serotype utilized as vector for gene therapy. This serotype offers a series of advantages for gene therapy: (i) they can efficiently transduce a wide variety of dividing and non dividing cells, e.g. cells of the CNS and muscle (ii) the vector genome persists for extended periods supplying long-term expression of the transgene (iii) AAV show low immunogenicity when injected *in vivo* and are not related to any pathogenicity (Tal, 2000). However, although the wild-type AAV specifically integrates into host genome, current recombinant AAV vectors have lost these characteristics (Berns and Linden, 1995). The packaging capacity of AAV is restricted to 5kb but since the genes encoding for scFv S18 and N3 are as small as 1kb, this factor is not a limitation for scFv expression. AAV2 based vectors have received increasing attention as candidates for gene therapy and currently 27 gene therapeutic approaches are under investigation worldwilde in clinical trials. Applications of AAV to treat neurodegenerative diseases are also actively studied in experimental models (Azzouz et al., 2000; Feng et al., 2004; Fu et al., 2002; Kirik et al., 2002).

Here we describe the first gene therapeutic attempt for prion diseases. To develop gene immunotherapy mediated by AAV delivery, we constructed an AAV specific vector containing anti-LRP scFv coding sequence comprising a secretion signal and driven by a cytomegalovirus (CMV) promoter. We further generated recombinant AAV particles carrying scFv sequences. To investigate the feasibility of AAV based gene therapy for TSEs, we delivered the viral vectors into the hippocampus of mice by direct stereotaxic microinjection. We show scFv expression in the brain of mice 30 days post recombinant AAV injection.

Materials and Methods

Construction of AAV vectors carrying scFv against LRP. The sequence encoding for each scFv (N3, S18 or C9) was subcloned from the mammalian expression vector pSecTag2B (Invitrogen) into the AAV vector plasmid pSub/CEP4 (Wendtner et al., 2002) together with a secretory sequence (Coloma et al., 1992), a myc tag and a

polyhistidine tag. Briefly, the sequences were amplified by PCR and inserted into the *XbaI* site of pSub/CEP4.

Production and purification of recombinant AAV vectors. For the generation of recombinant AAV vectors, 3 plasmids are required : the vector plasmid containing the transgene flanked by the viral ITRs (pSub/CEP4-N3, -S18 or –C9) the helper-plasmid pRC (Wendtner et al., 2002) carrying the two AAV specific open reading frames REP and CAP, the adenoviral plasmid pXX6-80 (Xiao et al., 1998) providing adenoviral function necessary for AAV replication. The production and the purification of recombinant AAV were done as previously described (Wendtner et al., 2002). Briefly, 293 cells were cotransfected with the 3 plasmids leading to the production of viral particles devoid of wild type AAV virus or adenovirus (Grimm et al., 1998). The lysate was purified by iodoxinol gradient followed by heparin affinity chromatography.

Titering of AAV stock. The genomic titer was determined by dot blot. An aliquot from the elution fractions obtained after purification was digested by proteinase K (1mg/ml) in 75mM Tris-HCl pH8, 25mM EDTA for 2h. The samples were denaturated in NaOH (0,5 N) prior to immobilization onto a nylon membrane along with the plasmid standard dilution using a dot-blot apparatus (GibcoBRL). The blots were probed with a transgene-specific digoxigenin-11-dUTP (Roche) probe synthetized by PCR. The membranes were further incubated with an anti-digoxigenin antibody coupled to HRP (Roche). For detection, an enhanced chemiluminescence kit was used. To determine the genomic titer, the signal obtained for the vector genome was compared with the signal generated from the plasmid DNA standard curve.

Cell culture and transfection. Hela, N2a and GT1 cells were grown in DMEM supplemented with 10% fetal bovine serum, streptomycin and penicillin at 37°C with 5% CO_2. For transfection, cells were seeded in 6-well plates and transfected the following day with 2µg DNA using Gene Porter (Peqlab) following manufacturer´s instructions. The cells were analysed by Westernblot or FACS analysis 48h after transfection.

***In vitro* transduction.** Cells were seeded one day prior to AAV transduction into 12-well plates ($1,8\times10^5$cells per well). They were infected with a multiplicity of infection (MOI) of 10000, the supernatant and the cell lysate were analysed 3 days after transduction by Werstern blot or FACS analysis when transduced with rAAV-GFP. For FACS, the cells were harvested, resuspended in 0,01% sodium azide, 20mM EDTA, 2% FCS and analysed directly.

Western blot analyses. To test the scFv expression after transfection or transduction, the medium was collected and the cells were lysed in 10mM Tris-Hcl, pH7,5, 100mM NaCl, 10mM EDTA, 0,5% Triton X-100 and 0,5% sodium deoxycholate. 25µL of supernatant or 25 µL of cell lysate were separated on a 12% SDS polyacrylamide gel and transferred on to PVDF membrane. The membrane was preincubated in a blocking solution (5% milk, 0,1% Tween in PBS) for 45 min and incubated with a murine anti-myc tag antibody (Santa Cruz). A horseradish peroxidase-conjugated anti-mouse IgG (Santa Cruz) was used as secondary antibody. The blot was developed with enhanced chemiluminescence (Western lightning, NEN).

Stereotaxic injection of AAV vectors into the brain of C57bl/6 mice. Animals were maintained and treated in accordance with ethical guidelines of Bavaria. Prior to microinjection, mice were anesthetized with an intraperitoneal injection of Xylazin, Ketamin and Vetranquil and were placed in a stereotaxic apparatus (SR-6N Narishige). Their head was immobilized using an adaptor. For injection into the hippocampus, a 5µL Hamilton syringe is placed 2 mm below the surface via a burr hole, 1,7 mm to the right and 2 mm posterior to point bregma according to Paxinos and Franklin (The mouse brain in stereotaxic coordinates). The vectors are injected through the syringe at a rate of 1µL /min. A total of 5×10^9 viral particles was delivered into each mouse brain in a volume of 5µL. At the end of injection, the needle was allowed to remain in the brain for 2 additional min before being retracted.

Pull-down Assays. Brain homogenate was diluted in 5 mL 6M guanidium-Hcl, 0,1M Na_2HPO_4/NaH_2PO_4 pH 8. The lysates were sonicated (30 secondes, 50% power) and

incubated for 2 hours at room temperature with 100 µL Ni^{2+} beads (Probond resin, Invitrogen) in presence of 10mM imidazole. Beads were extensively washed first with lysis buffer and then with phosphate buffer (0,1M Na_2HPO_4/NaH_2PO_4 pH 8). Bound proteins were eluted directly in SDS-sample buffer and analysed by immunoblotting.

Results

Secretion of functional scFvs by eukaryotic cells

In order to develop a therapy against prion diseases, we have previously selected scFv directed against the prion receptor, LRP/LR (Rey et al., submitted). The screening have been realized by phage display. Three rounds of selection were performed by panning on recombinant GST::LRP. Among the scFv fragments resulting from the third round of selection, N3 and S18 showed the best performance. The cDNA encoding for anti-LRP scFv N3 and S18 as well as a control scFv C9 directed against the coat protein preS1 of hepatitis B virus have been configurated into the pSecTag vector for eukaryotic expression. This vector contains a carboxy-terminal myc tag contributing to specific detection of the expressed protein and a polyhistidine tag for rapid purification. It also includes an Igκ leader sequence which routes proteins via cellular secretory pathway (Figure 1a).

To confirm that scFvs could be produced and secreted by mammalian cells, pSecTag plasmids carrying the different scFvs were transiently transfected into the neuronal cell line N2a. The expression was analysed in the medium and in the cellular fraction by western blotting 48h post transfection(Figure 2a). 25 µL of sample were separated on 12% SDS polyacrylamide gel and scFv were visualized with an anti-myc antibody. Western blot analysis demonstrated that desired proteins were expressed and secreted at significant levels and that the migration pattern was consistent with the expected molecular weight of approx. 35 kDa. This model system proves that single chain antibodies can be produced by neuronal cells which is the first step towards a gene

therapeutic approach. It is important to emphasize that the amount of secreted protein is really high since it can be visualized in a small fraction of supernatant (25 µL out of 1ml) without concentration or precipitation. To validate that the secreted antibodies retained their original specificity, scFv produced by mammalian cells have been used to detect LRP by westernblotting. Medium collected from transiently transfected cells was used. ScFv originated from mammalian expression specifically recognized moLRP::FLAG, as recombinant scFv produced in *E. coli* (Figure 2b). Together, these data provide the proof that mammalian cells are able to secrete high quantities of functional single chain antibody fragments.

AAV serotypes suitable to infect neuronal cells

To express the potential therapeutic antibodies *in vivo* within the brain, we decided to develop recombinant AAV vectors approach. In this regard, these vectors are able to infect a wide variety of cell types. Up to now, eleven AAV serotypes have been described with different tropism. To test which serotype was suitable for neuronal cells, four serotypes AAV1, AAV2, AAV3 and AAV5 carrying GFP gene have been used to transduce 2 neuronal cell lines: N2a and GT1. The transduction efficiency has been evaluated by FACS (Figure 3). AAV3 and AAV5 led to poor transduction efficiencies in both cell lines. On the contrary AA1 and AAV2 achieved high transduction rate: AAV1 resulted in approximatevely 72% and 39% positive cells respectively for GT1 and N2a cells. AAV2 generated 73% positive GT1 and 19% positive N2a cells. These analyses revealed that both serotype 1 and serotype 2 are appropriate candidates for neuronal transduction. Despite better results for AAV1 in N2a cells, we reasoned that AAV2 was a better choice regarding purification. Indeed, AAV2 is the better known serotype and can be purified via heparin affinity chromatography which is not the case for other serotypes (Zolotukhin et al., 1999).

Expression of scFv via AAV vector in neuronal cells

To develop an AAV-based gene therapy, we subcloned the sequence encoding for scFv as well as the myc and oligohistidine tags and the secretion sequence into AAV-based vector (pSub/CEP4) (Figure 1b). Transfection of N2a cells followed by analysis of cellular supernatants confirmed that this vector system achieved high level of the secretory scFvs (Figure 4a). The replication-defective AAV based vector pSub/CEP4 carrying transgene sequence was transfected in 293 cells together with 2 helper plasmids to produce recombinant AAV particles. The resultant recombinant viruses were purified by iodaxinaol gradient and heparin affinity chromatography. To verify that rAAV directed the expression of the desired protein, N2a cells and GT1 cells were transduced at a MOI of 10000, the supernatant was collected 3 days post-transduction and analysed for the presence of scFvs. The level of AAV-mediated expression was sufficiently hight to allow detection of scFv by western blot in both cell types. However since the transduction rate is low in N2a cells, high levels of scFv could be detected in the medium only after repeated transduction (Figure 4b).

In vivo expression of the recombinant AAV encoding scFv

PrP^{Sc} accumulates mainly in the central nervous system and particularly high amounts could be find in the hippocampus. For that reason, we decided to target this area of the brain directly by stereotaxic injection. 5×10^{9} genomic particles were injected to each mice and the presence of the secreted scFv was investigated by Western blotting 30 days after AAV treatment (Figure 5). ScFvs are detectable after concentration by pull-down assay.

The effect of anti-LRP antibodies against TSE is under investigation in a murine model. Mice have been microinjected with rAAV into the hippocampus, 2 weeks before intra cerebral scrapie inoculation (Figure 6b). The quality of rAAV injected is shown Figure 6a: rAAV have been used successfully to transduced hela cells.

DISCUSSION

Acting as the receptor for PrP (Gauczynski et al., 2001b), LRP/LR appears as a promising target for anti-prion therapy. Therefore, we searched for potentially neutralizing agents of LRP/LR. In the last decades, immunotherapy received growing attention although monoclonal antibodies have limited application in human clinical trials due to their immunogenicity.

These limitations have led to the development of alternative systems such as non-natural antibody fragments. Single shain antibodies are the smallest fragments engineered to date and are composed of variable regions of the heavy and light chains, respectively, joined via a short peptide linker. In a previous study, we have described the selection of anti-LRP scFvs. Using a passive transfer approach, one scFv termed S18 has been reported to reduce PrP^{Sc} deposition in the spleen of infected mice (Rey et al., submitted). However, i.p. injection of 1 mg of this antibody weekly for a period of eight weeks does not increase the survival time observed in treated animals compare to control animals injected with PBS. The efficacy of the antibody treatment might be dependent on the accessibility of highly infectious sites such as the brain and on the concentration of the scFv fragment. In addition, when the scFvs are applied i.p., it is unlikely that they can cross the blood brain barrier and therefore might fail to reach the brain were most of prions replicate. Furthermore, scFvs have short half-lives in the blood (approx. 2 days). To address limitations of the conventional delivery, we exploited a gene therapeutic approach based on the AAV vector system. In the present work, we demonstrate proof of principle for the first anti-prion gene therapy. We first showed that neuronal cells enable production and secretion of scFvs retaining their specificity. Following the generation of recombinant AAV vectors carrying a transgene encoding for scFv, C57BL/6 mice were intracerebrally injected with the virus. Detection of the scFv in the brain has been proven 30 days post injection.

Circulation of scFvs

We were not able to detect scFvs by immunohistochemistry, the localization of scFvs around the injection site in the hippocampus is then unknown. It has been shown

that AAV2 infects a restricted region near the injection site of the brain and this observation is believed to result from rapid HSPG uptake of AAV2 particles by neurons (Bartlett et al., 1998; Wang et al., 2003). However, our therapeutic proteins comprise a sequence specifying secretion, thus scFvs should circulate into tissue or using the brain microvasculature. Studies on diffusion in the brain report that proteins as big as BSA are able to diffuse efficiently in the brain extracellular space of the brain(Tao and Nicholson, 1996). Another group used AAV5 to deliver a naturally secreted lysosomal serine protease and could detect this enzyme up to 4 mm surrounding the injection site (Haskell et al., 2003). It is likely that our antibodies also diffuse in a similar way.

Improvement of the delivery system.

In this study, animals were treated with a single injection of rAAV2. One way to improve the strategy is to explore multiple injection approaches. For example, injection in the hippocampus in both hemispheres might increase the expression of the transgene. It is also possible to combine intracerebral treatment with systemic delivery to inhibit PrP^{Sc} invasion in peripheral organs and central nervous system in parallel. rAAVs are promising delivery vectors because they warrant long term expression of transgenes in absence of any toxicity and inflammatory responses. However, despite high titers the transduction efficiency is limited compared to other viral vector systems. An alternative is to employ adenovirus/AAV chimeras which combine efficient adenovirus-mediated gene transfer with stable gene expression by AAV (Goncalves et al., 2001). AAV1 appears to be a good candidate for brain transduction because it has been demonstrated that this serotype shows a wider distribution after transduction compared to AAV2 (Wang et al., 2003).

Improvement of antibodies.

Recombinant proteins that are smaller than 60kDa are taken up by the kidney and excreted into the urine (Maack et al., 1979). Therefore, scFvs tend to have a short half-

life. Increasing the size of the antibody fragment is thus an obvious strategy to prolong the half-life of antibodies. Multimerization or addition of groupment Fc or CH3 from IgG results in antibody fragments that are still suitable for gene therapy (Afanasieva et al., 2003). Altering the charge or isoelectric point (pI) can also change the pharmacokinetics of antibodies. For example, increasing cationic amino acids content (e.g., lysine) leads to prolonged half-life (Adams and Schier, 1999; Kobayashi et al., 1999).

It is reasonable to assume that the therapeutic effect is dictated by antigen affinity. Affinity maturation can be improve by site-directed mutagenesis that consists in substitution of amino acids in CDRs . The clones with higher affinity for the antigen are subsequently selected (Yang et al., 1995). Chain shuffling is another technique to select antibodies with increased affinity. In this methodology, a single V_H region from an antibody with affinity to a particular antigen is paired with an entire library of V_L. A new phage display library is constructed and panned on the antigen (Marks et al., 1992).

In conclusion, we initiated the first gene therapy model for prion diseases based on immunotherapy. We first demonstrated that our system was working *in vitro*: (i) anti-LRP single chain antibodies similar to those produced in a bacterial system can be secreted by mammalian cells due to a secretion signal. (ii) AAV2 is suitable to deliver the same scFv in neuronal cells. The preliminary results obtained in animals established that a single injection of rAAV carrying scFv sequence into the brain resulted in expression of the therapeutic protein. Further experiments that are in progress in our laboratory will reveal if the level of scFv produced in the brain is sufficient to prevent prion diseases.

Acknowledgements

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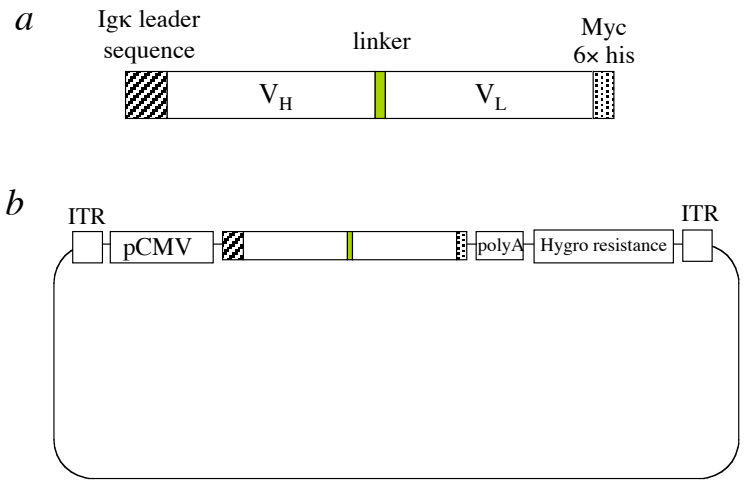

Figure 1. Schematic representation of scFv constructs. *a*: open-reading frame-derived scFvs were cloned into the plasmid pSecTagB. The coding sequence contains an Igκ leader sequence to allow secretion, the scFv cDNA consisting of variable part of heavy and light chain, a myc epitope tag and a polyhistidinne tag. *b*: a recombinant AAV vector encoding the secretory version of scFvs was constructed. ITR : Inverted Terminal Repeats.

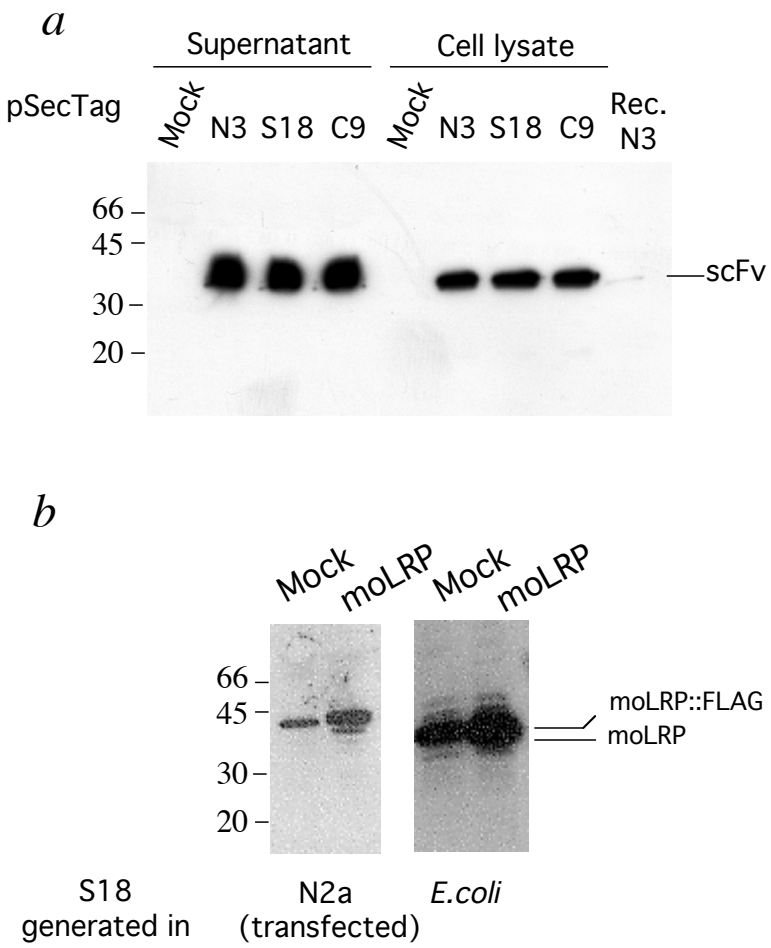

Figure 2.Extracellular and intracellular expression of functional scFvs in neuronal cells. *a*: N2a cells were transfected with pSecTag plasmids encoding secretory anti-LRP single-chain antibodies S18 and N3 or the control C9. Empty vector was used as negative control. After 48h, the supernatant and cell lysate were collected separately and analysed by SDS-PAGE, followed by Western blotting with a monoclonal anti-myc primary antibody. 5 ng of recombinant N3 produced in *E.coli* were used as control. *b*: supernatant collected from N2a cells transfected with pSecTag-S18 as well as S18 produced in *E.coli* were employed as primary antibodies to detect mouse LRP (moLRP) overexpressed in BHK cells by Western blotting.

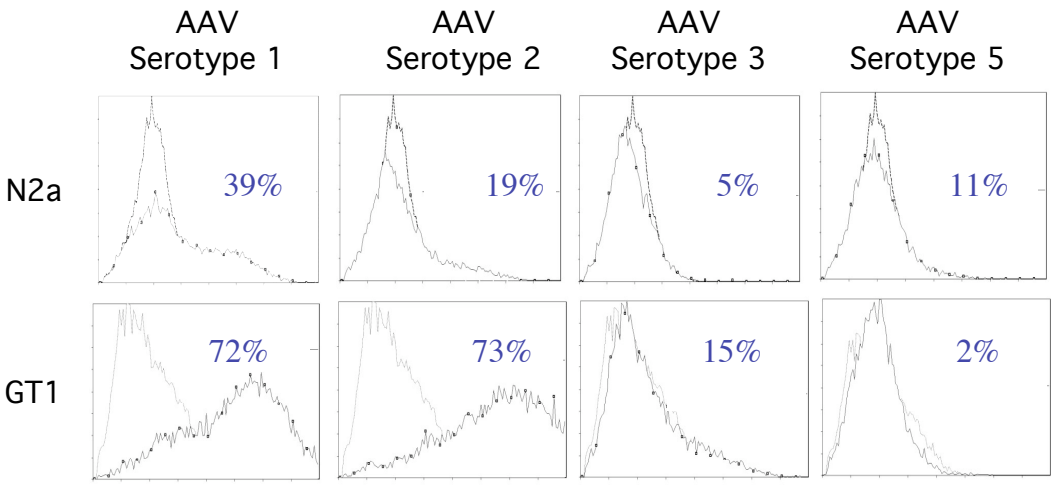

Figure 3. Comparison of neuronal transduction efficiency for AAVserotype 1, 2, 3 and 5

GT1 or N2a cells were transduced with different serotype of rAAV-GFP. After 72h, the cells were harvested and the fluorescence was measured by FACS. Merge of non-transduced cells and transduced cells (dashed profile) is shown with the percentage of positive cells. Fluorescence intensity (abscissa) is plotted against a relative cell numbers (ordinate).

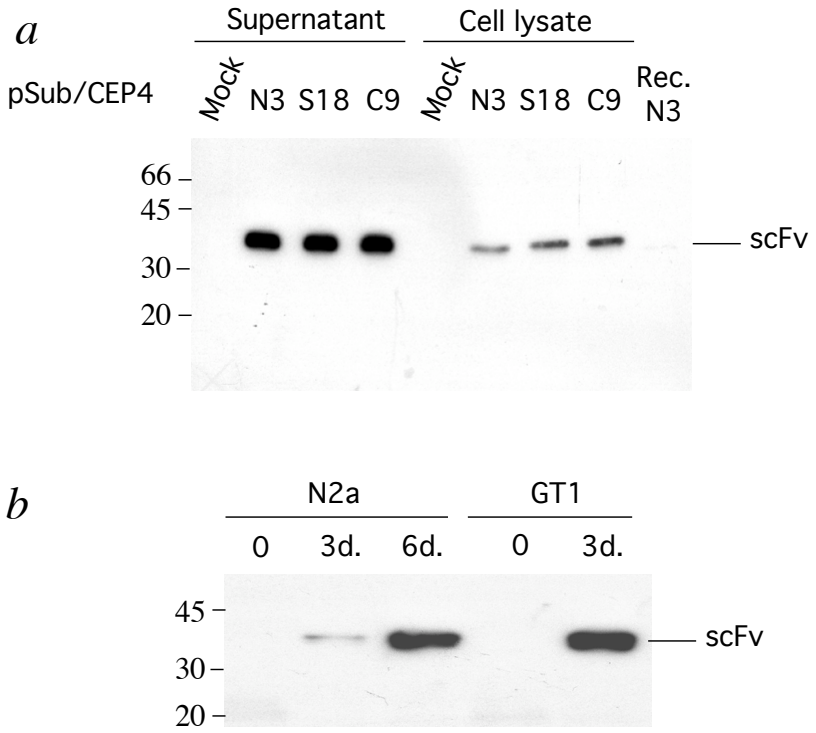

Figure 4. Expression of scFvs with an AAV based system.

a: N2a cells were transfected with the AAV based vector pSub/CEP4 encoding scFvs N3, S18 or C9. Empty vector was used as a mock control. 48 after transfection, medium and lysate were immunoblotted with an anti-myc antibody. *b*: N2a cells and GT1 cells were transduced with rAAV-S18. Supernatants were collected after 72h. In the case of N2a cells, the transduction was repeated after 72h and the supernatants collected again after 72h (6 days after the first transduction). The medium was analysed by Western blotting.

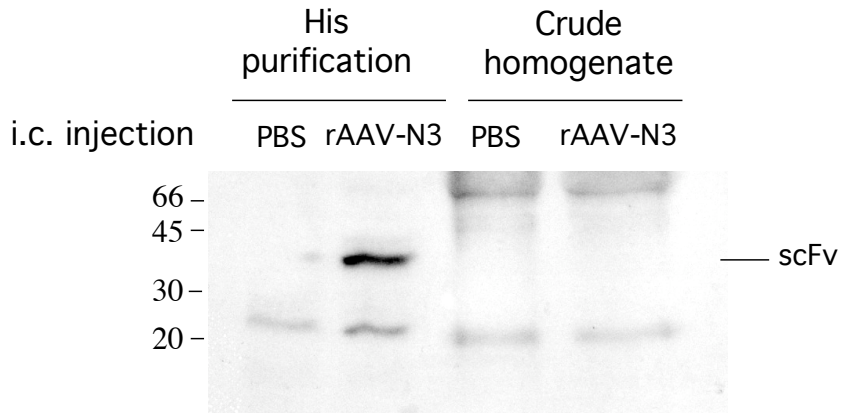

Figure 5. Detection of scFv in the brain after stereotaxic injection. Crude brain homogenates and IMAC purified eluate from brain homogenates were separated by SDS-PAGE. ScFv N3 expression was detected with an anti-myc antibody 30 days post injection.

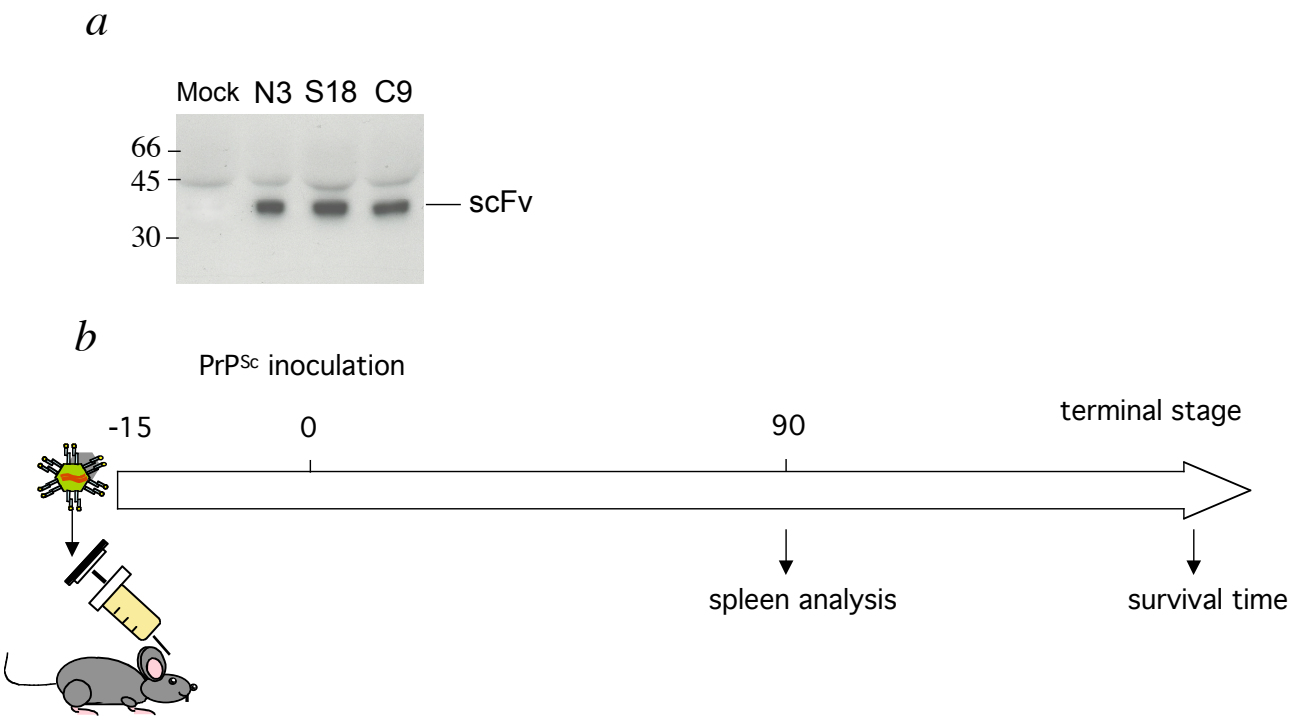

Figure 6. *In vivo* experiment schedule.

a: rAAV used for *in vivo* experiment were tested in Hela cells. Supernatant was analysed by immunoblotting 72h after transduction.

b: rAAV injections were performed into hippocampus at day –15 relative to the day of PrP^{Sc} inoculation. PrP^{Sc} content is determined in spleens at day 90 and the survival times are monitored.

CHAPTER VI

References

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Abbreviations

aa	amino acid

Amino acids

A (Ala)	alanine
C (Cys)	cysteine
D (Asp)	aspartate
E (Glu)	glutamate
F (Phe)	phenylalanine
G (Gly)	glycine
H (His)	histidine
I (Ile)	Isoleucine
K (Lys)	lysine
L (Leu)	leucine
M (Met)	methionine
N (Asn)	asparagine
P (Pro)	proline
Q (Gln)	glutamine
R (Arg)	arginine
S (Ser)	serine
T (Thr)	threonine
V (Val)	valine
W (Trp)	tryptophan
Y (Tyr)	tyrosine

AAV	adeno-associated virus
BCA	bicinchoninic Acid
BHK	baby hamster kidney cells
bp	base pair

BSA	bovine serum albumin
BSE	bovine spongiform encephalopathy
cDNA	complementary DNA
CDR	complementarity determining region
CJD	Creutzfeldt-Jakob disease
CMV	cytomegalovirus
CNS	central nervous system
DNA	deoxyribonucleic acid
DMEM	Dulbecco´s Modified Eagle Medium
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FACS	fluoresecence-activated cell sorting
FCS	fetal calf serum
FDC	follicular dendritic cell
Fv	fragment variable
GAG	glycoaminoglycan
Gal-3	galectin 3
GFP	green fluorescent protein
GPI	glycosyl phosphatidylinositol
GSS	Gerstmann-Sträussler-Scheinker (syndrome)
GST	gluthathione-*S*-transferase
GT1	murine hypothalamic neuronal cells
h	hour
HIV	human immunodeficiency virus
HRP	horse radish peroxidase
HS	heparan sulfate
Hsp	heat shock protein
HSPG	heparan sulfate proteoglycan
hu	human
Ig	immunoglobulin
i.p.	intra peritoneal

IPTG	isopropyl-beta-D-thiogalactopyranoside
ITR	inverted terminal repeat
kDa	kilodalton
kb	kilobase
LB medium	Luria-Bertani medium
LRP	laminin receptor precursor
LR	laminin receptor
M	molar
min	minutes
mo	mouse
MOI	multiplicity of infection
mRNA	messenger RNA
N2a	murine neuroblastoma cells
NMR	nuclear magnetic resonance
nvCJD	new variant CJD
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pI	isoelectric point
PK	proteinase K
PMSF	phenylmethylsulphonylfluoride
PrP	prion protein
PrP^{c}	cellular prion prtein
PrP^{Sc}	scrapie prion protein (pathogenic isoform of PrP)
PVDF	polyvinylidene fluoride
rAAV	recombinant AAV
RML	Rocky Mountain laboratory
RNA	ribonucleic acid
Rpm	rotations per minute
SAF	scrapie associated fibrils
ScFv	single chain antibody

SDS	sodium dodecyl sulfate
sCJD	sporadic CJD
SiRNA	small interfering RNA
SFV	Semliki Forest virus
TNF	tumor necrosis factor
TSE	transmissible spongiform encephalopathy
VLA-6	very late activation antigen-6

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Publications

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Patent

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