Role of PrP^{C} in neuronal differentiation and propagation of its infectious isoform PrP^{Sc} by microvesicles

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1 SUMMARY

Prion diseases or transmissible spongiform encephalopathies (TSEs) are rare neurological disorders that may be of genetic or infectious origin, but most frequently occur sporadically in humans. Their outcome is invariably fatal. The infectious agent has been defined as prion (from *pr*oteinaceous *i*nfectious *on*ly) in 1992 by Stanley B. Prusiner and represent mainly, if not solely, an abnormal, protease-resistant isoform (PrP^{Sc}) of a cellular protein, the prion protein or PrP^C. According to the "protein only" hypothesis, the prion is devoid of informational nucleic acids and consists of an "infectious" protein that is capable of converting the normal host protein PrP^C into a likeness of itself.

TSEs can be distinguished from other neurodegenerative diseases because of their infectivity and transmission capability. The only organ system in which severe histopathological damage can be demonstrated as a consequence of infection with prions is the nervous system. The communal lesions are neuronal loss, spongiosis and astrogliosis, accompanied by an intra- and extracellular accumulation of PrP^{Sc}, occasionally in form of amyloid plaques. Even if a strong activation of microglia and astrocytes occurs, no immunological response is usually detectable as consequence of prion infection.

Despite the considerable attention for its involvement in TSEs, the physiological role of the cellular, nonpathogenic isoform of PrP^C, has not yet been determined. In the last years, several putative cellular functions have been attributed to PrP^C: its localization in "lipid rafts" is consistent with a possible role in cell adhesion, transmembrane signalling or as a recognition molecule. Furthermore, PrP^C has been implicated in protection against oxidative stress, copper metabolism, apoptosis, cell proliferation and in the regeneration of blood precursors stem cells in the adult. It has also been shown that PrP^C interacts with the neuronal cell adhesion molecule NCAM, promoting neurite outgrowth. However, both the PrP^C-mediated effects and the role of PrP^C-dependent pathways on neuronal differentiation are still not elucidated.

First objective of this Ph.D thesis was the establishment of a novel *in vitro* cellular model for the study of the role of PrP^C in neuronal differentiation and neurite outgrowth. Furthermore, an additional goal of this project was the indentification of the PrP^C domains responsible for the induction of neuronal differentiation.

A novel PrP^C-depleted cell line (PrP$^{0/0}$ ML) was derived from murine primary PrP-knockout neuronal cells by SV40 large T antigen-mediated immortalization. A temperature sensitive

form of this oncogenic protein was used, allowing a temperature-mediated regulation of its expression. This cell line was then characterised for its growth potential, for the expression of specific cellular markers and for its ability to differentiate. It was found that, under culture conditions promoting the expression of the temperature-sensitive SV40 large T antigen, the cells expressed nestin, a specific marker of neuronal precursor cells. Therefore, the PrP$^{0/0}$ ML cell line was identified as a potential neuronal stem cell line. In fact, under nonpermissive culture conditions when the expression of the temperature-sensitive SV40 large T antigen is downregulated, the PrP$^{0/0}$ ML cells differentiated into neurons. Noteworthy, maintenance of the cells in conditions that promote cell differentiation induced a progressive reduction in the expression levels of nestin, an event that strongly correlated with the appearance of the specific neuronal markers MAP-2b and NeuN.

In order to investigate the role of PrP^C in the process of neuronal differentiation, the PrP$^{0/0}$ ML cells were then reconstituted for the expression of either the full-length PrP or a N-terminal truncated PrP^C form (PrPΔ32-134). The differentiation potential of both reconstituted cell lines under nonpermissive culture conditions was then compared with that of the parenteral PrP$^{0/0}$ ML cells. This *in vitro* study clearly highlights that PrP^C expression in the PrP$^{0/0}$ ML cell line accelerates neuronal differentiation and that the N-terminal domain of the prion protein is not necessary for this PrP-mediated function.

Prion diseases like BSE, vCJK, Kuru and the majority of iatrogenic cases of CJK are caused by a peripheral infection. Infectious prions accumulate in the central and peripheral nervous system as well as in extracerebral tissues, such as the secondary lymphoid organs and muscles. The prion pathogenesis is a dynamic process which can be defined temporary and spatially in different phases: i) infection and peripheral replication, ii) neuroinvasion, transport of prions from the periphery to the central nervous system (CNS), and iii) neurodegeneration. In the last years, progresses in the elucidation of the peripheral prion pathogenesis were achieved. The identification of the cell types involved in the lymphoreticular prion replication phase and the recognition of the role of the peripheral nervous system in the process of prion spread from the periphery to the CNS have elucidated some of the cellular mechanisms that are involved in prion uptake, replication and propagation. However, relatively little information is available about the mechanism(s) underlying intercellular prion transfer and tissue-to tissue prion spread.

Microvesicles (MVs) are submicron vesicles (0,03-1 μm) with a single membrane and are shed from most eukaryotic cells undergoing activation or apoptosis. The segregation of specific proteins is followed by blebbing of the membrane surface, leading to the formation of MVs and their release in the extracellular environment. MVs can be also secreted upon fusion of multivesicular endosomes with the plasma membrane (exosomes). The secretion of MVs is the result of a complex cellular process involving changes in the metabolism of lipids and proteins.

The functional role of MVs is still largely unknown. However, there is evidence showing that they are important modulators of cell-to-cell communication, participate in a variety of intracellular adhesion processes and are able to induce cellular response(s).

The release of PrP^C and infectious PrP^{Sc} by prion infected epithelial, neuroglial and neuronal cells in association with exosomes has recently been highlighted. Furthermore, it has been shown that exosomes can propagate prion infectivity both *in vitro* and *in vivo*, suggesting that PrP^{Sc}-bearing exosomes may provide a mechanism for intercellular transmission of infectious prions in addition to cell-to-cell contact.

Second objective of this Ph.D thesis was to determine the possible role of plasma membrane-derived microvesicles in the propagation and transmission of prions. The release of MVs was first studied in different murine neuronal cell lines. Here it is shown for the first time that neurons also shed plasma membrane derived MVs, in addition to exosomes.

Immunoelectron microscopy and immunoblot analyses clearly demonstrated the presence of PrP^C on the membrane of MVs released from PrP^C-expressing cells. Characterization of lipid rafts components in MVs highlighted the presence of the ganglioside GM2, the tyrosine kinase p59Fyn, flotillin-2 and the neuronal protein GAP-43.

In order to investigate whether MVs are involved in the intercellular transmission of prions, MVs were first isolated from two prion infected murine neuronal cell lines, namely the Neuro-2a PK1 and the N2a58 cells, and then used for *in vitro* and *in vivo* infection assays. Immunoblot analyses after proteinase K treatment demonstrated the association of PrP^{Sc} with the secreted MVs. The PrP^{Sc}-bearing MVs were then used to perform infection experiments on noninfected cells. By the use of cell blot assay, a method that allows the detection of PrP^{Sc}-amplification and -accumulation in cultured cells, the kinetic of prion infection in the *de novo* infected cells was followed. Noteworthy, it was found that PrP^{Sc}-bearing MVs were capable to transmit prions *in vitro* and to stably infect the recipient cells. In order to investigate the role of MVs in the transmission of infectivity *in vivo*, PrP^{Sc}-bearing MVs as well as MVs isolated from noninfected cells (as negative control) were injected intracerebrally in PrP^C-

overexpressing indicator mice (*tga20*). The development of clinical disease was followed in a time-dependent manner. Clinical symptoms could be observed only in the group of indicator mice inoculated with the PrP^{Sc}-bearing MVs, which then succumbed to desease. These findings clearly demonstrated that MVs are biological carriers of both PrP^{Sc} and prion infectivity. MVs could therefore participate *in vivo* in the processes of intercellular prion transmission and propagation.

ZUSAMMENFASSUNG

Die transmissiblen spongiformen Enzephalopathien (TSEs) oder Prionenerkrankungen bilden eine Gruppe fataler neurodegenerativer Erkrankungen, welche einen weiten Wirtsbereich (Mensch und Tier) aufweisen. Sie treten in vererbbarer, sporadischer sowie infektiöser Form auf. Der infektiöse Erreger wurde als „Prion" (*pr*oteinaceous *i*nfectious *on*ly) in 1992 von Stanley B. Prusiner gennant und ist auf die enge Korrelation von Infektiösität und PrP^{Sc}, einer partiell Protease-resistenten Isoform (Scrapie-Isoform) des zellulären, physiologischen Prion Protein (PrP^C), zurückzuführen. Gemäss der „protein only" Hypothese bezeichnen Prionen infektiöse Einheiten, welche sich durch das Fehlen einer Erbinformation von konventionellen Erregern unterscheiden und in der Lage sind, das normale endogene PrP^C in der infektiöse Form umzuwandeln. TSEs unterscheiden sich von anderen neurogenerativen Erkrankungen durch Ihre Infektiosität und Übertragbarkeit.

Prionen verursachen im Gehirn einen herdförmigen Neuronenzelluntergang, spongiforme Veränderungen des Nervengeflechts (Neuropil), Astrozytenvermehrung (Astrozytose) und charakteristische intra- und extrazelluläre PrP^{Sc}-Ablagerungen. Außer einer mitunter massiven Mikroglia- und Astrozytenaktivierung sind üblicherweise keine zellulären immunologischen Reaktionen erkennbar.

Die Funktion des zellulären, nicht pathogenen PrP^C ist trotz intensiven Anstrebungen während der letzten Jahre noch nicht eindeutig identifiziert worden. Verschiedene putative Funktionen wurden zu PrP^C zugeschrieben: die Lokalisierung von PrP^C in „lipid rafts" lässt eine Rolle in der Zelladhäsion, in der transmembranären Signalkaskaden sowie als Ligand/Rezeptor vermuten. Es wurde weiterhin gezeigt, dass PrP^C in der Regulierung von oxidativem Stress, in dem Kupfer-Stoffwechsel, in der Apoptose, in der Regenerierung von adulten blutbildenden Stammzellen und in der Regulierung der Zellvermehrung involviert ist. Die nachgewiesene Interaktion von PrP^C mit dem zellmembranären Protein NCAM (neuronal cell adhesion molecule) hat zur Postulierung geführt, dass PrP^C in der neuronalen Differenzierung eine wesentliche Rolle spielt. Die PrP^C-abhängigen zellulären Mechanismen, die in der Induktion der neuronalen Differenzierung involviert sind, sind bis heutzutage noch nicht vollständig aufgeklärt.

Erstes Ziel dieser Dissertation war, ein neues zelluläres Modell für die Untersuchung der Rolle von PrP^C in der neuronalen Differenzierung zu etablieren. Dieses neue *in vitro* Modell wurde dann verwendet, um die PrP^C-bedingten Einflüsse auf diesen Prozess zu charakterisieren. Die Identifizierung der Domäne des Prionproteins, die für die Induktion der

neuronalen Differenzierung notwendig sind, war ein zusätzliches Ziel dieses ersten Projektes. Um diese biologische Fragenstellungen experimentell zu bearbeiten, wurde erstens eine neue PrP-depletierte Zelllinie (PrP$^{0/0}$ ML) durch SV40-Large T Antigen-bedingte Immortalisierung aus murinen primären PrP-knockout neuronalen Zellen generiert. Die biologischen Eigenschaften dieser Zelllinie wurden dann vollständig charakterisiert: Wachstum, Expression von spezifischen zellulären Antigenen und Differenzierung wurden untersucht. Es stellte sich heraus, dass unter Kulturbedingungen, bei welcher die temperatursensitive Form des SV40-Large T Antigen exprimiert wird, die PrP$^{0/0}$ ML Zellen die Expression des Antigens Nestin vorwiesen. Da Nestin ein zellulärer Marker ist, der spezifisch in neuronalen Vorläuferzellen vorkommt, wurden die PrP$^{0/0}$ ML Zellen als potenziellen neuronalen Stammzellen identifiziert. Unter nicht permissive Kulturbedingungen, differenzierten sich die PrP$^{0/0}$ ML Zellen ausschliesslich zur Neuronen.

Es wurde in der Tat nachgewiesen, dass in einem zeitabhängigen Prozess die Unterregulierung von Nestin der Hochregulierung der spezifischen neuronalen Markern Map-2b und NeuN vorausgeht.

Um die Rolle des Prionproteins in der neuronalen Differenzierung zu untersuchen, wurden dann die PrP$^{0/0}$ ML Zellen für die Expression entweder von „wild Type" PrP^{C} oder von einer N-terminalen trunkierten Form von PrP^{C} (PrPΔ32-134) rekonstituiert. Der Prozess der Differenzierung der beiden rekonstituirten Zelllinien wurde mit jenem der PrP$^{0/0}$ ML Zellen verglichen. Die durchgeführten Experimente zeigten eindeutig, dass die Expression von PrP^{C} in der PrP$^{0/0}$ ML Zelllinie die neuronale Differenzierung beschleunigt und dass der N-Terminus des Prionproteins für diese Funktion nicht notwendig ist.

Prionenerkrankungen, wie BSE, vCJK, Kuru und die meisten Fälle von iatrogener CJK, entstehen normalerweise durch eine peripherale Infektion. Infektiöse Prionen akkumulieren sich nicht nur innerhalb des zentralen und peripheren Nervensystems aber auch in extrazerebralen Geweben, wie in den sekundären lymphatischen Organen und in den Muskeln. Die Pathogenese der Prionerkrankung ist ein dynamischer Prozess, den man in sowohl zeitlich als auch räumlich getrennten Phasen unterteilen kann: i) Infektion und periphere Replikation, ii) Neuroinvasion, der Transport von Prionen von der Peripherie zum Zentralnervensystem, und iii) Neurodegenerierung. In den letzen Jahren wurden grosse Vorschritte in der Aufklärung der periphere Pathogenese erzielt. Die Identifizierung von den Zellen, welche die Prion-Propagation im lymphoretikulären System unterstützen, und die

Erkenntnisse über die wichtige Rolle des peripheren Nervensystems bei der Ausbreitung von Prionen von der Peripherie ins Zentralnervensystem haben einige der zellulären Mechanismen aufgeklärt, die bei der Prionen-Aufnahme, -Replikation und –Ausbreitung beteiligt sind. Die molekularen Mechanismen der Prionenübertragung von Zelle zu Zelle und von Gewebe zu Gewebe sind jedoch noch nicht vollständig aufgeklärt.

Mikrovesikel (MVs) sind submikroskopische (0,03-1 µm in Durchmesser) Vesikel, die von einer einfachen Membran umgeben sind und von eukaryotischen Zellen nach Aktivierung oder während der Apoptose freigesetzt werden. Mikrovesikel werden sowohl von der Plasmamembran als auch nach der Verschmelzung von multivesikulären Endosomen mit der Plasma Membran (Exosomen) sezerniert. Die Freisetzung der Mikrovesikel ist das Resultat eines sehr komplexen zellulären Vorgangs, der unter anderem Veränderungen in dem Metabolismus sowohl von Lipiden als auch von Proteinen beinhaltet.

Die biologischen Funktionen von freigesetzten MVs sind heutzutage noch nicht aufgeklärt worden. Es wurde postuliert, dass MVs wichtige Modulatoren für die Kommunikation zwischen Zellen sein könnten. MVs spielen auch eine Rolle in verschiedenen intrazellulären Adhäsionsprozessen und in der Induktion zellulärer Antworten.

Es wurde schon gezeigt, dass mit Prionen infizierten Zellen PrP^{Sc} und Prionen-Infektiösität in Assoziation mit Exosomen freisetzen. Zusätzlich hat man nachgewiesen, dass solche Exosomen in der Lage sind, Prionenerkrankungen sowohl *in vitro* als auch *in vivo* zu übertragen. Diese Befunde führten zur Hypothese, dass Vesikel für die Übertragung und Verbreitung von Prionen verantwortlich sein könnten.

Zweites Ziel dieser Dissertation war, die Rolle von Plasmamembran-stammende Mikrovesikel in der Ausbreitung und Übertragung von Prionen festzustellen. Die Freisetzung von MVs wurde zuerst in verschiedenen neuronalen murinen Zelllinien untersucht. Es wurde für das erste Mal gezeigt, dass Neuronen, zusätzlich zur Exosomen, auch Mikrovesikel sezernieren. Immunoelektronenmikroskopische- und Immunoblot-Analysen von isolierten MVs haben eindeutig nachgewiesen, dass PrP^{C} einen Bestandteil von MVs ist. Die Charakterisierung von „lipid rafts" Komponenten hat ergeben, dass in MVs das Gangliosid GM2, die Tyrosinkinase p59Fyn, flotillin-2 und das neuronale Protein GAP-43 vorhanden sind. Um die Involvierung von den MVs in der interzelluläre Übertragung von Prionen nachzuweisen, wurden MVs aus zwei unterschiedlichen stabil infizierten murinen neuronalen Zelllinien, die Neuro-2a PK1 und die N2a 58, isoliert und für *de novo in vitro* und *in vivo* Infektionsexperimenten verwendet. Immunoblot Analysen nach Proteinase K Behandlung haben gezeigt, dass PrP^{Sc}

mit den freigesetzten MVs assoziiert ist. Die PrP^{Sc}-tragenden MVs wurden in den daran folgenden Experimenten zur Infektion von uninfizierten Zellen angesetzt. Mittels „cell blot assay", ein Verfahren dass den Nachweis von PrP^{Sc}-Amplifikation und -Akkumulierung in kultivierten Zellen ermöglicht, wurde der Verlauf der *in vitro* Prioneninfektion verfolgt. Man hat eindeutig gezeigt, dass PrP^{Sc}-tragenden MVs für die Übertragung von Prionen und für eine stabile Prioneninfektion *in vitro* der Empfängerzellen verantwortlich sind. Um die *in vivo* MV-bedingte Prionenübertragung zu untersuchen, wurden PrP^{Sc}-tragenden MVs und MVs gewonnen aus nicht infizierten Zellen (als negativ Kontrolle) in PrP^{C} überexprimierenden Mäusen (tga20) intrazerebral inokuliert. Die Entwicklung einer klinischen manifestierenden Prionerkrankung wurde in diesem „Bioassy" zeitlich verfolgt. Ausschliesslich Indikator Mäuse, die mit PrP^{Sc}-tragenden MVs inokuliert wurden, entwickelte die Erkrankung. Diese Ergebnisse zeigten eindeutig, dass MVs biologische Trägern von PrP^{Sc} und Infektiösität sind. MVs könnten deswegen *in vivo* in der Prozesse der interzellulären Prionen-Ausbreitung und -Übertragung beteiligt sein.

2 INTRODUCTION

2.1 Transmissible Spongiform Encephalopathies (TSEs)

The subacute transmissible spongiform encephalopathies (TSE), or prion diseases (Prusiner, 1982a), are a complex group of fatal neurodegenerative diseases that occur in a wide variety of mammal, exemplified by scrapie (Kimberlin, 1990; Prusiner, 1982b; Prusiner and DeArmond, 1990) in animals and Creutzfeldt-Jakob disease in humans. These disorders can be distinguished in genetic, sporadic or infectious forms. The genetic form is dominantly inherited whereas the infectious form is acquired exogenously. When there is no source of exogenous infection or genetic aetiology, the cases are defined sporadic. The transmission of this kind of diseases to experimental animals led to the recognition of the unusual properties of the pathogenic agent, like the extremely long incubation time and resistance to high temperatures, formaldehyde treatment and UV irradiation (Alper et al., 1966; Gordon, 1946; Pattison, 1965). The association of the infectivity with a protein led to designation "prion" (for proteinaceous infectious particle) to distinguish it from conventional pathogens such as bacteria and viruses or viroids (Prusiner, 1982a) according to the *"protein only"* hypothesis.

The *"protein only"* hypothesis as proposed by Prusiner suggests that the infectious agent causing TSE, the prion, is devoid of nucleic acid and is identical to a posttranslationally modified form (PrP^{Sc} or an infectious subspecies PrP^*) of a host protein (PrP^C), possibly differing only in its conformation from the latter (Cohen et al., 1994). Two different theories for the mechanism of PrP^{Sc}-induced PrP^{Sc} formation, the heterodimer or "refolding" model (Prusiner, 1991; Prusiner and DeArmond, 1990) and the "seeded polymerization" model (Come et al., 1993; Jarrett and Lansbury, 1993; Lansbury and Caughey, 1995) were presented.

The "refolding" model proposes that upon infection of an appropriate host cell, the incoming infectious agent (PrP^{Sc} or PrP^*) starts a catalytic cascade using PrP^C or PrP^U, a partially unfolded intermediate arising from stochastic fluctuations in PrP^C conformations, as substrate, converting it upon a conformational change into new infectious PrP, which in turn will convert more PrP^C into the infectious entity. The formation of PrP^C/PrP^U-PrP^{Sc} heteromeric complex may lower catalytically the activation energy barrier to the formation of new PrP^{Sc} from PrP^C/PrP^U, inducing a positive feedback and further recruitment of PrP (autocatalytic process). The extensive unfolding and refolding is believed to require chaperone activity and energy.

The underlined nucleated or "seed polymerisation" model proposes that the conformational change is thermodynamically controlled: the conversion process between PrP^C and PrP^{Sc} is reversible. Converted PrP^{Sc} is stabilised only when it adds onto a crystal-like seed or aggregate of PrP^{Sc}. Once a seed is present, further monomer addition may be accelerated.

Recently, significant milestones in support of the prion hypothesis were achivied. For the first time the conversion of PrP^C to protease-resistant forms similar to PrP^{Sc} was reported in a cell-free system composed of substantially purified constituents (Kocisko et al., 1994). This conversion was selective and required the presence of preexisting PrP^{Sc}, providing direct evidence that PrP^{Sc} derives from specific PrP^C-PrP^{Sc} interactions. Noteworthy, the generation of *de novo* infectious PrP^{Sc} *in vitro* was demonstrated by the use of independent experimental approaches (Castilla et al., 2005; Legname et al., 2002). Recombinant mouse prion protein produced in Escherichia coli was *in vitro* polymerized into amyloid fibrils that represent a subset of beta sheet-rich structures. Mice intracerebrally inoculated with fibrils consisting of recombinant PrP developed neurologic dysfunction typical for prion diseases (Legname et al., 2004; Legname et al., 2005), providing compelling evidence that prions are infectious proteins. Additionally, a procedure involving cyclic amplification of protein misfolding (PMCA) has been established that allows a rapid conversion of large excess PrP^C into a protease-resistant, PrP^{Sc}-like form in the presence of minute quantities of PrP^{Sc} template (Saborio et al., 2001). In this procedure, conceptually analogous to polymerase chain reaction cycling, aggregates formed when PrP^{Sc} is incubated with PrP^C are disrupted by sonication to generate multiple smaller units for the continued formation of new PrP^{Sc}. After cyclic amplification, more than 97% of the protease-resistant PrP present in the sample corresponds to newly converted protein (Saborio et al., 2001). Interestingly, the *in vitro*-generated PrP^{Sc}-like form share similar biochemical and structural properties with PrP^{Sc} derived from sick brains. Furthermore, the PMCA-produced PrP^{Sc}-like form was shown to be infectious *in vivo* (Castilla et al., 2005). These data provide strong evidence in support of the protein-only hypothesis of prion transmission.

Five human neurodegenerative diseases that are caused by prions had been described, namely Kuru, Creutzfeldt-Jakob disease (CJD), new variant CJD (vCJD) (Will et al., 1996), Gerstmann-Sträussler-Scheinker syndrome (GSS) (Gajdusek, 1977), and fatal familial insomnia (FFI) (Medori et al., 1992).

Kuru was an epidemic, slowly progressing neurodegenerative disease in the eastern highlands of Papua New Guinea in the first decades of last century. It was hypothesised that its propagation occurred by ritual cannibalism and may have originated with the consumption of the remains of a sporadic CJD sufferer (Alper and Haig, 1968).

Creutzfeldt-Jakob disease may arise spontaneously (so-called sporadic form) or it can be familial (so-called inherited form) or occur in acquired form (so-called iatrogenic form). Gerstmann-Sträussler-Scheinker syndrome and fatal familial insomnia arise only as familial diseases. All familial forms of CJD, GSS and FFI have been linked to mutations of the *PRNP* gene (Brown and Gajdusek, 1991; Brown et al., 1991a; Brown et al., 1991b; Brown et al., 1992; Collinge et al., 1989; Doh-ura et al., 1989; Goldfarb et al., 1990; Goldfarb et al., 1991a; Goldfarb et al., 1991b; Haltia et al., 1991; Owen et al., 1990). Aetiology of sporadic cases can be explained by somatic mutations of *PRNP*, the human form of the PrP gene, while iatrogenic CJD is transmitted via medical treatment, e.g. by the use of contaminated neurosurgical instruments or treatment with drugs or tissues of human origin. The new form of CJD (vCJD) affecting unusually young people reveals a unique clinical and pathological pattern (Chazot et al., 1996; Will et al., 1996), being caused by the consumption of contaminated meet with BSE. BSE or bovine spongiform encephalopathy is one of at least six disorders of animals which are included in the ensemble of prion diseases. Like the transmissible mink encephalopathy (Marsh and Hadlow, 1992), chronic wasting disease of captive mule deer and elk (Williams and Young, 1993), feline spongiform encephalopathy (Pearson et al., 1992; Wyatt et al., 1991), and exotic ungulate encephalopathy (Kirkwood et al., 1990; Wells and McGill, 1992), BSE (Hope et al., 1989) is thought to result from ingestion of scrapie-infected animal products (Wells, 1993; Wilesmith et al., 1992). Scrapie is a naturally occurring affliction of sheep and goats and it was the first prion disease described more than 250 years ago.

However, it is considered equally possible that BSE originated as a sporadic case in cattle and was then spread by contaminated cattle offal (Weissmann, 1996).

All human and animal TSEs show a characteristic electroencephalogram (EEG) (Court and Bert, 1995). The pathological lesions are found in the brain, in the medulla and in the spinal cord, and vary in location and intensity. In the brain, these include neuronal degeneration in the cerebral cortex, striatum, thalamus and cerebellum, followed by vacuolation of neurons, neuronal cell death and gliosis (singly or in combination) (DeArmond et al., 1993; Marsh and Kimberlin, 1975; Zlotnik and Rennie, 1965). Accumulation of PrP^{Sc} (DeArmond et al., 1985;

Kitamoto et al., 1986; Kretzschmar et al., 1991; Prusiner et al., 1987; Wiley et al., 1987), a protease-resistant isoform of an endogenous protein (PrP^C), is a hallmark of TSEs.

2.2 PrP-gene structure and expression

The PrP gene, termed *Prnp*, consists of two exons in human (Basler et al., 1986; Puckett et al., 1991) and of three exons in mice, hamster (or, differentially spliced, two) and sheep (Basler et al., 1986; Bueler et al., 1992; Goldman et al., 1993; Li and Bolton, 1997; Westaway et al., 1994) (Fig. 1). In all species examined, the open reading frame (ORF) and the 3' untranslated mRNA region are entirely contained within the last exon. This exon has a size of about 2350 bp in human (Kretzschmar et al., 1986b; Liao et al., 1986; Puckett et al., 1991), and 2000 bp in rodents (Basler et al., 1986; Locht et al., 1986; Oesch et al., 1985; Westaway et al., 1994; Westaway et al., 1987), whereas ruminants possess a much larger ORF-exon (about 4000 bp) (Goldmann et al., 1990). These differences reside within the 3' untranslated region of PrP mRNA, whereas all ORFs are of similar length (about 740 bp) (Goldmann et al., 1990) and encode proteins of 253 to 257 amino acids (Basler et al., 1986; Chesebro et al., 1985; Kretzschmar et al., 1986b; Liao et al., 1986; Locht et al., 1986; Oesch et al., 1985; Prusiner, 1986; Westaway and Prusiner, 1986). PrP promoter regions exhibit some characteristics of so-called house-keeping genes. The major feature is the lack of typical regulatory elements found in most other genes.

PrP^C is a glycosylphosphatidylinositol-anchored cell-surface protein mainly localized in "lipid rafts" (Gorodinsky and Harris, 1995; Sarnataro et al., 2002; Taraboulos et al., 1995; Vey et al., 1996) and expressed constitutively in both neuronal and non-neuronal cells, with the highest expression level found in neurons of the central nervous system (CNS) (Kretzschmar et al., 1986a; Moser et al., 1995).
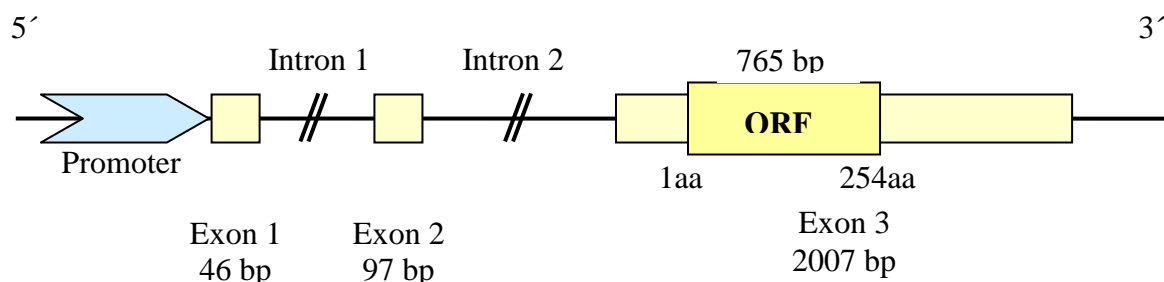
Figure 1. Schematic representation of the murine *Prnp* gene (drowing not in scale)

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The primary sequence of PrP protein is highly conserved among mammals (identity ≥ 85%) (Chesebro et al., 1985; Kretzschmar et al., 1986b; Oesch et al., 1985; Westaway and Prusiner, 1986) (Fig. 2). A N-terminal hydrophobic signal sequence of 22 amino acids co-translationally guides PrP through the membrane of the endoplasmatic reticulum and then is cleaved off to generate the N-terminus Lys-Lys-Arg- found in mature PrP protein (Bolton et al., 1987; Hay et al., 1987; Hope and Hunter, 1988; Hope et al., 1986; Turk et al., 1988). This is followed by a domain containing five octarepeats responsible for copper binding and two hexarepeats that are rich in proline and glycine (Bazan et al., 1987). A hydrophobic stretch is present between amino acids 113-135, which may, as demonstrated so far only *in vitro*, serve as transmembrane domain (Lopez et al., 1990; Yost et al., 1990). A second hydrophobic stretch is present between amino acids 231-253, which can be regarded as the signal sequence for attachment of a glycosylphosphatidylinositol (GPI) moiety (Stahl et al., 1990a; Stahl et al., 1987; Stahl et al., 1990b).

Modified from (Aguzzi and Heikenwalder, 2006)
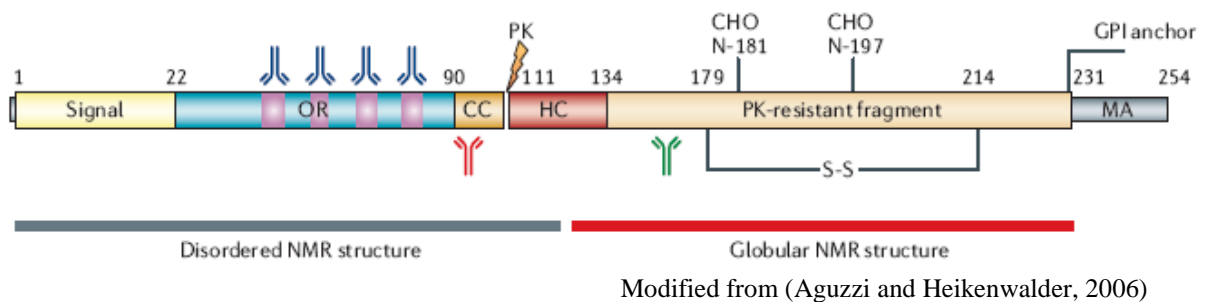
Figure 2. Outline of the primary structure of the cellular prion protein including post-translational modifications.

The cellular prion protein consists of a flexible N-terminal and a C-terminal globular domain. A secretory signal peptide resides at the extreme N terminus. The numbers describe the position of the respective amino acids. OR (prurple) defines the copper binding octarepeats. CC (orange) defines the charged cluster. HC (red) defines the "hydrophobic core". S-S indicates the single disulfide bridge. The protein is post-translationally modified by addition of two glycosylations (CHO) as well as of a glycosylphosphatidylinositol (GPI) membrane anchor (MA, membrane anchor region) The proteinase K (PK) resistant core of PrP^{Sc} is depicted in gold and the approximate cutting site of PK within PrP^{Sc} is indicated by the lightening symbol.

Purification of a C-terminal domain of the mouse PrP (*m*PrP(121-231)) allowed the determination of the three-dimensional structure by NMR (Hornemann and Glockshuber, 1996; Riek et al., 1996). This PrP fragment showed an overall globular shape with two short antiparallel β-pleated sheets (residue 128-131 and 161-164) and three α-helices (residue 144-154, 179-193 and 200-217). Meanwhile the three-dimensional structure of the full-length protein as determined by NMR revealed closed similarities between different species

(Calzolai et al., 2005; Hornemann et al., 1997; Lopez Garcia et al., 2000; Lysek et al., 2005; Riek et al., 1997; Zahn et al., 2000).

The PrP polypeptide begins its metabolic cycle in the endoplasmic reticulum where the glycosylphosphatidylinositol (GPI) moiety and high mannose glycans are attached and both signal sequences (the 22 N-terminal and the 23 C-terminal amino acids peptides) are cleaved off. In the Golgi apparatus maturation of the Asn-linked glycans (residues 181 and 197) occurs (Bolton et al., 1985; Caughey et al., 1989; Endo et al., 1989; Haraguchi et al., 1989; Turk et al., 1988). The mature PrP^C is then presumably transported within secretory vesicles to the external cell surface where it is anchored by the GPI-anchor. Like other GPI-proteins, most of PrP^C is found in detergent-resistant microdomains of the plasma membrane (Harmey et al., 1995; Naslavsky et al., 1997; Taraboulos et al., 1995).

From (Aguzzi and Heikenwalder, 2006)
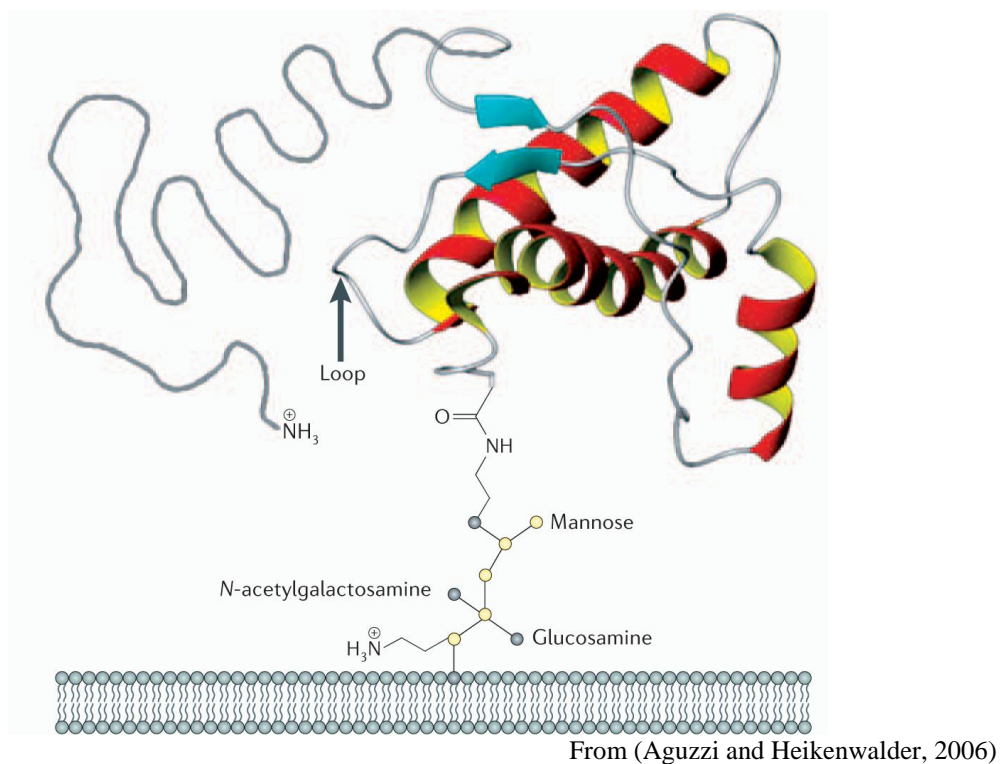
Figure 3. Structural features of the cellular prion protein

Tertiary structure of the cellular prion protein inserted into a lipid bilayer, as deduced from NMR spectroscopy, including the "unstructured" N-terminal tail (grey) and the glycosyl phosphatidyl inositol (GPI) anchor. The globular C-terminal domain contains a two stranded anti-parallel beta-sheet and three alpha-helices. The loop connecting the second β-sheet and the third α-helix is indicated by the black arrow. OR, octarepeat region.

2.3 Normal cellular function of PrP

In the last years, several putative cellular functions have been attributed to PrP^C: its localization in "lipid rafts" is consistent with a possible role in cell adhesion, transmembrane signalling or as a recognition molecule.

As PrP^C protein ligands, the anti-apoptotic molecule Bcl-2 (Kurschner and Morgan, 1995; Kurschner and Morgan, 1996), the chaperone Hsp60 (Edenhofer et al., 1996), the 37 kDa laminin receptor precursor LRP (Rieger et al., 1997), as well as synapsin Ib, the adaptor protein Grb2 and the unknown protein Pint 1 (prion interactor 1) (Spielhaupter and Schatzl, 2001) were identified.

The PrP^C interacting protein synapsin I is associated with small synaptic vesicles and both synapsin I and Grb2 copurify with PrP^C in neuronal microsomal vesicles (Spielhaupter and Schatzl, 2001). This would suggest a role for PrP^C in the recycling of the vesicles or a more direct role in synaptic activity (Herms et al., 1999). Electrophysiological studies on *Prnp$^{o/o}$* mice generated by Büeler et al. (Bueler et al., 1992) showed weakened GABA$_A$ (gamma-aminobutyric acid type A) receptor-mediated fast inhibition and impaired long-term potentiation in the hippocampus, which could be both complemented by reintroduction of high copy numbers of the mouse or human PrP gene into PrP null mice (Collinge et al., 1994; Whittington et al., 1995). However, no electrophysiological abnormalities were found by others in the same line (Herms et al., 1995; Lledo et al., 1996). Interestingly, *Prnp$^{o/o}$* mice generated by Manson et al. (Manson et al., 1994) showed short term rather than long term potentiation in the CA1 region of the hippocampus. Furthermore, both PrP knock-out mouse lines showed an alteration in both circadian activity rhythm and sleep, suggesting that PrP might be involved in the regulation of circadian motor activity and in the maintenance of sleep continuity and regulation (Tobler et al., 1997; Tobler et al., 1996).

It was shown that primary neurons isolated from *Prnp$^{o/o}$* mice are less resistant to serum deprivation than their PrP^C expressing counterparts, but this hypersensitivity could be counteracted by the overexpression of Bcl-2 (Kuwahara et al., 1999). Moreover, PrP^C could protect human primary neurons against Bax-mediated cell death to levels equal to the neuroprotective function of Bcl-2 (Bounhar et al., 2001). This neuroprotective function requires the presence of the octapeptide repeats of PrP^C. Other data demonstrated that when overexpressed, PrP^C sensitizes cultured cells to staurosporine-induced death and increases caspase 3 activity, an enzyme involved in the apoptotic cascade (Paitel et al., 2002). A similar toxicity was shown by the accumulation of cytosolic PrP^C due to proteasome dysfunction (Ma et al., 2002).

The fact that mice devoid of PrP^C harbour 50% lower copper concentrations in synaptosomal fractions than their PrP^C expressing counterparts suggests that PrP^C could regulate the copper concentration in the synaptic region of the neuron (Kretzschmar, 1999). It is known that PrP^C is a metal ion-binding protein and binds copper via histidines of the N-terminal region. The N-terminal part of the prion protein harbours high structural flexibility and is involved in the binding of PrP^C to a number of ligands. Therefore copper binding could influence the binding of PrP^C to other proteins. For example, it has been shown that PrP^C binds to the laminin receptor via two binding sites, one of them being located in the N-terminal octarepeat domain of PrP^C (Hundt et al., 2001). Otherwise, PrP^C could mediate the transport of copper from the extra- to the intracellular compartment.

Another feature attributed to the prion protein is that of the activity as copper/zinc-dependent superoxide dismutase (SOD1), which endows PrP^C with antioxidant activity (Brown et al., 1999a).

It has been shown, that through interaction with stress-inducible protein 1 (STI1), a membrane 66 kDa protein, PrP^C can induce neuroprotective signals that rescue cells from apoptosis (Zanata et al., 2002). Moreover, recombinant STI1 added to hippocampal cultures interacts with PrP^C at the neuronal surface and elicits neuritogenesis in wild-type neurons but not in PrP^C- null cells (Lopes et al., 2005).

Recently, it has been shown that PrP^C interacts with the neuronal cell adhesion molecule NCAM as well as with the extracellular matrix glycoprotein laminin, the 67-kDa high affinity laminin receptor and the 37-kDa laminin receptor precursor implying a potential role of PrP^C in neuronal differentiation, neurite outgrowth and neuronal survival. It has been proposed that interaction of PrP^C with NCAM at the neuronal cell surface of hippocampal neurons induces activation of p59fyn kinase promoting neurite outgrowth (Santuccione et al., 2005). Nevertheless the effect of PrP^C on neuronal differentiation is currently unclear.

2.4 Neurotoxic effects of PrPΔ32-134

Common to all knockout mice is resistance to scrapie and inability to propagate the scrapie agent, or prion, as predicted by the "protein only" hypothesis. In the past, in order to understand the structure-activity relationship of PrP regarding its capacity to support scrapie pathogenesis and prion propagation, different recombinant PrP genes with a series of deletions were expressed into a knockout background. In that study, it was shown that PrP

lacking residues 32-121 or 32-134, but not with shorter deletions, caused severe ataxia and neuronal death limited to the granular layer of the cerebellum as early as 1-3 months after birth (Shmerling et al., 1998). The investigators demonstrated the complete abolishment of the defect after having introduced one copy of a wild-type PrP gene.

2.5 Peripheral prion pathogenesis

As mentioned previously, TSEs can occur in genetic, sporadic or infectious forms. The infectious form is acquired exogenously and is represented by vCJD, kuru, iatrogenic CJD in humans and BSE in animals. In some of these disorders, a process called peripheral pathogenesis precedes the pathology found in the CNS. The most relevant routes followed by the infectious agent to entry the host organism are the oral one and the experimentally related i.p. injection. In the case of oral transmission, prions have to cross an epithelial barrier present in the gastroenteric tract in order to reach the mucosa-associated lymphoid tissue system (MALT). It was suggested that the membranous epithelial cells (M cells) could play a role in this process by performing the transepithelial transfer of orally consumed prion infectivity (Heppner et al., 2001). Otherwise, the migratory bone marrow-derived dendritic cells (DCs) could migrate from the blood vessel compartment to the inner surface of the intestinal wall and trapping prions in the enteric tube. Subsequently the infectious agents could be transported to the local lymphoreticular system (LRS) including mesenteric lymph nodes and probably more distant sites (Huang et al., 2002).

Of note is the possibility for prions to get into direct contact with the lymphoreticular system at the site of the palatine tonsils, thereby perhaps bypassing the intestinal pathway. Supporting this hypothesis is the finding of PrP^{Sc} in tonsils biopsies of patients with vCJD (Hill et al., 1999). After oral challenge, prion infectivity usually colonizes the lymphoreticular tissue early in the incubation period. It was shown that many sites of the LRS including spleen, Peyer`s patches, lymph nodes and tonsillar tissue are involved in PrP^{Sc} replication (Eklund et al., 1967; Fraser and Dickinson, 1970; Fraser and Dickinson, 1978; Hill et al., 1997; Kimberlin and Walker, 1979; Mould et al., 1970), after both intracerebral and intraperitoneal inoculation. The nature of the cells that support prion replication within the lymphoreticular system is still uncertain. It seems that besides FDCs (Mabbott et al., 2000; McBride et al., 1992; Montrasio et al., 2000), other cells of the immune system could be involved.

Another issue highlighted by the work of Blattler and colleagues (Blattler et al., 1997) is how the infectious agent transfers from the lymphoreticular system to the central nervous system.

They reported that transplantation of PrP-positive hematopoietic cells into PrP$^{0/0}$ mice sufficed to support prion propagation in the spleen after i.p. inoculation, but failed to induce spongiform encephalopathy in PrP-producing neurografts implanted into the brains of these mice, indicating that a further PrP-expressing tissue compartment, such as the peripheral nervous system (PNS), is necessary for neuroinvasion of prions.

The transport of prions to the PNS as well as from the CNS to the periphery (Koperek et al., 2002) could be performed by two kind of mobile cells, namely macrophages (Prinz et al., 2002) and DCs (Huang et al., 2002). Other studies revealed a supportive role of the complement system in the early phase of peripheral prion pathogenesis. The results obtained suggested that opsonization of the infective agent with C3d/C4b facilitated transport to and uptake of infectivity by the LRS (Klein et al., 2001; Mabbott et al., 2001).

The PNS represents the second system (apart from the immune system) necessary for peripheral prion pathogenesis (Blattler et al., 1997) and it bridges the gap between the periphery and the CNS. To some degree it could also be involved in the replication and accumulation of infectivity (Glatzel et al., 2001). A group documented granules of PrP^{Sc} within axons, so that intra-axonal transport of prions might be important for their spread not only in the CNS, but also in the PNS (Kovacs et al., 2004).

2.6 Prion transmission

As mentioned above, different cell types, including immune cells, contribute to the replication and transfer of infectious prions from peripheral sites of replication to the brain (Glatzel and Aguzzi, 2000a). The mechanisms underlying this intercellular transfer are not elucidated (Harris, 1999b), but close cell contact may be involved (Kanu et al., 2002). Nevertheless, cell-free conversion data (Baron et al., 2002) indicate that additional pathways involving in non-cell-associated forms of infectious agent may participate in the propagation of prions. It has been already demonstrated that the culture medium of infected cells is infectious (Schatzl et al., 1997) suggesting that PrP^{Sc} may be released from cells. PrP^{C} could be released at the same manner from uninfected cells (Liu et al., 2002). It has been reported that both PrP^{C} and PrP^{Sc} can be present in the culture medium in a non-cell-associated form and they were both found associated with exosomes, secreted intralumenal contents of multivesicular bodies (MVB) (Fevrier et al., 2004). More recently, others have reported that platelets activation led to the transient expression of PrP^{C} on the platelet surface and its subsequent release on both microvesicles and exosomes. The presence of PrP^{C} on platelet-derived exosomes suggests a

possible mechanism for PrP^C transport in blood and for cell-to-cell transmission (Robertson et al., 2006).

2.7 Outline of the work described in this thesis

2.7.1 Role of PrP^C in neuronal differentiation

Accumulation within the brain of infected animals of an abnormal, protease-resistant, prion protein, PrP^{Sc}, is a hallmark of scrapie and other related neurodegenerative diseases. Despite the considerable attention for its involvement in transmissible spongiform encephalopathies, the physiological role of the cellular, nonpathogenic isoform of PrP, termed PrP^C, has not yet been determined. Among the several functions attributed to PrP^C as a cell surface molecule, an involvement in neurite outgrowth has been proposed through its interaction with NCAM at the neuronal cell surface of hippocampal neurons (Santuccione et al., 2005). However, both the PrP^C-mediated effects and the role of PrP^C-dependent pathways on neuronal differentiation are still not elucidated.

The aim of this work was to address the role of the cellular prion protein in the process of neuronal fate specification and neurite outgrowth by establishing a novel *in vitro* model as well as to identify the domain of PrP^C responsible for such activities.

A novel PrP^C-depleted cell line ($PrP^{0/0}$ ML) was derived from murine primary PrP-knockout neuronal cells by SV40 large T antigen-mediated immortalization. This cell line was then characterised for its growth potential, for the expression of specific cellular markers and for its ability to differentiate. It was found that, under culture conditions promoting the expression of the temperature-sensitive SV40 large T antigen, the cells expressed nestin, a specific marker of neuronal precursor cells. Therefore, the $PrP^{0/0}$ ML cell line was identified as a potential neuronal stem cell line. In fact, under nonpermissive culture conditions when the expression of the temperature-sensitive SV40 large T antigen is downregulated, the $PrP^{0/0}$ ML cells differentiated into neurons.

In order to investigate the role of PrP^C in the process of neuronal differentiation, the $PrP^{0/0}$ ML cells were then reconstituted for the expression of either the full-length PrP or a N-terminal truncated PrP^C form (PrPΔ32-134), which cause severe ataxia and neuronal death limited to the granular layer of the cerebellum and to Purkinje cells when expressed *in vivo* on a PrP-knockout background (Shmerling et al., 1998). The differentiation potential of both reconstituted cell lines under nonpermissive culture conditions was then compared with that

of the parenteral PrP$^{0/0}$ ML cells. This *in vitro* study clearly highlights that PrP^C expression in the PrP$^{0/0}$ ML cell line accelerates neuronal differentiation and that the N-terminal domain of the prion protein is not necessary for this PrP-mediated function.

2.7.2 Plasma membrane released microvesicles as carriers of infectious prions

Like other GPI-anchored proteins, PrP^C as well as PrP^{Sc} localize in glycosphingolipid-enriched microdomains (or lipid rafts) of neural and lymphocytic plasma membrane. The association of PrP^C with lipid rafts is required for its conversion to PrP^{Sc}. Particulary enriched in lipid rafts are microvesicles (MVs) which are released during membrane blebbing of normal healthy or damaged cells. Microvesicles are 100 nm-1 μm in size and contain numerous proteins and lipids similar to those present in the membranes of the cells from which they originate but they may also contain proteins derived from the cytoplasm and mRNA.

Microvesicles can also originate from the endosomal membrane compartment after fusion of secretory granules with the plasma membrane: in this case exosomes are formed which are more homogenous in size (30-100 nm) as compared to the plasma membrane-derived microvesicles. It has previously been shown that both PrP^C and PrP^{Sc} are present in cell culture supernatants in a secreted, exosome-associated form and that exosomes bearing PrP^{Sc} are infectious *in vivo* and *in vitro*. These findings suggest that PrP^{Sc}-bearing exosomes may provide a mechanism for intercellular transmission of infectious prions in addition to cell-to-cell contact.

Second objective of this Ph.D thesis was to determine the possible role of plasma membrane-derived microvesicles in the propagation and transmission of prions and in the mechanism(s) of PrP^C paracrine diffusion. The release of MVs was first studied in different murine neuronal cell lines. Here it is shown for the first time that neurons shed plasma membrane derived MVs, in addition to exosomes.

Immunoelectron microscopy and immunoblot analyses clearly demonstrated the presence of PrP^C on the membrane of MVs released from PrP^C-expressing cells. Characterization of lipid rafts components in MVs highlighted the presence of the ganglioside GM2, the tyrosine kinase p59Fyn, flotillin-2 and the neuronal protein GAP-43.

In order to investigate whether MVs are involved in the intercellular transmission of prions, MVs were first isolated from two prion infected murine neuronal cell lines and then used for *in vitro* and *in vivo* infection assays. Immunoblot analyses after proteinase K treatment

demonstrated the association of PrP^{Sc} with the secreted MVs. The PrP^{Sc}-bearing MVs were then used to perform infection experiments on noninfected cells and the kinetic of prion infection in the *de novo* infected cells was followed. Noteworthy, it was found that PrP^{Sc}-bearing MVs were capable to transmit prions *in vitro* and to stably infect the recipient cells. *In vivo* bioassays clearly demonstrated that PrP^{Sc}-bearing MVs were capable of eliciting a fatal prion disease in recipient indicator mice. These findings clearly demonstrated that MVs are biological carriers of both PrP^{Sc} and prion infectivity. MVs could therefore participate *in vivo* in the processes of intercellular prion transmission and propagation.

The establishment of a novel *in vitro* prion replication system for murine prions was also achieved by the use of one of the clonal cell lines previously reconstituted for PrP^C expression. This finding indicates that reconstitution of homologous or heterologous prion protein expression in PrP-depleted cell lines is a powerful approach to develop novel *in vitro* prion replication models.

3 MATERIALS AND METHODS

3.1 Cell culture

3.1.1 Reagents

Amphotericin B	Sigma, Munich, Germany
Antibiotic-Antimycotic	Sigma, Munich, Germany
B27 Supplement	Gibco BRL, Eggstein, Germany
BDNF (brain-derived neurotrophic factor)	R&D, Wiesbaden, Germany
bFGF (basic fibroblast growth factor)	Invitrogen, Eggstein, Germany
CMF-PBS (calcium- and magnesium-free PBS)	Gibco BRL, Eggstein, Germany
Dulbecco´s Modified Eagle Medium (DMEM)	Sigma, Munich, Germany
Dimethylsulfoxid (DMSO)	Sigma, Munich, Germany
DNase	Sigma, Munich, Germany
EDTA 0.5 mM	Sigma, Munich, Germany
Fetal calf serum (FCS)	Gibco BRL, Eggstein, Germany
Ham´s F12 nutrient	Sigma, Munich, Germany
Hank´s balanced salt solution	Sigma, Munich, Germany
L-glutamine	Sigma, Munich, Germany
Neurobasal Medium (NB)	Gibco BRL, Eggstein, Germany
N2 Supplement	Gibco BRL, Eggstein, Germany
Opti-MEM	Invitrogen, Eggstein, Germany
Penicillin-Streptomycin	Sigma, Munich, Germany
Polybrene	Sigma, Munich, Germany
Trypan blue	Sigma, Munich, Germany
Trypsin	Sigma, Munich, Germany

3.1.2 Media and buffers

Dulbecco´s Modified Eagle Medium (DMEM) was supplemented with 10% fetal calf serum (FCS), 4 mM L-Glutamin and antibiotics (20.000 U/ml penicillin, 20 mg/ml streptomycin and 25 ug/ml Amphotericin B).

Serum-free medium (SFM) was prepared by mixing 250 ml DMEM and 250 ml Ham´s F12 nutrient and supplemented with 1% N2 Supplement, 1% FCS , 4 mM L-Glutamine and antibiotics (20.000 U/ml penicillin, 20 mg/ml streptomycin and 25 ug/ml Amphotericin B).

Neurobasal Medium (NB) supplemented with B27 (50x, 10mlGibco)

Opti-MEM was supplemented with 4 mM L-Glutamine only. Neither antibiotics nor fungicides were added.

Opti-MEM with 4 mM L-Glutamine was supplemented with 10% fetal calf serum (FCS) and antibiotics (20.000 U/ml penicillin, 20 mg/ml streptomycin).

CMF-PBS (PBS w/o Ca^{2+}/Mg^{2+}): 120 mM NaCl, 17 mM Na_2HPO_4, 3 mM KH_2PO_4, pH 7.2

Trypsin-EDTA: 0.05% (w/v) Trypsin, 0.5 mM EDTA in CMF-PBS

All media and buffers were sterilised by filtration (0.2 um)

3.1.3 Cell lines

3.1.3.1 Generation and maintenance of the PrP$^{0/0}$ ML cell line

Prnp$^{0/0}$ Zurich I cerebella (Bueler et al., 1992) were dissected into ice-cold calcium- and magnesium-free PBS (CMF-PBS). Meninges were removed and the tissue was minced in small pieces. Cerebella were digested with 1% trypsin for 3 minutes at room temperature. Trypsin was replaced with 0.05% DNase and tissue was triturated with fire-polished Pasteur pipettes. Cells were centrifuged and resuspended in CMF-PBS. Supernatant was removed, cells were resuspended in Neurobasal medium supplemented with B27 and plated on 35 mm Petri dishes at a density of 2 x 105 cells/dish. Twenty-four hours after plating, cells were infected with a retrovirus vector carrying the temperature-sensitive SV40 large T antigen in 8 ug/ml polybrene for 2 hours at 37°C, as previously described (Jat and Sharp, 1989). After infection, the virus-containing medium was replaced with fresh medium consisting of DMEM supplemented with 10% FCS. Heterogeneous PrP$^{0/0}$ cell populations were then cultivated in DMEM, 10% FCS, 4 mM L-glutamine, 200 U/ml Penicillin, 0,2 mg/ml Streptomycin and

0,25 μg/ml Amphotericin B and immortalized cells were selected by their capability to grow at the permissive temperature of 33°C. The PrP$^{0/0}$ ML cell line was isolated from one of these heterogeneous preparations by limiting dilution; it was expanded and frozen down in large stocks of vials. Each experiment was performed by thawing a new vial of cells propagated for limited time.

To induce differentiation, cells were shifted at the nonpermissive temperature of 39°C under low serum concentration (Serum Free Medium (SFM): DMEM:Ham´s F12 Medium in a ratio 1:1 supplemented with 1% N2 Supplement, 1% FCS, 4 mM L-glutamine, 200 U/ml Penicillin, 0,2 mg/ml Streptomycin and 0,25 μg/ml Amphotericin B).

3.1.3.2 Maintenance of N2a cells

Murine Neuro-2a cells (American Type Culture Collection ATCC CCL 131) were maintained in DMEM supplemented with 10% FCS, 4 mM L-Glutamin and antibiotics (200 U/ml penicillin and 0,2 mg/ml streptomycin). The cell lines were maintained at 37° C in a humidified 5% CO_2 atmosphere.

3.1.3.3 Maintenance of Neuro-2a PK1 cells

Murine Neuro-2a PK1 cells and Rocky Mountain Laboratory strain (RML)-infected Neuro-2a PK1 cells (Klohn et al., 2003) were maintained in Opti-MEM supplemented with 10% FCS, 4 mM L-Glutamin and antibiotics (200 U/ml penicillin and 0,2 mg/ml streptomycin). The cell lines were maintained at 37° C in a humidified 5% CO_2 atmosphere.

3.1.3.4 Maintenance of N2a58/22L cells

Murine Neuro-2a58 cells infected with the prion strain 22L (N2a58/22L) (Nishida et al., 2000) were maintained in Opti-MEM supplemented with 10% FCS, 4 mM L-Glutamin and antibiotics (200 U/ml penicillin and 0,2 mg/ml streptomycin). The cells were maintained at 37° C in a humidified 5% CO_2 atmosphere.

3.1.4 Thawing procedure

Cells were thawed in a water bath at 37°C. After being gently resuspended, cells were immediately transferred to a T75-flask in complete DMEM medium. To reduce the cytotoxic

effect of DMSO the medium was replaced with fresh DMEM or Opti-MEM after 20 hours of cultivation.

3.1.5 Splitting cells

$PrP^{0/0}$ precursor cells were cultivated at 33°C, 5% CO_2 in 95% humidity (Heraeus incubator, Hanau, Germany). By a 80% - 90% confluency, cultures were split as follows: the medium was removed, the monolayers were washed with CMF-PBS and the cells were detached by trypsin/EDTA (0.5 mM EDTA, 0.05 % trypsin in CMF-PBS) for 3 minutes at 33°C. Complete medium was then added to neutralize Trypsin/EDTA. Cells were then resuspended by pipetting up and down several times, avoiding excessive foaming. One fifth of the suspension was transferred into a new T75-fask or T25-flask. Cells were split twice a week.

Neuro2-a PK1 and N2a58/22L cells were cultivated at 37°C, 5% CO_2 in 95% humidity (Heraeus incubator, Hanau, Germany). The cells were grown in Opti-MEM and split as above.

3.1.6 Freezing procedure

Cells were washed once with CMF-PBS, trypsinised and resuspended in DMEM or Opti-MEM. Afterwards, cells were transferred into a 15 ml Falcon tube and then centrifuged (Haraeus, Hanau, Germany, 1100 rpm for 5 minutes at 4°C). The pellet was resuspended in freezing medium (90% FCS, 10% DMSO) (approximately 1.5×10^6 cells/ml). Cells were frozen at -80°C for one day, and then transferred to liquid nitrogen.

3.1.7 Subcloning (limited dilution) of $PrP^{0/0}$ cells (heterogeneous population)

Several 96-wells plates were prepared by adding 100 µl of complete DMEM medium containing 10ng/ml bFGF and incubating them at 33°C for 30 min in order to prevent a pH-shift in the wells during pipetting ("buffering"). 80% confluent T75-fläsks were treated with trypsin as usually to detach the cells. The cells were then resuspended in 10 ml of complete DMEM medium and counted. The cell suspension was serially diluted performing 1:10 dilution steps to achieve a final cell concentration of either 1 cell/300 µl or 1 cell/100 µl. To each well of a 96 well-plate 100 µl of each cell suspension were added.

3.1.8 Proliferation Assay

The cells were seeded at a density of 6 X 10^4 cells into each well of a six-well plate and incubated in DMEM/10% FCS or in SFM at the appropriate temperature. Cells growth was monitored at different time points by counting the number of live cells by Coulter Counter (Z1 Coulter® Particle Counter, Beckman Coulter™)

3.1.9 Stable transfection of PrP$^{0/0}$ ML cell line

Transfection of PrP$^{0/0}$ ML cells was performed using the FuGENE 6 Transfection Reagent Kit (Roche, Mannheim, Germany). This lipofection method is based on a complex formation between a multi-component reagent and the plasmid DNA, followed by transportation into animal cells.

1.4 x 10^6 cells were seeded into each 10 cm petri dish 24 hours before transfection. After cultivation overnight, the cell monolayer was 50% confluent. The complete DMEM medium was replaced with 10 ml of serum-free medium Opti-MEM (w/o antibiotics).

The transfection mix was prepared by adding 72 μl FuGENE 6 Transfection Reagent to 728 μl serum-free medium Opti-MEM (w/o antibiotics), followed by an incubation of 5 minutes at RT. Thereafter, 24 μg DNA was added to the transfection mix (ratio of FuGENE 6 Transfection Reagent [μl] to DNA [μg] was 3:1), and the mixture was again incubated for 20 minutes at RT. The DNA-Reagent solution was then added in a drop-wise manner to the cells. After 6 hours incubation at 33°C, FCS was added to a final concentration of 10%. The next day, Opti-MEM medium was removed, the cells were washed with CMF-PBS and complete DMEM was added.

In order to select for transfected cells, 48 hours post-transfection the medium was replaced with fresh complete medium containing 2 μg/ml puromycin.

48 hours after selection start, bFGF in a concentration of 10 ng/ml was added to improve the growth potential of the cells.

3.1.9.1 Isolation of single cell-derived clones

Two weeks post-transfection visible clones were present on the plate. The medium was replaced with 10 ml CMF-PBS and the clones were isolated by using a 10μl pipette.

The isolated clones were then transferred into 96-wells previously incubated at 33°C for 30 minutes with 150 µl complete medium containing 1 µg/ml puromycin and 10 ng/ml bFGF. The growing clones were further expanded in DMEM complete medium containing 1 µg/ml puromycin.

3.1.10 Isolation of microvesicles

Cells (2-4x10^7 cells) were cultured for 2-3 days prior to MVs isolation by sequential centrifugation protocol (adapted from Baj-Krzyworzeka et al., 2002; Raposo et al., 1996; Thery et al., 2001). Cellular debris was removed by two consecutive centrifugation steps at 4500x g for 5 min at RT. Supernatants were centrifuged at 20000x g for 1 h at 4°C. MVs were pooled, washed in either CMF-PBS or Opti-MEM, repelleted and then either resuspended in Opti-MEM for *in vitro* or in CMF-PBS for *in vivo* infection experiments or lysed [20 mM Tris-HCl (pH 7,5), 1 mM EDTA, 100 mM NaCl, 1% Triton X-100, 0,5% sodium deoxycholate, 0,5% SDS] for immunoblot analyses. Exosomes were then enriched from the 20000x g supernatants by a 100000x g ultracentrifugation for 1 h at 4°C. Exosomes were pooled, washed in CMF-PBS, repelleted and then lysed in lysis buffer (see above).

3.1.11 Infection of cells with microvesicles and brain homogenates

The day before infection, cells were seeded in two wells of a six-well plate so that they reached 70% confluency in 24 hours. For infection, either MVs isolated from Neuro-2a PK1 infected cells (MVs$^{\text{Inf}}$) or those isolated from N2a58/22L cells (MVs$^{\text{22L}}$) or 1% of scrapie-brain homogenate (Chandler) were used as inoculum. Briefly, cells were at first washed with CMF-PBS and then overlaid either with 0,5 ml medium + 0,5 ml resuspended MVs or with 1 ml brain homogenate. As negative controls, samples incubated with MVs derived from Neuro-2a PK1 noninfected cells (MVs$^{\text{Noninf}}$) and those incubated with wild-type brain homogenate were included. When cells reached confluency (about 24-48 hours p.i), the twin wells were pooled and transfer into a T75-fläsk. Cells were then passaged as usual and at each passage a cell blot assay (see below) was performed to test for infectivity.

3.2 Molecular and biochemical analysis

3.2.1 Reagents

Agarose	Invitrogen life technologies, Karlsruhe, Germany
Ampicillin	Serva, Heidelberg, Germany
Guanidinium thiocyanate	Roth, Karlsruhe, Germany
HRP-conjugated cholera toxin,	
B subunit (HRP-CTxB)	Sigma, Munich, Germany
Proteinase K	Roche Diagnostics NL, Germany
PMSF (Protease Inhibitor)	Roche Diagnostics NL, Germany
Roti-Quant	Roth, Karlsruhe, Germany
Tween-20	Sigma, Munich, Germany
Yeast extract	Invitrogen life technologies, Karlsruhe, Germany

All other chemicals were purchased from Merck (Darmstadt, Germany)

3.2.2 Media and buffers

3.2.2.1 General buffers

Guanidinium thiocyanate: 3 M guanidinium thiocyanate, 10 mM Tris-Cl (pH 8.0)

Lysis buffer I: 50 mM Tris-Cl, pH 8.0; 150 mM NaCl; 0.5% sodium deoxycholate; 0,5% Triton X-100

Lysis buffer II: 20 mM Tris-HCl (pH 7,5), 1mM EDTA, 100mM NaCl, 1% Triton X-100, 0,5% sodium deoxycholate, 0,5% SDS, 1mM Na-Vanadate, 1mM Na-Molybdate, 20 mM NaF, 10mM Na-phyrophosphat, 20mM ß-Glycerophosphat and one complete protease inhibitor tablet (Roche)

CMF-PBS (PBS w/o Ca^{2+}/Mg^{2+}): 120 mM NaCl, 17 mM Na_2HPO_4, 3 mM KH_2PO_4. The pH value was adjusted to 7.2

TBST: 20 mM Tris-HCL, 500 mM NaCl, 0.1% Tween-20. The pH value was adjusted to 7.5

1x TAE: 40 mM Tris-Acetat, 2 mM EDTA. The pH value was adjusted to 8.0

20x SSC: 3 M NaCl, 300 mM Na_3-citrate. The pH value was adjusted to 7.0

3.2.2.2 Media for bacteria and supplements

SOC medium was prepared using 2% Tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose. The pH value was adjusted to 6.8-7.2

LB-medium: 1% Tryptone, 0.5% yeast extract, 1%NaCl, 1.5% Agar. The pH value was adjusted to 7.2

LB-agar: 1% Tryptone, 0.5% yeast extract, 1%NaCl, 1.5% Agar

Ampicillin was added in a concentration of 100µg/ml

3.2.2.3 Bacterial strain

The following bacteria strain was used: DH5α-T1[®] strain (Invitrogen life technologies, Karlsruhe, Germany): F^- φ80*lac*ZΔM15(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^-) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *ton*A

3.2.3 Vectors

pIRESpuro2 (Clontech Laboratories, Inc)
pPrPHG plasmid (Fischer et al., 1996)
pPrPHG.F vector (Shmerling et al., 1998)

3.2.4 Molecular size markers

1 kb DNA marker (NEB Biolabs, Schwalbach, Germany): 500, 1000, 1500, 2000, 3000, 4000, 5000, 6000, 8000, 10000 bp.

100 bp DNA marker (NEB Biolabs, Schwalbach, Germany): 100, 200, 300 400, 500, 517, 600, 700, 800, 900, 1000, 1200, 1517 bp.

<u>2-Log bp DNA marker</u> (NEB Biolabs, Schwalbach, Germany): 100, 200, 300 400, 500, 517, 600, 700, 800, 900, 1000, 1200, 1517, 2017, 3001, 4001, 5001, 6001, 8001, 10002 bp.

<u>SeeBlue 2 pre-stained</u> (Invitrogen): 3, 6, 14, 17, 28, 38, 49, 62, 98, 188 kDa

<u>MultiMark Multi-Colored</u> (Invitrogen): 3, 6, 11, 17, 19, 31, 52, 98, 185 kDa

3.2.5 Antibodies

3.2.5.1 Primary antibodies

Alix (3A9), mouse (Cell Signaling, USA)

Flotillin-2, goat (Santa Cruz, CA, USA).

Fyn (FYN3), rabbit (Santa Cruz Biotechnology, Inc)

GAP-43, goat (Santa Cruz Biotechnology, Inc)

GM2 (MK 1-16), mouse (Seikagaku, Corp. Chuo-ku,Tokyo, Japan)

MAP-2B IgG1, mouse (BD Biosciences),

Nestin IgG$_1$, mouse (BD Biosciences)

NF-H, rabbit (Sigma)

Nonphospho–Src (Tyr416), mouse (Cell Signaling)

PrP-6H4 IgG$_{1,k}$, mouse (Prionics)

SAF-32, mouse (Cayman)

SV40 Large T Antigen IgG$_{2a}$, mouse (BD Biosciences)

tsg 101 (M-19), goat (Santa Cruz Biotechnology, USA)

3.2.5.2 Secondary antibodies

HRP-conjugated anti-mouse IgM (Sigma)

HRP-conjugated anti-mouse IgG (Zymed-Invitrogen)

HRP-conjugated anti-rabbit IgG (Sigma)

HRP-conjugated anti-goat IgG (Dianova)

3.2.6 PCR genotyping

The genotype of the PrP$^{0/0}$ ML cell line was confirmed by PCR. *Prnp^0* and *Prnp$^+$* alleles were detected using the primers P3 (specific for the *Prnp^0* allele), P10 (specific for the *Prnp$^+$* allele), and prPrP 3´nc (specific for both the *Prnp^0* and *Prnp$^+$* alleles) according to Fischer et al. (Fischer et al., 1996). Genomic DNA extracted from either PrP$^{0/0}$ ML cells or mouse tail lysates was used as DNA template.

3.2.7 Cell viability assay

The activity of LDH released in the supernatant from damaged cells was measured using the Cytotoxicity Detection Kit according to the manufacturer's instructions (Roche).
LDH release after treatment of the cells with 1% Triton-X100 was set as 100% and relative LDH release values of the probes were then calculated.

3.2.8 Immunoprecipitation

Cells were lysed in cold lysis buffer II. Samples were pelleted at 14,000 rpm at 4 °C for 10 minutes, and supernatants were transferred to a clean microfuge tube. Protein concentration was determined by BCA assay as recommended by the manufacturer (Pierce). Two hundred fifty micrograms of each cell lysate sample were adjusted to a final volume of 500 µl with lysis buffer II. To the samples 2,5 µg rabbit polyclonal anti-Fyn antibody (FYN3) were added, and samples were incubated overnight at 4°C on a rocking platform. After incubation, 20 µl Protein A-Sepharose beads (Amersham Pharmacia) were added, and samples were incubated for 2 hours at 4°C on a rocking platform. Sepharose beads were then washed three times with lysis buffer II. Pellets were boiled in SDS sample buffer and analyzed by immunoblotting.

3.2.9 High-performance-thin-layer-chromatography and gangliosides immunostaining

Gangliosides were extracted according to the method of Svennerholm and Fredman (Svennerholm and Fredman, 1980). The eluted glycosphingolipids were dried and separated by high-performance-thin-layer-chromatography (HPTLC) aluminium-backed silica gel 60 (20x20) plates (Merck, Darmstadt, Germany). Chromatography was performed in chloroform:methanol:0.25% aqueous KCl (5:4:1) (v:v:v). Plates were immunostained for 1h

at room temperature with HRP-CTxB (to detect GM1), or, alternatively, with anti-GM2 and then with anti-mouse IgM-HRP. Immunoreactivity was assessed by chemiluminescence reaction using the ECL Western blocking detection system (Amersham, Buckinghamshire, UK).

3.2.10 Western blot analysis

Cell- and microvesicles lysates were prepared in cold lysis buffer II. Cell lysates only were pelleted at 14,000 rpm at 4 °C for 10 minutes and their supernatants were transferred to a clean microfuge tube. Protein concentration was determined by BCA assay as recommended by the manufacturer (Pierce) or according to Bradford´s.

80 μg of total protein of each cell lysate and the equivalent of 250 μg of each cell lysate after immunoprecipitation (see above) were electrophoresed through a NuPAGE 12.0% SDS polyacrylamide gel (Invitrogen) or 7% Tris-Acetat-Gels (Invitrogen). Otherwise a self-prepared 12% SDS-Page gel was used. In the case of microvesicles lysates, 20 μg of total protein were loaded. When a proteinase K digestion was performed, the digested protein amounts loaded corresponded to 80 or 250 μg for cell lysates and 20 μg for microvesicles lysates.

Proteins were transferred to PVDF membranes by semidry blotting. Membranes were blocked at RT for 1 hr with Tris-buffered saline/0.05% Tween 20 (TBST)/5% nonfat dry milk, incubated with the appropriate primary antibody diluted in Tris-buffered saline/0.05% Tween 20 (TBST)/1% nonfat dry milk (for PrP, SAF-32, 1:500 or 6H4, 1:5000; for GAP-43, anti-GAP-43, 1:500; for flotillin-2, anti-flotillin-2, 1:750; for GM2, anti-GM2, 1:500; for Alix, Alix (3A9), 1:1000; for Tsg101, tsg 101 (M-19), 1:750; for Nestin detection, anti-Nestin, 1:250; for MAP2B, anti-MAP2B, 1:1000 and for NF-H, anti-NF-H, 1:2000) overnight at 4°C. After washing with TBST, membranes were incubated for 1 hr at room temperature to HRP-conjugated secondary antibody (1:10000) diluted in the same buffer as above. Bands were visualized by enhanced chemiluminescence (Amersham Pharmacia).

3.2.11 Cell blot assay

Cells were cultured on glass coverslips (Roth, Germany) until confluence, washed with CMF-PBS, and placed cell side down on lysis buffer I-soaked nitrocellulose membranes (Hybond-C-Extra, Amersham, Germany) that were previously laid on a lysis buffer-soaked filter paper. Membranes were then processed as described (Klohn et al., 2003). Briefly, membranes were

dried at RT, coverslips were carefully removed and then the membranes were incubated in a Proteinase K solution [5 µg/ml PK in lysis buffer] for 90 min at 37°C. After twice wash in dH₂0, PK digestion was stopped by incubating the membranes in 2 mM PMSF for 10 min at RT. Membranes were than treated with 3 M guanidinium thiocyanate in 10 mM Tris-Cl (pH 8.0) for 10 min. After extensive washing in dH₂0, membranes were processed for PrP^C/PrP^{Sc} detection by the use of the monoclonal antibody 6H4 as described above (3.2.10).

3.2.12 PCR using the pPrPHG plasmid and the pPrPHG.F vector as templates

In order to get the bicistronic pPrP-IRESpuro expression vector and the pPrPΔ32-134-IRESpuro construct coding for either the full-length PrP or the PrPΔ32-134 ORF plus the puromycin antibiotic resistance gene, the pPrPHG plasmid (Fischer et al., 1996) or the pPrPHG.F vector (Shmerling et al., 1998) were used respectively as templates. The same primers were used in both cases, namely 5'-P-ORF (EcoRI site italicized) and 3'-P-ORF (BamHI site italicized). The PCR product obtained for the PrP ORF corresponded to a 765 bp fragment.

Name	Sequences of designed primers
5'-P-ORF	(5'- CCG*GAATTC*ACCATGGCGAACCTTGGCTAC)
3'-P-ORF	(5'-CGC*GGATCC*TCATCCCACGATCAGGAAGATGAGG)

3.2.13 Agarose gel electrophoresis

1% agarose gels were prepared by adding 1.5 g agarose to 150 ml 1x TAE buffer. The resulting emulsion was then heated in a microwave oven until the solid agarose became solved. Then ethidium bromide (10 mg/ml) was added to a final concentration of 0.5 µg/ml and the gel solution was poured into the horizontal tray (Invitrogen life technologies, Karlsruhe, Germany) with inserted comb (NEB Biolabs, Schwalbach, Germany). DNA samples were mixed with 1/10 volume of 10x sample buffer (0.5% Bromophenol blue, 0.5% Xylene cyanol FF, 30% Ficoll Type 400 in 10xTAE buffer) and applied to the gel. As marker, either the 100 bp, 1 kb or the log-2 ladder was used depending on the fragment size amplified. Electrophoresis was then performed at 100 V for 1 hour. Afterwards bands were photographically documented (Program: *BioCap Version 97.03s f. Windows*, Printer: Mitsubishi P91) under UV light and, if desired, bands were cut out for purification.

3.2.14 Extraction of DNA fragments from agarose gels

After electrophoresis, the DNA bands of interest were cut out from the gel and eluted using the Gel Extraction Kit (Qiagen, Hilden, Germany) as described by the manufacturer.

3.2.15 Ligation of PCR products with pIRESpuro2 vector

The bicistronic pPrP-IRESpuro expression vector coding for both the full-length PrP and the puromycin antibiotic resistance gene under the control of the human cytomegalovirus (CMV) major immediate early promoter/enhancer was generated by inserting the PrP ORF into the multiple cloning site of the pIRESpuro2 vector (Clontech). Briefly, the 765 bp PCR product obtained as described above was cleaved with EcoRI and BamHI and cloned into the EcoRI-BamHI-digested pIRESpuro2 backbone (Fig. 5). At the same manner, the PrPΔ32-134 amplicon was cleaved and inserted to get the pPrPΔ32-134-IRESpuro construct.

3.2.16 Chemical transformation of bacteria

For transformation *Escherichia coli* (*E.coli*) bacteria strain DH5α-T1® (Invitrogen life technologies, Karlsruhe, Germany) was used.
10 µl of ligation-mixture were added to 100 µl of DH5α-T1® cells and after a gently mixing the suspension was incubated 30 min on ice. Afterwards the sample was submitted to an heat-shock at 42°C for 45 sec. followed by an incubation of 2 min. on ice. Then, 250 µl SOC medium were added and the cells were incubated at 37°C for 1 hr on a shaker. Subsequently, they were spread in different concentrations on prewarmed LB-plates with suitable antibiotics. The plates were incubated at 37°C o.n.

3.2.17 Plasmid preparation

Preparation of plasmids from bacteria was performed using the Qiagen plasmid kits (Qiagen, Hilden, Germany) according to the manufacture instructions.

3.2.18 Restriction enzyme analyses

The presence of the insert and its correct size was checked by restriction enzyme analysis. All restrictions were performed using commercially available restriction enzymes from New England Biolabs (NEB, Schwalbach, Germany) in line with the manufactures instructions.

Usually, 5U of enzyme should be enough to cut 1 µg DNA. The volume of enzyme should not be greater than 1/10 of the total volume of the restriction reaction, composed also by 10x proper buffer and sometimes 100x BSA depending on the enzyme. ddH_20 is added to adjust the final volume. The duration and the temperature of the restriction digestion depends on the enzyme(s) used.

3.2.19 DNA sequencing

After confirmation of the DNA fragment size by restriction analyses, the inserted DNA fragments were sequenced to confirm their identity. Sequencing was performed at MWG Biotech (Ebersberg, Germany). For this purpose, samples containing approximately 2 µg of DNA were lyophilized using a speedvac centrifuge (Eppendorf, Hamburg, Germany). The resulting lyophilized samples were sent to the company, where they were sequenced using the designed primer pairs.

3.3 Immunological analysis

3.3.1 Reagents

Acetone	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
DAKO mounting medium	DAKO Corporation, Carpinteria, USA
DAKO Pen	DAKO Corporation, Carpinteria, USA
Goat serum	Zymed Laboratories, South San Francisco, USA
bisBENZIMIDE/Hoechst	Sigma, Munich, Germany
Methanol	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Triton X-100	Sigma, Munich, Germany
Tween-20	Sigma, Munich, Germany

All other chemicals were purchased from Merck (Darmstadt, Germany)

3.3.2 Media and buffers

3.3.2.1 General buffers

CMF-PBS (PBS w/o Ca^{2+}/Mg^{2+}) was generated using 120 mM NaCl, 17 mM Na_2HPO_4, 3 mM KH_2PO_4, and the pH value was adjusted to 7.2

1% paraformaldehyde (PFA)
1% (w/v) paraformaldehyde
in CMF-PBS pH 7.4

4% paraformaldehyde (PFA)
4% (w/v) paraformaldehyde
in CMF-PBS pH 7.4

2,5% glutaraldehyde
2,5% (w/v) glutaraldehyde
in CMF-PBS pH 7.4

1% osmium tetroxide
1 % (w/v) osmium tetroxide
in Veronal acetate buffer pH 7.4

Blocking buffer
4% normal goat serum or 10% normal goat serum
0.1% Triton X-100
0.02% NaN3
in CMF-PBS pH 7.4

FACS buffer
0.25% BSA
0.05% NaN3
in CMF-PBS pH 7.4

3.3.3 Antibodies

3.3.3.1 Primary antibodies

MAP2B IgG1, mouse (BD, Biosciences)

GFAP IgG$_{2b}$, mouse (BD, Biosciences)

TNP-IgG$_{1,k}$, mouse, monoclonal immunoglobulin isotype standard (BD Biosciences)

Nestin IgG1, mouse (BD, Biosciences)

PrP-6H4 IgG$_{1,k}$, mouse (Prionics)

PrP-8G8 IgG$_{1,k}$, mouse (Cayman)

PSA-NCAM IgM, mouse (Chemicon)

SV40 Large T Antigen IgG$_{2a}$, mouse (BD, Biosciences)

NeuN IgG1, mouse (Chemicon)

3.3.3.2 Secondary antibodies

FITC - conjugated anti-mouse IgM (Santa Cruz Biotechnology)

RPE - conjugated anti-mouse IgG (DAKO)

Alexa Fluor$^{®}$488 - conjugated anti-mouse IgG, F(ab')$_2$ Fragments (A-11017, Invitrogen life technologies, Karlsruhe, Germany)

10 nm colloidal gold - conjugated anti-mouse immunoglobulin (1:100, Biocell, UK)

3.3.4 Immunofluorescence analysis of specific cell markers

Cells were plated onto poly-D-lysine overlaid glass coverslips (BD BioCoat^{TM} Cellware Poly-D-Lysine 12 mm round Coverslips) and grown under different conditions at 33°C or at 39°C for 1 to 6 days. In order to perform a staining after 4 days, 30'000 cells were seeded per 24-well dish for the 33°C condition and 60'000 cells/well for the 39°C condition. The staining was avoid in case of a 80-90% cells confluency.

At the time-point (24 hours, 48 hours, 72 hours or 96 hours) of investigation, cells were rinsed gently with CMF-PBS and fixed either 20 min. at RT with 4% PFA (for GFAP, Nestin, SV40 Large T Antigen and NeuN staining) or 10 min. at RT with 4% (PFA) followed by 10 min. at –20°C in Methanol:Acetone (1:1) (for MAP2B staining). Cells were then washed again with CMF-PBS for 5 min. and permeabilised with 0.2% Triton X-100 in CMF-PBS for 5 min. at

RT. Cell nuclei were stained with Hoechst dye during an incubation time of 15 min. at RT. The dye was washed out 3 times (5min/each) with CMF-PBS and the cells were pre-incubated for 1 h at RT in blocking buffer (4% normal goat serum, 0.1% Triton X-100, 0.02% NaN3). After blocking buffer removal, primary antibodies (MAP2B 1:300, GFAP and Nestin 1:250, SV40 Large T Antigen 1:100, NeuN 1:50 in blocking buffer) were added to the cells followed by incubation o.n. at 4°C.

The day after, cells were washed 3 times in CMF-PBS for 5min./each and incubated at RT for 2 h with the secondary antibody (goat α-mouse Alexa Fluor®488, 1:300 in blocking buffer) in a humidified chamber. After washing 3 times in CMF-PBS for 5min./each, the coverslips were mounted upside down on slides with a drop of DAKO Fluorescent mounting medium. A nail polish sealing allowed the storage of the slides at 4°C in complete darkness until the epifluorescence microscope analysis.

3.3.5 Immunofluorescence analysis of PrP^C

Cells were fixed 20 min. at RT with 4% PFA, washed with CMF-PBS for 5 min and then permeabilised with 0.2% Triton X-100 in CMF-PBS for 5 min. at RT. Cell nuclei were stained with Hoechst dye during an incubation time of 15 min. at RT. The dye was washed out 3 times (5min/each) with CMF-PBS and the cells were pre-incubated for 1 h at RT in blocking buffer (4% normal goat serum, 0.1% Triton X-100, 0.02% NaN3). After blocking buffer removal, primary antibodies against PrP^C (6H4 1:400, Prionics or 8G8 1:200, Cayman) were added to the cells followed by incubation o.n. at 4°C. As negative control, the isotype antibody anti-IgG$_{1,k}$-TNP (1:400) was used.

The day after, cells were washed 3 times in CMF-PBS for 5min./each and incubated at RT for 2 h with the secondary antibody (goat α-mouse Alexa Fluor®488, 1:300 in blocking buffer) in a humidified chamber. After washing 3 times in CMF-PBS for 5min./each, the coverslips were mounted upside down on slides with a drop of DAKO Fluorescent mounting medium. A nail polish sealing allowed the storage of the slides at 4°C in complete darkness until the epifluorescence microscope analysis.

3.3.6 Fluorescent activated cell sorting (FACS)

Cells were detached with CMF-PBS/1mM EDTA, resuspended in 5 ml DMEM/10% FCS and counted.

Single staining for PrP was achieved by incubating the cells (0.5×10^6) with 100 μl of monoclonal 6H4 antibody (1:250; Prionics AG) for 30 minutes at 4°C.

Staining for PSA-NCAM was achieved by incubating the cells (0.5×10^6) with 100 μl of monoclonal PSA-NCAM IgM (1:200, Chemicon) for 30 minutes at 4°C.

Cells were then washed in cold FACS buffer (CMF-PBS, 1% (w/v) BSA, 10 mM EDTA (pH 8), 0.1% sodium azide), incubated for 30 minutes at 4°C with 100 μl of goat anti-mouse IgG-RPE (1:200) for PrP detection or with 100 μl of goat α-mouse IgM-FITC (1:50) for PSA-NCAM detection.

One step wash was performed before cytofluorimetry (FACScan, Becton Dickinson).

3.3.7 Immunoelectron microscopy

Microvesicles were isolated as described previously, resuspended in the appropriate volume of Opti-MEM and incubated with the anti-PrP monoclonal 6H4 antibody (1:200) for 1h at 4°C. Samples were then absorbed by nickel-grid before extensive washing with water. A drop of immunogold labelled secondary antibody (anti-mouse immunoglobulin-colloidal gold 10 nm, 1:100 in CMF-PBS or medium) was then applied on the grid and incubated for 20 minutes at RT. After a three-time washing step, the grid was incubated with one drop of uranyl acetate for 10 sec at RT. Samples were then analysed under an electron microscope (Zeiss, Germany).

Neuro-2a PK1 cells were incubated with the anti-PrP monoclonal 6H4 antibody (1:200) for 1h at 4°C. Cells were washed in CMF-PBS and then fixed with 1% paraformaldehyde for 1h at 4°C, washed and labelled with anti-mouse immunoglobulin-colloidal gold 10 nm (1:100, Biocell, UK) for 3h at 4°C. Cells were post-fixed first in 2, 5% glutaraldeyde for 45 min at RT and then in osmium tetroxide 1% in Veronal acetate buffer, pH 7.4, for 2h at 4°C, stained with uranyl acetate (5 mg/ml), dehydrated in acetone and embedded in Epon 812. Samples were then sectioned and examined under an electron microscope (Zeiss, Germany).

3.4 In vivo infectivity bioassay

MVs were isolated both from noninfected and RML-infected Neuro-2a PK1 cells as previously described and resuspendend in 0,2 ml PBS. Noninfected and infected Neuro-2a

PK1 cells were collected and resuspended in 1 ml PBS. MVs and cells were subjected to five consecutive cycles of freeze and thawing. Protein concentration for cell lysates and MVs was determined by Bradford assay as recommended by the manufacturer's guidelines. Samples were then adjusted to 10 µg total protein per 30 µl with PBS/5%BSA. Different dilutions of mouse brain homogenate infected with the Rocky Mountain Laboratory (RML) scrapie strain (passage 5.0, 1 x 10^8 LD_{50}/ml 10% brain homogenate) (Prinz et al., 2004) in PBS/5% BSA were used as positive controls, whereas mock infected brain homogenate was included as negative control.

Thirty microliters for each sample were administered intracerebrally to groups of four *tga20* mice (Fischer et al., 1996). The indicator *tg*a20 mice were anaesthetized at 4-20 weeks of age with metofane (Pitman-Moore, USA) and inoculated with 30 µl inocula using a 300-µl insulin syringe and a 26-gauge hypodermic needle inserted 3-4 mm into the right parietal lobe.

Disease in animals was diagnosed when at least three of the following symptoms were observed: foot clasping of hindlegs when mice were lifted by the tail, plastic tail; decreased motor activity; mincing gait, disorientation; mild hind leg paresis, ataxia; kyphosis. Incubation time to terminal scrapie sickness was determined and infectivity titers were calculated by using the relationship $y = 11.45 - 0.088\,x$, where y is $logLD_{50}$/ml homogenate and x is incubation time in days to terminal disease (Prusiner et al., 1982). The presence of a protease-resistant isoform of PrP (PrP^{Sc}) in the infected brains was investigated on proteinase K-treated (20 µg/ml; 30 min; 37°C) homogenates by Western blot analysis, as described above.

4 RESULTS

4.1 The role of the cellular prion protein in the process of neuronal differentiation

Four different PrP-knockout cell lines, named HpL 3-4, F14, Zpl and NpL2, respectively, have already been generated to expand studies on the normal function of PrP^C (Holme et al., 2003; Kim et al., 2005; Kuwahara et al., 1999; Nishimura et al., 2007). However, the HpL 3-4 cell line (Kuwahara et al., 1999) has been made from PrP-deficient animals of the Nagasaki-type and, therefore, expresses the neurotoxic protein Doppel (Dpl) (Anderson et al., 2004; Genoud et al., 2004; Li et al., 2000; Moore et al., 1999; Moore et al., 2001; Rossi et al., 2001). The recently established F14, Zpl and NpL2 cell lines (Holme et al., 2003; Kim et al., 2005; Nishimura et al., 2007) have been derived from neuronal primary cells of Zurich I-type mice (Bueler et al., 1992; Manson et al., 1994). These cells express neither PrP nor Dpl but, being already differentiated into neurons, might not be suitable for studying the possible role of PrP^C in processes of cell differentiation.

The temperature-sensitive tsA58 mutant of the simian virus 40 (tsSV40) large T antigen has been previously used to generate several neuronal cell lines (Barber et al., 2000; Cattaneo and Conti, 1998; Eves et al., 1992; McManus et al., 1999; Son et al., 1999; White et al., 1994; Whittemore and White, 1993). Cells immortalized with this oncogene proliferate at the permissive temperature (33°C), but arrest their growth and differentiate upon shift to the nonpermissive temperature (39°C), i.e. when the thermolabile large T antigen is inactivated (Jat and Sharp, 1989). Therefore, this system is particularly useful to study signalling pathways potentially involved in processes of cell differentiation without the interference by the transforming oncogene.

4.1.1 Generation of a novel PrP-knockout cell line by SV40 Large T Antigen-mediated immortalization

In order to address the role of the cellular prion protein in the process of neuronal fate specification and neurite outgrowth as well as to identify the domain of PrP^C responsible for such activities the approach outlined above was used to generate a novel PrP-deficient cell line deriving from cerebellar neuroepithelial precursor cells of the PrP-knockout Zurich I mice (*Prnp$^{0/0}$* Zurich I, Bueler et al., 1992).

Primary cells prepared from the cerebella of *Prnp$^{0/0}$* Zurich I animals (Bueler et al., 1992) were infected with a retrovirus transducing the tsA58 SV40 large T antigen (Jat and Sharp, 1989). The derived immortalized heterogeneous cell populations were then selected by their ability to grow at the permissive temperature of 33°C in serum-containing medium. Colonies that could proliferate in these conditions were subsequently isolated by repeated proliferation and limiting dilution of the cells. One clonal line, named PrP$^{0/0}$ ML, was selected in consideration of morphology, growth rate and ability to adhere to the plates and was characterized in detail.

The presence of two *Prnp* knockout alleles in the PrP$^{0/0}$ ML cells was confirmed by PCR analysis (Fig. 4A). Furthermore, the expression of the SV40 Large T antigen and its characteristic nuclear localization was corroborated by immunofluorescence analysis of cells maintained at the permissive temperature (Fig. 4, upper panels). Switching the cells to the nonpermissive temperature (39°C) caused a strong reduction of the large T-antigen as revealed by both immunoflorescence (Fig. 4B, lower panels) and western-blot analyses (data not shown) indicating that the expression of the oncogene was controlled in a temperature-sensitive manner.
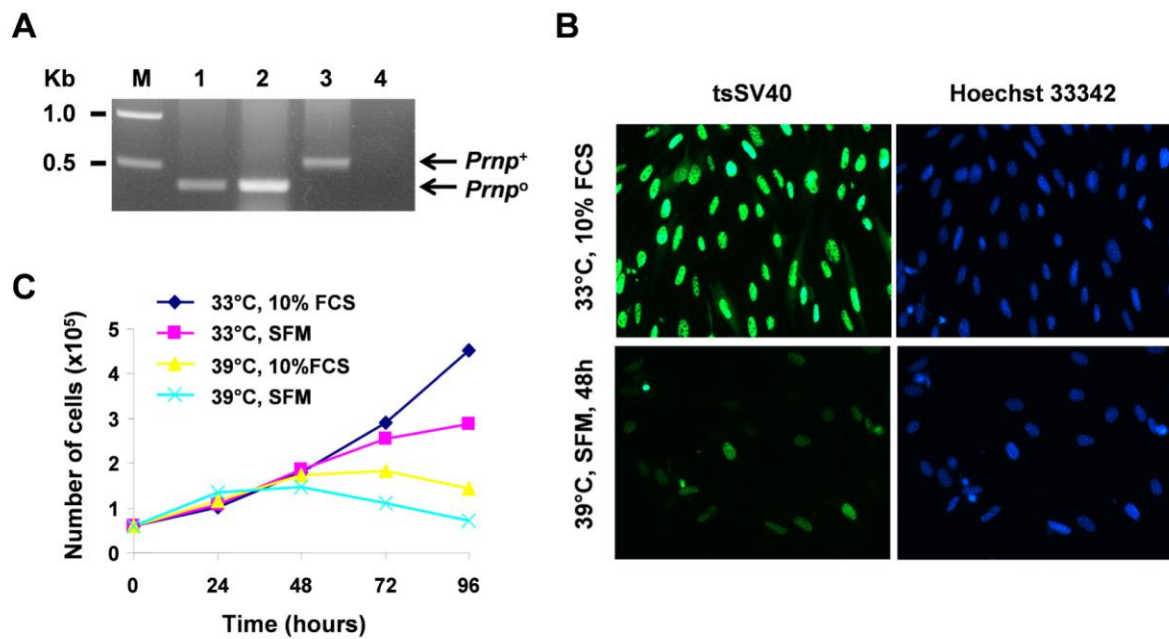
Figure 4. Characterization of the PrP$^{0/0}$ ML cell line

(A) The genotype of the PrP$^{0/0}$ ML cells was confirmed by PCR analysis using primers that allow to amplify simultaneously both the wild-type *(Prnp$^+$)* and the *Prnp* knockout *(Prnp^0)* alleles. PrP$^{0/0}$ ML cells are homozygous for the *Prnp* knockout allele (lane 2). Genomic DNAs from *Prnp* knockout (lane 1) and *Prnp* wild-type (lane 3) mice were used as positive controls. A negative control, run in the absence of DNA template (lane 4), was included to exclude the presence of contaminants in the reaction mixture. (B) Immunoreactivity for the SV40 large T antigen in PrP$^{0/0}$ ML cells grown in permissive (upper panels) and nonpermissive culture conditions (lower panels). The characteristic nuclear localization of the oncogene product (tsSV40, green) was detected in the cells maintained in permissive conditions (upper panels). Strong reduction of large T antigen expression was noticed after cultivation of the PrP$^{0/0}$ ML cells in nonpermissive condition for 48 hours (lower panels). Nuclei were counterstained with Hoechst 33342 (blue). Scale bar, 50 μm. (C) Growth curves of the PrP$^{0/0}$ ML cells maintained at 33°C or 39°C in high (10% FCS) or low serum concentration (SFM). At 33°C in serum-containing medium, the cells proliferated with a doubling time of approximately 30 hours. Withdrawal of the serum significantly decreased the growth rate. At the nonpermissive temperature of 39°C the cells exhibited decreased proliferation potential even in serum-containing medium. This effect is further enhanced with serum deprivation that led to progressive stoppage of the cell division and reduction in the number of cells.

4.1.2 Proliferative potential of the PrP$^{0/0}$ ML cell line

The proliferative potential of the PrP$^{0/0}$ ML cell line was subsequently evaluated under different culture conditions and the number of viable cells at selected time-points was plotted as growth curves (Fig. 4C). At 33°C in serum-containing medium (10% FCS), the cells proliferated with a doubling time of approximately 30 hours. Under these conditions, cell growth is sustained by the presence of both the immortalizing oncoprotein and the serum. Withdrawal of the serum (SFM) significantly decreased the growth rate and extended the cell doubling time to 35 hours. At the nonpermissive temperature of 39°C, when the oncogene expression is reduced, the cells exhibited decreased proliferation potential even in serum-containing medium, with a doubling time of approximately 46 hours. At the same temperature, serum deprivation led to progressive stoppage of the cell division with the consequent reduction in the number of cells (Fig. 4C). At nonpermissive conditions of maintenance, the addition of the growth factor basic fibroblast growth factor (bFGF) (10 ng/ml) rescued the proliferative capability of the cells and extended cell survival beyond 96 hours. Furthermore, at 33°C in serum-containing medium, bFGF reduced the cell doubling time at approximately 12 hours (data not shown). Thus, PrP$^{0/0}$ ML cells responded to bFGF stimulation by undergoing cell proliferation, a cellular response to this growth factor already been shown for cultured CNS progenitor cells derived from the embryonic rat striatum (Cattaneo and McKay, 1990; Gage et al., 1995; Gensburger et al., 1987; Kilpatrick and Bartlett, 1993; Murphy et al., 1990; Reynolds et al., 1992; Temple and Qian, 1996; Vescovi et al., 1993).

Based on these data, the two most opposite situations (33°C with high serum vs. 39°C in low serum concentration) were adopted in further experiments.

4.1.3 Temperature - and growth factor-dependent phenotypic changes of the PrP$^{0/0}$ ML cells

The switch from proliferating precursors to differentiated neurons *in vivo* is typically associated with changes in specific traits of the cell. In particular, this process is defined by progressive stages that can be identified on the basis of the expression of key markers and cell morphology (Kempermann et al., 2004). Immortalized CNS progenitor cells were reported to efficiently differentiate into neuronal and glial lineages *in vitro* upon growth factor withdrawal from the culture medium (Cattaneo and Conti, 1998; Eves et al., 1992). In these

experimental conditions, the different steps of the differentiation process are featured by progressive expression from immature to mature neuronal markers and by morphological changes that reasonably recapitulate the developmental progression occurring during the formation of the CNS (Eves et al., 1992).

Based on these considerations, the immortalized PrP$^{0/0}$ ML cells were characterized in terms of cellular lineage and differentiation potential. The expression of specific markers for different cell lineages and for different stages of neuronal development was evaluated by immunofluorescence analyses. Nestin is an intermediate filament protein that is only transiently expressed during the development of the CNS and is abundantly expressed in neuroepithelial stem cells, radial glial cells and oligodendrocyte precursors. It is therefore considered to be a marker of CNS precursor cells (Frederiksen and McKay, 1988; Gallo and Armstrong, 1995; Hockfield and McKay, 1985; Lendahl et al., 1990) (for review, see Wiese et al., 2004). The microtubule-associated protein (MAP)-2b is a protein associated with microtubules and plays an essential role in the outgrowth of dendrites (Heidemann, 1996; Izant and McIntosh, 1980). It is normally expressed in differentiating neurons. The neuron-specific nuclear protein NeuN (Mullen et al., 1992) is a protein specifically localized in the nucleus of CNS cells and in cells that are terminally differentiated in neuronal phenotypes. The neuronal filament (NF)-H (200 kDa) is one of the three subunits which form by copolymerisation the neurofilaments (NFs), the most abundant cytoskeletal intermediate filaments in neurons of the central and peripheral nervous system (Hirokawa et al., 1984; Liem, 1990). During neuronal differentiation there is a temporal regulation of the expression of the three neurofilament subunit genes. The NF-L (68 kDa) and NF-M (145 kDa) mRNAs and proteins accumulate during the embryonic period but detectable levels of the NF-H mRNA and protein are not observed until the post-natal period (Julien et al., 1986; Shaw and Weber, 1982; Willard and Simon, 1983).

As shown in Figure 5 and Figure 6, upon cultivation at the permissive condition of temperature the PrP$^{0/0}$ ML cell line was highly immunoreactive for nestin (Fig. 5A). However, the cells growing at 33°C were negative for neuronal antigens, such as the neuron-specific nuclear protein NeuN (Fig. 5B), the microtubule-associated protein (MAP)-2b (Fig. 6A) and the neuronal filament (NF)-H (data not shown), as well as for the astroglial marker glial fibrillary acidic protein (GFAP) (Debus et al., 1983) (data not shown), and the oligodendrocyte marker myelin basic protein (MBP) (Mirsky et al., 1980) (data not shown). Interestingly, maintenance of the cells in serum-free medium at 39°C for up to 96 hour,

conditions that promote cell differentiation, induced a progressive reduction in the expression levels of nestin (Fig. 5D, G), an event that strongly correlated with the appearance of NeuN (Fig. 5H and Table 1), MAP-2b (Fig. 6D, G, J and Table 1) and NF-H (data not shown). The PrP$^{0/0}$ ML cells thus resulted positive for MAP-2b after 24 hours cultivation in nonpermissive conditions (Fig. 6D, G; J and Table 1), and became immunopositive for NeuN (Fig. 5H and Table 1) and NF-H (data not shown) after 96 hours at the same conditions. The expression of markers for distinct subtypes of cerebellar neuronal populations, such as calbindin for the Purkinje cells and the gammaaminobutyric acid type A receptor α 6 subunit (GABAA Rα6) for the granule cells, however, remained negligible for up to 96 hours (data not shown). Similarly, antigens for other cell lineages, including GFAP for astrocytes and MBP for oligodendrocytes, were undetectable at all time-points (data not shown). Moreover, maintenance of the cells in serum-free medium at 39°C in the presence of brain-derived neurotrophic factor (BDNF) induced an acceleration in the process of neurodifferentiation, as shown by both the earlier detection and the stronger expression levels of MAP-2b and NeuN (Fig. 7).

Besides the expression of cell lineage-specific markers, the extent of cell differentiation can be figured out also by morphological criteria. Polarization of neuronal cells, with a single axon and several dendrites, is crucial for the proper function of neurons. This process normally occurs via the differentiation of one immature neurite to generate a single axon whilst the remaining neurites elongate to become dendrites. Therefore, neurite formation and specialization is essential to define the shape of neurons and their synaptic connections. Based on this, the morphological changes the PrP$^{0/0}$ ML cells undergo under the different conditions of culture were next examined in detail. After labelling the cells with a generic anti-MAPs antiserum that allows to appreciate both axons and dendrites by immunoreacting with the cytoskeletal proteins Tau (axonal protein) and MAP2 (dendritic protein), it was possible to note that, upon cultivation at 39°C in SFM for 48 or 96 hours, the PrP$^{0/0}$ ML cells began to acquire an asymmetrical morphology and extended longer neurites (Fig. 5F, I) than the same cells maintained at the permissive temperature (Fig. 5C). The neuritogenic response of the PrP$^{0/0}$ ML cells was subsequently quantified by measuring both the number of cells harbouring neurites and the mean length of the longest neurites per cell at different time-points upon cultivation in either permissive or nonpermissive growth conditions. As shown in Table 2, the proportion of cells with neurites was significantly lower after cultivation in the presence of serum at 33°C than in SFM at 39°C for 48 hours. Their number further increased from 48 to 96 hours at the nonpermissive temperature (Table 2). Similarly, the mean length of

the longest neurites per cell was about 50,9 ± 5,3 μm for the cells maintained at 33°C and gradually increased by 16% for cells grown at 39°C for 48 hours and by 44% for cells cultivated at the nonpermissive temperature for 96 hours (Table 2).

Thus, these results indicate that the PrP$^{0/0}$ ML cells are lineage determined precursor cells. The switch to nonpermissive conditions of maintenance then impels their differentiation toward a phenotype with biochemical features of neurons, though no antigenic traits of specific cerebellar neuronal subtypes are attained.

Table 1. Quantification of the percentage of cells expressing neuron - specific markers at different time - points under nonpermissive conditions of culture.

Culture conditions	39°C in SFM				
Genotype	PrP$^{0/0}$ ML		PrPA117		PrPA57**
Hours	48	96	48	96	48
MAP-2b$^+$ cells*	23,1 ± 2,6	28,8 ± 5,9	28,9 ± 5,5	25,1 ± 5,1	37,3 ± 3,8
NeuN$^+$ cells*	<1	17,3 ± 2, 6	17,0 ± 7,3	32,2 ± 9,8	36,1 ± 6,4

*Values are expressed as percentage ± s. e. m.
**PrPA57 cells showed a high cell death rate at the latest time- point with a significant reduction in the cell number. This did not allow comparing this line with the other two genotypes at 96 hours after the temperature switch.

Table 2. Quantification of the cell differentiation as determined by morphological analysis

	PrP$^{0/0}$ ML			PrPA57		PrPΔ32-134	
	33°C, 10% FCS	39°C, SFM		33°C, 10% FCS	39°C, SFM	33°C, 10% FCS	39°C, SFM
Hours		48	96		48		48
Cells with neurites*	6,5±3,1	27,8±3,4	41,7±12,1	17,7±3,8	47,0±3,4	15,7±5,1	35,1±0,8
Mean length of the longest neurites per cell**	50,9±5,3	58,9±4,7	73,6±6,6	71,7±2,5	75,6±5,1	71,2±2,6	73,6±5,7

*Values are expressed as percentage ± s. e. m.
**Values are expressed in μm ± s. e. m.
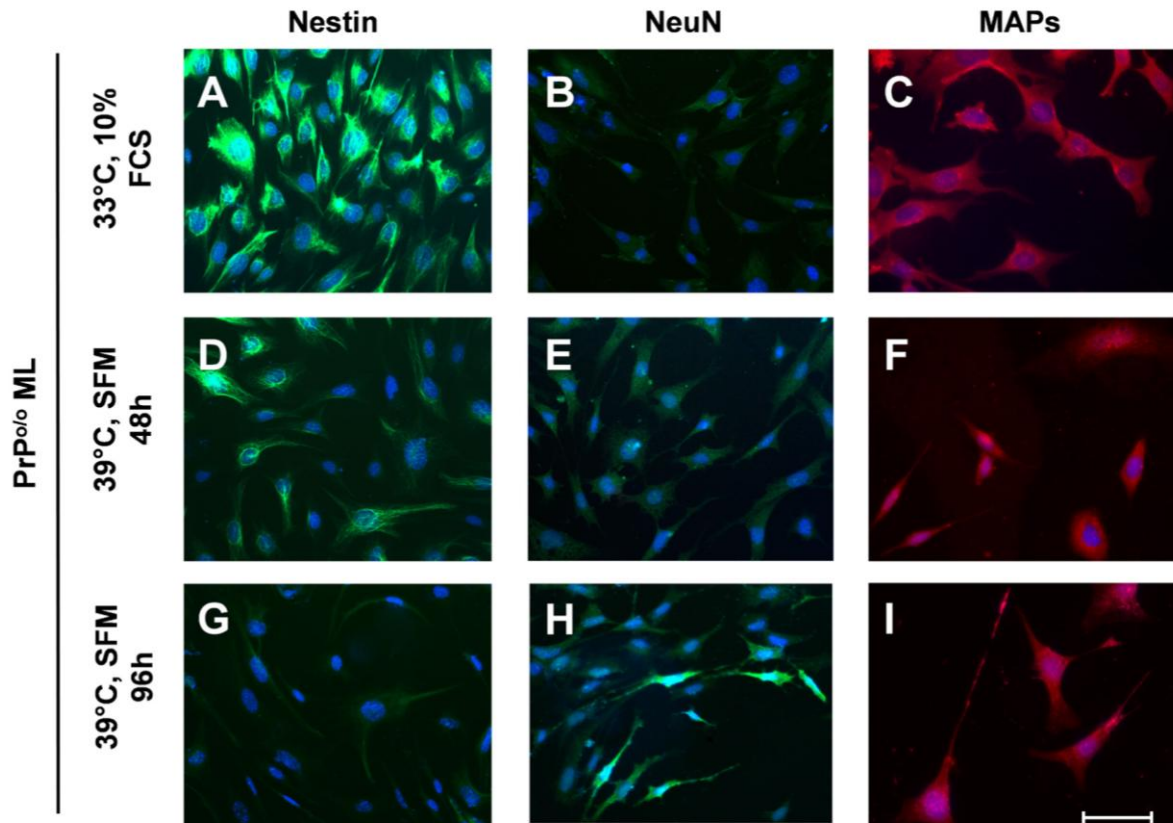
Figure 5. The PrP$^{0/0}$ ML cell line is a unipotent neuronal precursor line which can differentiate into neurons when cultivated under specific conditions

Immunofluorescence analyses of cell specific markers in PrP$^{0/0}$ ML cells cultivated under both permissive (A-C) and nonpermissive (D-I) conditions. PrP$^{0/0}$ ML cells maintained in permissive conditions were immunoreactive for nestin (A) and negative for neuron-specific markers, such as the neuronal nuclei antigen (NeuN, B). Immunostaining for MAPs revealed the polygonal morphology of the cells and the presence of rare and short processes (C). Under conditions that promote cell differentiation, a time-dependent expression of NeuN (E and H) accompanied the reduction in the expression levels of nestin (D and G). This switch towards a neuronal phenotype is accompanied by progressive neuritogenesis, as revealed by anti-MAPs immunolabelling (F and I). Cell nuclei were visualized by Hoechst 33342 staining (blue). Scale bar: 50 µm
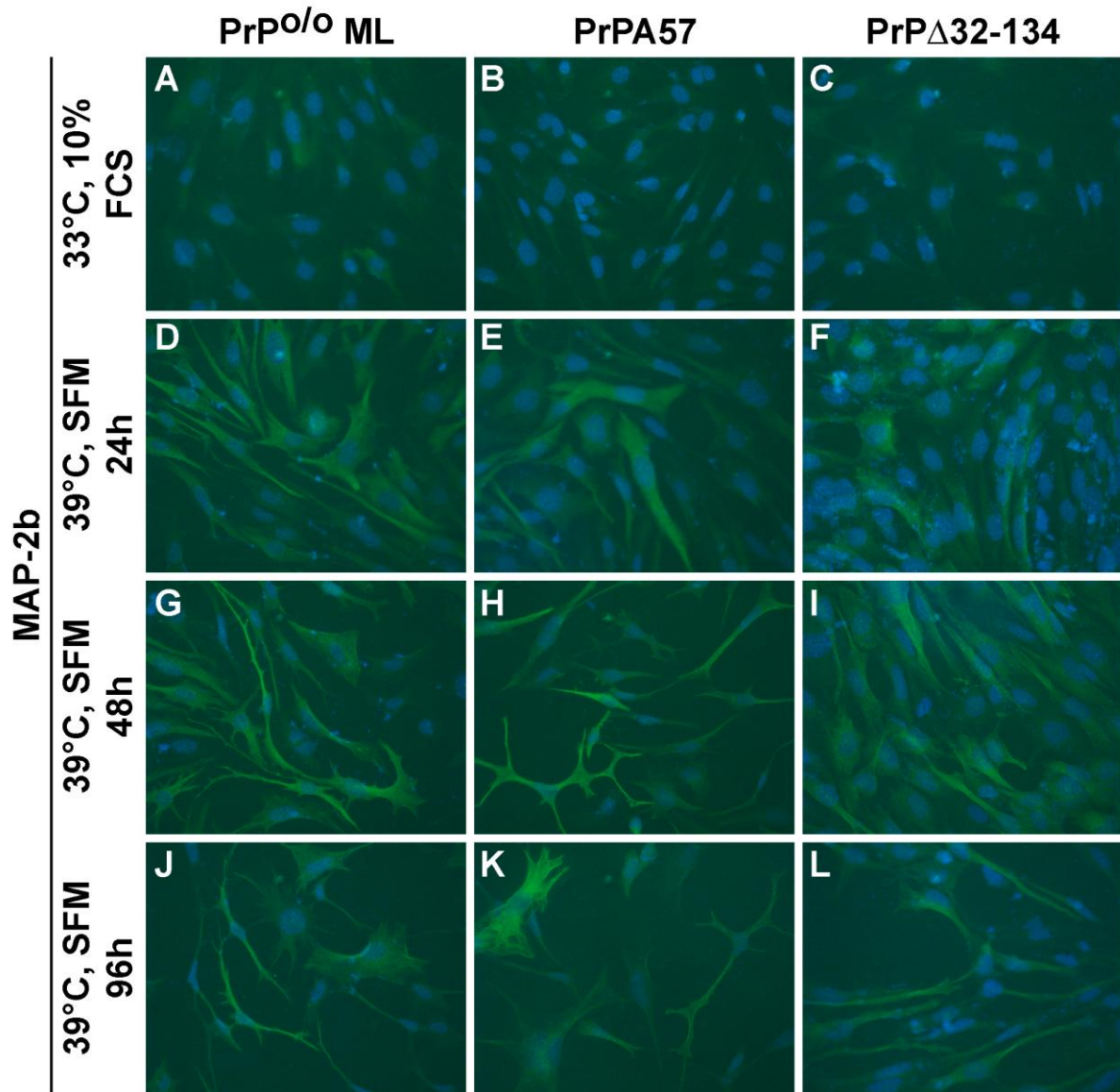
Figure 6. Time-dependent expression of the neuronal marker MAP-2b in the PrP$^{0/0}$ ML, PrPA57 and PrPΔ32-134 cell lines

Immunofluorescence analyses of the neuronal marker MAP-2b in the PrP$^{0/0}$ ML, PrPA57 and PrPΔ32-134 cells cultivated under both permissive (A-C) and nonpermissive (D-L) conditions. PrP$^{0/0}$ ML (A), PrPA57 (B) and PrPΔ32-134 cells (C) maintained in permissive conditions were negative for MAP-2b. Under conditions that promote cell differentiation, a time-dependent expression of MAP-2b in all the three cell lines was observed (D-L). Cell nuclei were visualized by Hoechst 33342 staining (blue).
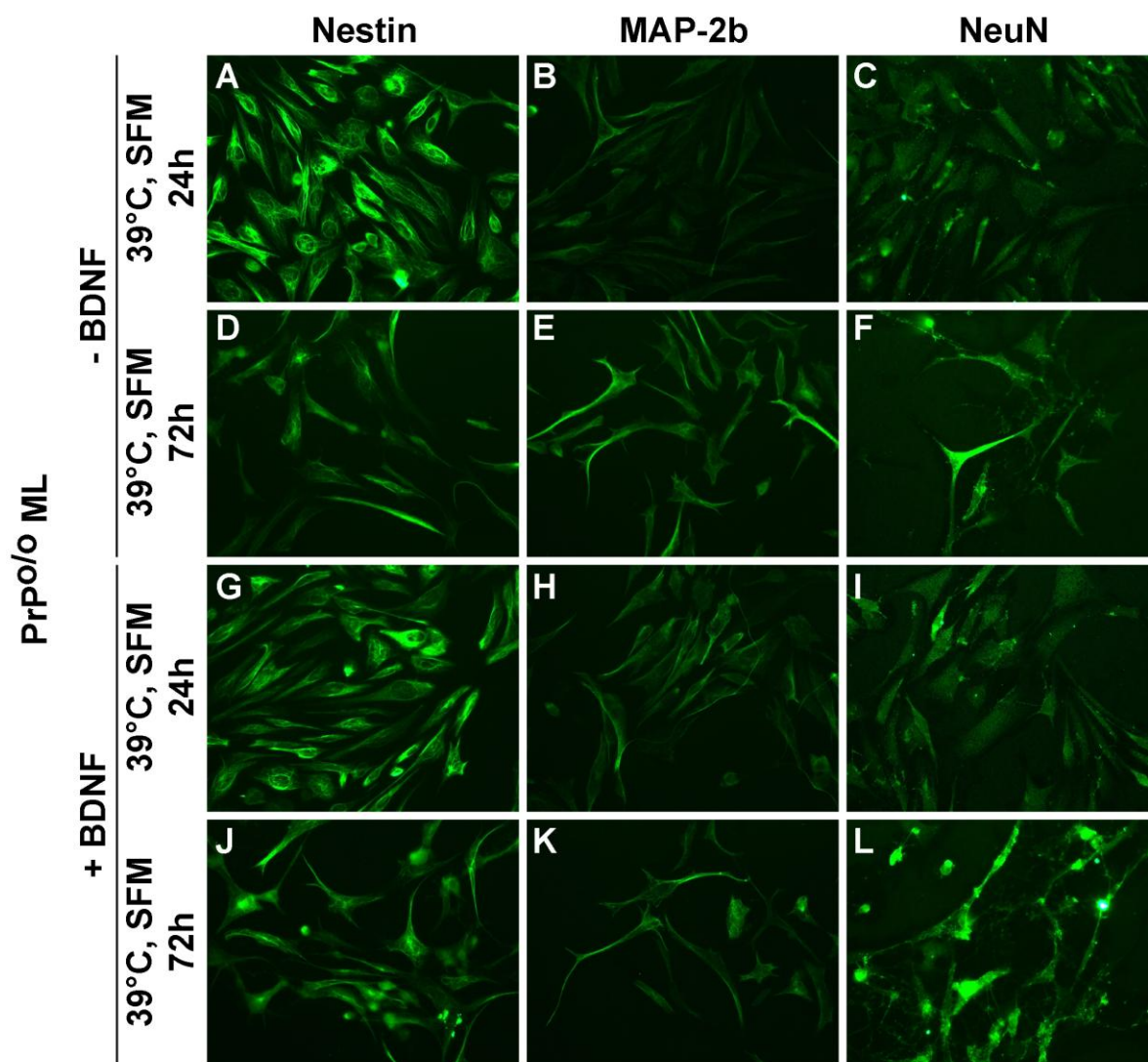
Figure 7. Brain derived neurotrophic factor accelerates the differentiation process of the PrP$^{0/0}$ ML cell line

Immunofluorescence analyses of cell specific markers in PrP$^{0/0}$ ML cells cultivated under nonpermissive conditions both in the absence (A-F) and presence (G-L) of BDNF. PrP$^{0/0}$ ML cells maintained in nonpermissive conditions for 24h were immunoreactive for nestin (A) and negative for neuron-specific markers, such as MAP-2b (B) and NeuN (C). Under conditions that promote cell differentiation for 72h, expression of MAP-2b (E) and NeuN (F) accompanied the reduction in the expression levels of nestin (D). The presence of BDNF accelerates the switch towards a neuronal phenotype as revealed by the earlier and stronger expression of the neuronal markers MAP-2b (H and K) and NeuN (I and L).

4.1.4 Reconstitution of the PrP$^{0/0}$ ML cell line with the full-length prion protein or PrPΔ32-134

In spite of considerable experimental efforts invested during the last decade, the cellular function of the prion protein remains obscure. To generate a useful cell system for studying the physiological role of this enigmatic protein, the expression of either wild-type PrP^C or its truncated form PrPΔ32-134 was reconstituted into the PrP$^{0/0}$ ML cells.

4.1.4.1 Construction of the pPrP-IRESpuro or the pPrPΔ32-134-IRESpuro vector

The bicistronic expression vector pIRESpuro2 (Clontech, USA) was chosen to drive expression of the murine *Prnp* gene in the PrP$^{0/0}$ ML cells. In this vector, the transcriptional regulation of the transgene is under the control of the human cytomegalovirus (CMV) major immediate early promoter/enhancer. A multiple cloning site sequence (MCS) that precedes synthetic stop codons in all the three reading frames allows insertion of the gene of interest. A synthetic intron known to enhance the stability of the mRNA, the encephalomyocarditis virus (ECMV) internal ribosome entry site (IRES) sequence, a sequence encoding for the puromycin-N-acetyl-transferase, a protein which confers puromycin resistance to cells, and the polyadenylation signal of the bovine growth hormone gene complete the expression cassette of the pIRESpuro2 vector (Fig. 8). Ribosomes can enter the bicistronic mRNA at the 5´end to translate the gene of interest and at the ECMV IRES to translate the antibiotic resistance marker. Therefore, the selection of transfected cells with puromycin ensures a stable expression of the gene of interest over time in culture.

For the construction of the pPrP-IRESpuro vector, the sequence encoding for the full-length PrP (PrP ORF) was inserted into the multiple cloning site of the pIRESpuro2 vector. A 765 bp PCR product of the PrP ORF was amplified using the pPrPHG plasmid (Fischer et al., 1996) as template and the primers 5'-P-ORF and 3'-P-ORF (see Materials and Methods). The amplicon was cleaved with EcoRI and BamHI and cloned into the EcoRI-BamHI-digested pIRESpuro2 backbone to yield the plasmid pPrP-IRESpuro (Fig. 9A). The PrPΔ32-134 ORF was prepared by PCR using the same primers and the pPrPHG.F vector (Shmerling et al., 1998) as template. The PCR product was then ligated into the pIRESpuro2 vector as described above to yield the pPrPΔ32-134-IRESpuro construct (Fig. 9A). Resulting clones were screened for the presence of the *Prnp* fragments by restriction analysis. The entire open reading frames of two positive clones for each expression vector were analysed by sequencing to exclude the presence of mutations.
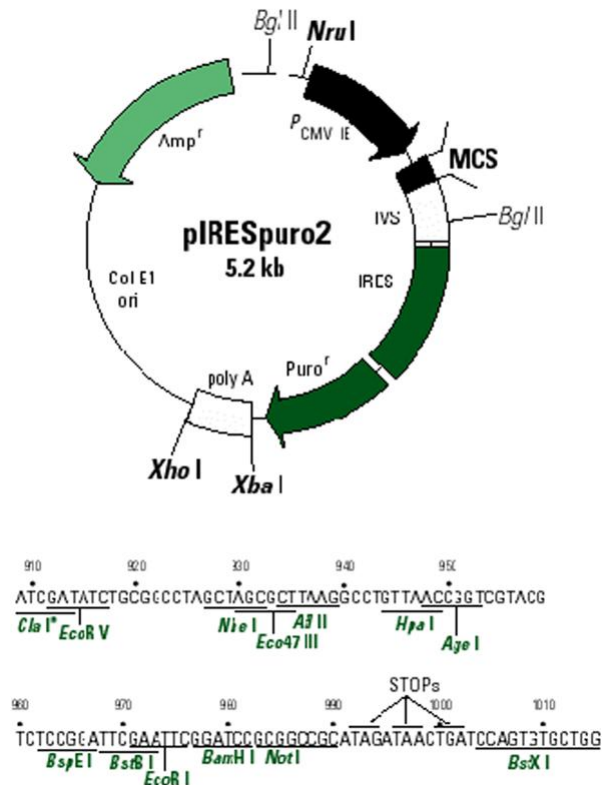
Figure 8. Schematic representation of the plasmid pIRESpuro2

Schematic representation of the plasmid pIRESpuro2 used for construction of the pPrP-IRESpuro and pPrPΔ32-134-IRESpuro transgenic expression vectors. The nucleotide sequence of the multiple cloning site (MCS) region in which the PrP coding sequences were inserted is also depicted. Positions of restriction enzymes' recognition sites are illustrated.

4.1.4.2 Generation of full-length prion protein- or PrPΔ32-134-reconstituted PrP$^{0/0}$ ML cells

PrP$^{0/0}$ ML cells were transfected by lipofection (see Materials and Methods) with the pPrP-IRESpuro or the pPrPΔ32-134-IRESpuro vectors (Fig. 9A), respectively, and clones that stably expressed the protein of interest were isolated. Furthermore, PrP$^{0/0}$ ML cells stably transfected with an empty vector were generated as controls for possible insertional effects of the transgene. Cells transfected with the plasmid coding for the full-length PrP^{C} produced several clones. Three of these, named PrPA117, PrPA57 and PrPA109 (data not shown), were chosen as representative of low, average and high PrP^{C} expression levels, respectively. The PrPA57 line was taken into particular consideration, due to morphology and growth rate comparable with PrP$^{0/0}$ ML cells, and was extensively characterized. By contrast, cells transfected with the plasmid coding for PrPΔ32-134 produced only one clone with high levels of PrP expression. However, additional PrPΔ32-134 expressing clones may have been missed

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during the screening procedure because of the use of a weakly reacting antibody against the truncated PrP form (data not shown). Expression of the proteins in all cell lines was confirmed by Western blotting (Fig. 9B) and their presence on the outer surface of cell clones, because of the GPI-anchor, was detected by FACS analysis (Fig. 9C). Considering that PrPΔ32-134 induced degeneration of cerebellar neurons *in vivo* when expressed into a $PrP^{0/0}$ background (Flechsig et al., 2003; Shmerling et al., 1998), one investigated whether the truncated PrP similarly caused detrimental effects on the $PrP^{0/0}$ ML cells. Surprisingly, the reconstitution of the $PrP^{0/0}$ ML cells with PrPΔ32-134 was not cytotoxic up to 48 hours in culture at both permissive and nonpermissive conditions, as demonstrated by the activity of the lactate dehydrogenase (LDH) released into the culture media (Fig. 10).
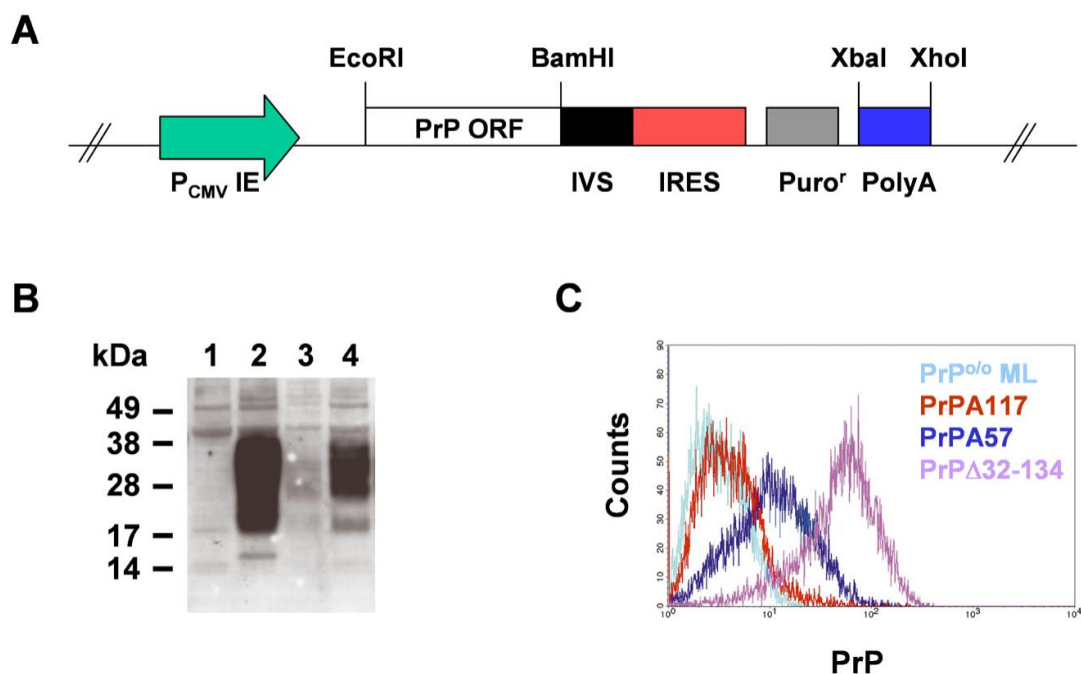
Figure 9. Reconstitution of wild-type PrP^C and PrPΔ32-134 expression in the $PrP^{0/0}$ ML cells

Cells were stably transfected with expression vectors encoding either the wild-type PrP^C or the amino-terminally deleted protein PrPΔ32-134. Clones that stably expressed the protein of interest were isolated and characterized. (A) Schematic representation of the plasmid pPrP-IRESpuro that codes for either the full-length PrP^C or PrPΔ32-134. (B) PrP expression was evaluated on the reconstituted cell lines by immunoblot using the 6H4 monoclonal antibody. The cell lines PrPA117 (lane 3) and PrPA57 (lane 4) express wild-type PrP^C at different levels whereas PrPΔ32-134 line (lane 2) expresses the N-terminally truncated form of PrP. As expected, no PrP^C expression is detected in $PrP^{0/0}$ ML cells (lane 1). (C) FACS analysis for cell surface PrP^C-expression using the 6H4 monoclonal antibody. PrP^C–expression of the different reconstituted cell lines is directly compared to the negative control, i.e. the $PrP^{0/0}$ ML cells.
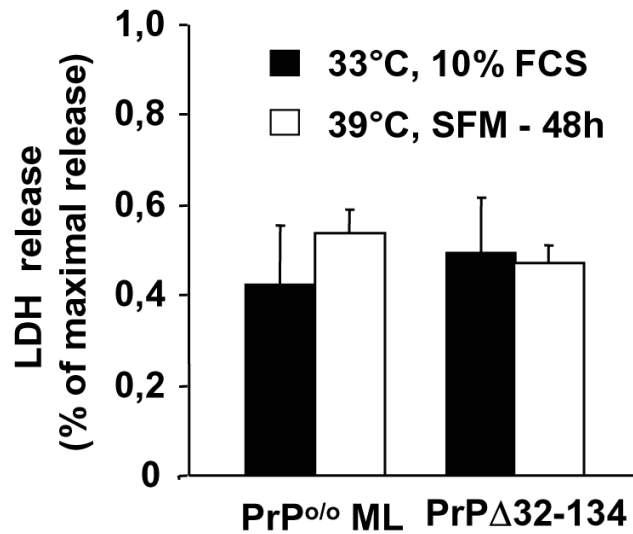
Figure 10. Expression of the N-terminally truncated PrP^C is not cytotoxic for the PrP$^{o/o}$ ML cells

The release of LDH in the supernatant of PrP$^{0/0}$ ML and PrPΔ32-134 cells cultivated either at 33°C in 10%FCS or at 39°C in SFM was monitored 48 hours after the temperature switch. The percentage of LDH release was obtained by comparing to the maximal release of cells treated with 1% Triton-X 100. No significant difference was detected between each experimental group ($p < 0.05$, ANOVA followed by Scheffè's F-test). Values are the mean ± s.e.m. of four independent experiments.

4.1.5 Neuronal differentiation process in PrP- and PrPΔ32-134-reconstituted PrP$^{0/0}$ ML cells

A line of evidence suggests that PrP^C plays a role in cell differentiation, neurite outgrowth and synaptogenesis likely via the interaction with NCAMs, laminin or STI1 (Chen et al., 2003; Graner et al., 2000a; Kanaani et al., 2005; Lopes et al., 2005; Santuccione et al., 2005; Steele et al., 2006). Thus, in order to investigate whether the introduction of either wild-type PrP^C or its amino-terminally deleted form PrPΔ32-134 affected the differentiation potential of the PrP$^{0/0}$ ML cells, immunocytochemical analyses, aiming to visualize both the expression of neuron-specific antigens and the structural changes cells undergo under different growth conditions, were performed on the PrPA57 and PrPΔ32-134 cell lines. First, the expression of the CNS precursor marker nestin was evaluated and it was found that, at 33°C in serum-containing medium, this protein was present in both the PrPA57 and PrPΔ32-134 cell lines (Fig. 11A, G), although at lower levels than in the PrP$^{0/0}$ ML cells (Fig. 5A). This difference in nestin expression could be possibly due either to the fortuitous selection of clones that originally expressed lower levels of the intermediate filament protein or to a possible role of PrP in cell differentiation, which causes a reduction in nestin expression even under normal

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growth conditions. As described for the PrP$^{0/0}$ ML cell line, the partial removal of the serum in association with the shift of temperature to 39°C for 48 hours caused a strong downregulation of the neuronal progenitor marker in both cell lines (Fig. 11D, J). Similarly to the PrP knockout cells, this reduction in nestin expression was accompanied by the appearance of the NeuN (Fig. 11E, K and Table 1) and MAP-2b (Fig. 6E, H, K, Fig. 6F, I, L and Table 1) antigens in both cell types. However, while the PrP$^{0/0}$ ML cells expressed NeuN only after 96 hours from the growth condition switch (Fig. 5H and Table 1), the PrP-expressing cells showed NeuN expression already at 48 hours (Fig. 11E, K and Table 1). Furthermore, an incremented proportion of cells expressing the neuronal marker MAP-2b was observed in the PrP-expressing lines when compared to the PrP$^{0/0}$ ML cells (Fig. 6E, H, K, Fig. 6F, I, L and Table 1).

Neuronal differentiation in PrP$^{0/0}$ ML and PrPA57 cells was further investigated by monitoring the expression levels of nestin, MAP-2b (Fig 12A-B) and NF-H (data not shown) at 24 hours and 96 hours (Fig. 12C-D) after growth conditions switch by immunoblot analyses. As shown in Figure 12, Nestin expression in PrPA57 cells was reduced as compared to the PrP$^{0/0}$ ML cells under all the four cell growth conditions analyzed. However, nestin expression levels detected in both cell lines at 24 hours of cultivation did not differ significantly among the different culture conditions used (Fig. 12A). On the other hand, prolonged cultivation at the nonpermissive temperature of 39°C caused a marked decrease in Nestin expression as compared to the cells grown at the permissive temperature: the downregulation in the PrPA57 cells was so strong that no signal was detectable anymore by immunoblot analysis (Fig. 12C). MAP-2b expression was detected as early as 24 hours of cultivation in both cell lines. However the expression levels in PrPA57 cell were higher than those detected in the PrP$^{0/0}$ ML cells (Fig. 12B). After 96 hours of cultivation, MAP-2b expression was upregulated in PrP$^{0/0}$ ML cells grown under both low serum and nonpermissive conditions, indicating that the neuronal precursor cells were in the process of differentiation. In contrast, PrPA57 cells expressed MAP-2b only at low serum concentration and 33°C but not if they were cultivated at the nonpermissive temperature (Fig. 12D). Furthermore, the expression of the late neuronal antigen NF-H was not detected at the 24 hours time point in both cell lines and under all the four conditions of cultivation (data not shown). However, NF-H expression was specifically detected in PrPA57 cells cultivated at the nonpermissive temperature for 96 hours but not in the PrP$^{0/0}$ ML cells (data not shown).

Noteworthy, comparative analyses of lines $PrP^{0/0}$ ML, PrPA117 and PrPA57 revealed a positive correlation between the degree of PrP^C expression and the number of cells expressing both NeuN and MAP-2b (Table 1). Moreover, it is interesting to note that $PrP^{0/0}$ ML cells stably transfected with an empty vector ($PrP^{0/0}$ ML EV cells) differentiated into mature neurons under nonpermissive conditions with the same kinetic as the parenteral cells (Fig. 13). Therefore, the identified effect cannot be ascribed to the accidental selection of PrP-expressing clones with fast-differentiating properties but it is rather the prion protein that facilitates the process of neuronal differentiation of the PrP knockout precursor cells.

To determine whether the switch to nonpermissive culture conditions influenced also the structure of the cells, one decided to analyze the morphology of both the PrPA57 and PrPΔ32-134 cell lines and to compare them to the $PrP^{0/0}$ ML cells. Immunostaining analyses performed with an anti-MAPs antiserum (that points out axons and dendrites) revealed that, at 33°C in serum-containing medium, some of the cells of both reconstituted cell lines already showed a polar morphology (Fig. 11C, I). Consistent with this, the proportion of cells with neurites resulted about three-fold higher than that of the cells lacking the prion protein (Table 2). Such effect was reflected also on the length of the processes as both cell lines showed about 30% increase in the mean length of the maximal neurites per cell when compared to the $PrP^{0/0}$ ML cells maintained in the same conditions of culture (Table 2). Switching the cells to 39°C in SFM for 48 hours dramatically incremented the process of neuritogenesis, as demonstrated by the fact that the number of PrPA57 cells harbouring neurites increased significantly (Table 2). Also the expression of PrPΔ32-134 boosted the proportion of cells with processes, but to a lower extent (Table 2). By contrast, no difference was seen in the mean length of the longest neurites per cell in both lines when compared to the same cells grown at 33°C in the presence of serum (Table 2).

Altogether, these data indicate that the expression of PrP^C propels differentiation to the neuronal phenotype and neuritogenesis even under permissive conditions of culture. The switch of temperature associated with serum withdrawal further accelerates the process. The deletion of the N-terminal domain of the cellular prion protein does not impair this activity, although it makes the event less efficient.
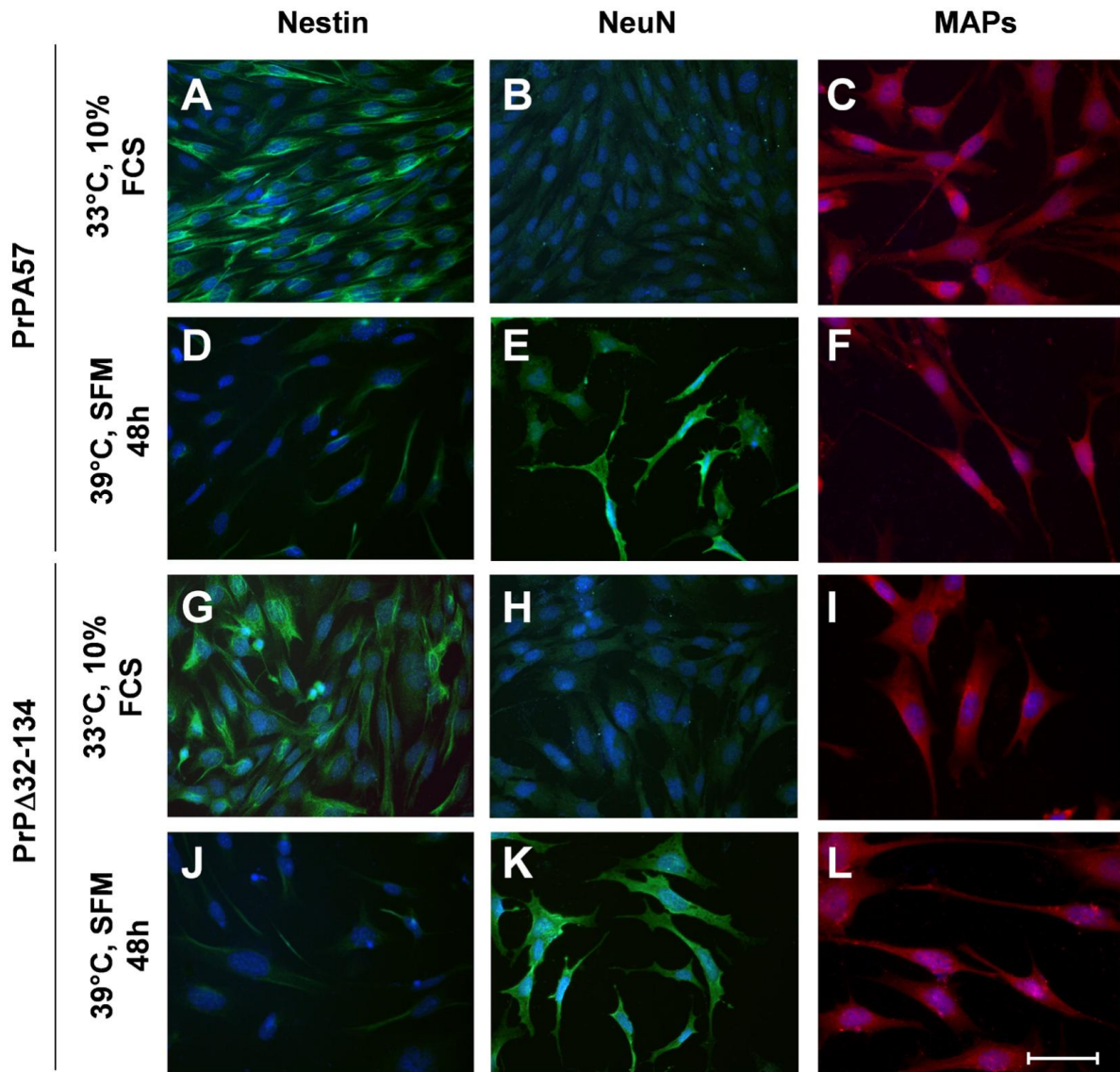
Figure 11. Accelerated neuronal differentiation of the cells expressing the wild-type or the truncated PrP[C]

Immunofluorescence analyses of cell specific markers in the PrPA57 and PrPΔ32-134 cells cultivated under both permissive (A-C and G-I, respectively) and nonpermissive (D-F and J-L, respectively) conditions. At the permissive temperature, both the PrPA57 and PrPΔ32-134 cell lines were immunoreactive for nestin (A and G) but were negative for the neuronal marker NeuN (B and H). The morphological remodelling of the cytoskeleton was appreciated by anti-MAPs immunostaining (C, F, I, L). This revealed more numerous cells with neuritis as well as the presence of longer processes per cell (C and I) when compared to the knockout counterpart (Fig. 2C). In nonpermissive conditions, a strong downregulation of nestin was observed 48 hours after the switch of temperature (D and J). At the same time-point, the specific neuronal marker NeuN was detectable in both the PrPA57 (E) and PrPΔ32-134 cell lines (K). Anti-MAPs immunostainings revealed an increase in the proportion of cells with processes (F and L) when compared to the permissive conditions of culture. Cell nuclei were visualized by Hoechst 33342 staining (blue). Scale bar: 50 μm.
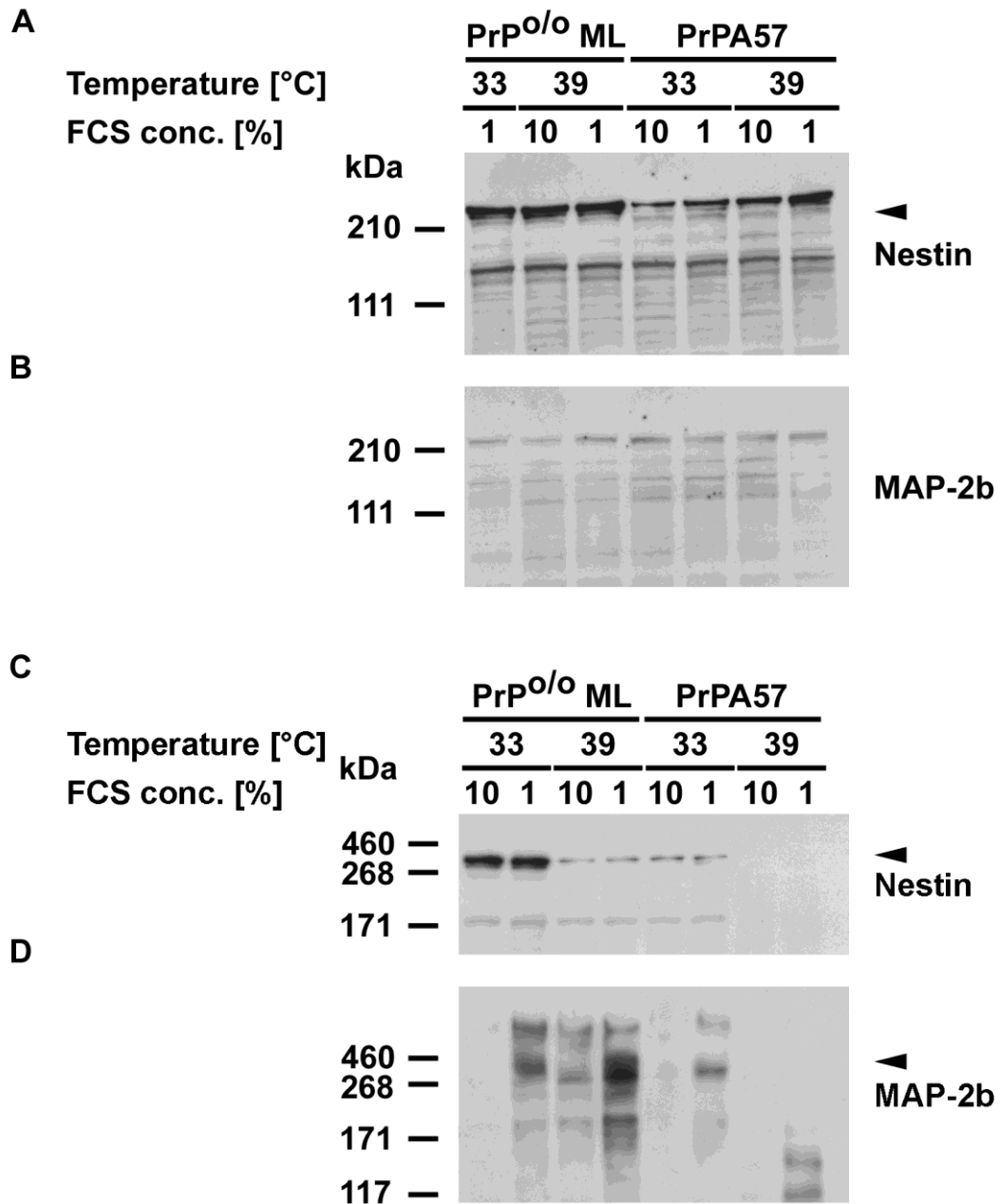
Figure 12. Upregulation of the neuronal marker MAP-2b after neuronal differentiation induction

Immunoblot analyses of cell specific markers in the PrP$^{0/0}$ ML and PrPA57 cells cultivated under both permissive and nonpermissive conditions for either 24 hours (A and B) or for 96 hours (C and D). At the permissive temperature, both the PrP$^{0/0}$ ML and PrPA57 cell lines were immunoreactive for nestin (220 kDa) (A) but were negative for the neuronal marker MAP-2b (280kDa) (B).Twenty-four hours of cultivation under nonpermissive conditions were not sufficient for changing the expression pattern of both neuronal markers (A and B). In nonpermissive conditions, a strong downregulation of nestin was observed 96 hours after the switch of temperature (C). At the same time-point, the early specific neuronal marker MAP-2b was detectable in the PrP$^{0/0}$ ML but not in the PrPA57 (D), which were accelerated in the neuronal differentiation process.

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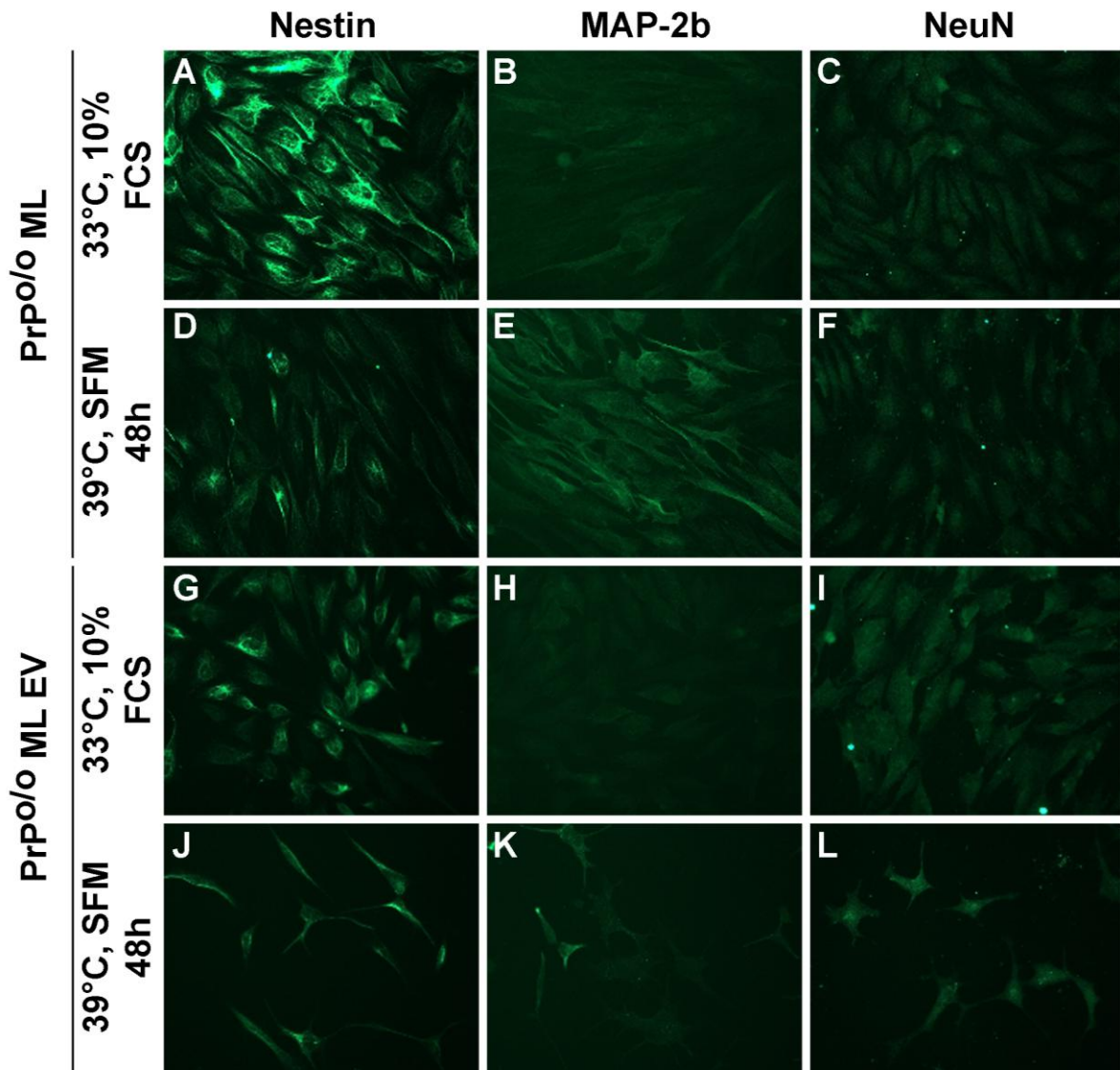
Figure 13. PrP$^{0/0}$ ML EV cells differentiate into mature neurons with the same kinetic as the parenteral PrP$^{0/0}$ ML cell line

Immunofluorescence analyses of cell specific markers in the PrP$^{0/0}$ ML and PrP$^{0/0}$ ML EV cells cultivated under both permissive (A-C and G-I, respectively) and nonpermissive (D-F and J-L, respectively) conditions. At the permissive temperature, both the PrP$^{0/0}$ ML and PrP$^{0/0}$ ML EV cell lines were immunoreactive for nestin (A and G) but were negative for the neuronal markers MAP-2b (B and H) and NeuN (C and I). In nonpermissive conditions, a strong downregulation of nestin was observed 48 hours after the switch of temperature (D and J). At the same time-point, the specific neuronal marker MAP-2b was detectable in both the PrP$^{0/0}$ ML (E) and PrP$^{0/0}$ ML EV cell lines (K) but not the neuronal marker NeuN (F and L).

4.1.6 Activation of p59Fyn kinase in PrP-mediated neuronal differentiation

Nonreceptor protein tyrosine kinases of the Src family regulate the survival, proliferation, differentiation and motility of many cell types (Erpel and Courtneidge, 1995; Kalia et al., 2004; Parsons and Parsons, 1997; Parsons and Parsons, 2004). The p59Fyn kinase, a member of this family (Resh, 1998) has been reported to play a role in PrP-mediated neurite outgrowth, neuronal polarization and neuronal survival (Chen et al., 2003; Kanaani et al., 2005; Mouillet-Richard et al., 2000; Santuccione et al., 2005). In order to assess whether the observed PrP-mediated acceleration of neuronal differentiation in the PrP$^{0/0}$ ML cells involves the activation of the p59Fyn kinase, the activation state of this kinase by specific detection of the p59Fyn inactive form after immunoprecipitation was determined. As shown in Fig. 14, in PrPA57 and PrPΔ32-134 cells the levels of Tyr-416 dephosphorylated p59Fyn decreased as compared to the PrP$^{0/0}$ ML cells already when the cells were cultivated under permissive conditions, indicating that in these two cell lines the steady-state activation of the p59Fyn kinase was increased. When neuronal differentiation was induced by switching the growth conditions for 72 hours, strong reduction in non-phosphorylated p59Fyn kinase levels was detected in the PrPA57 cells. On the other hand, no change was detected in the phosphorylation state of p59Fyn kinase in both PrP$^{0/0}$ ML and PrPΔ32-134 cells under nonpermissive conditions as compared to the activation state observed under permissive conditions. To further assess the role of p59Fyn kinase signal pathways in PrP-mediated acceleration of neuronal differentiation, PP1, an inhibitor of the Src kinase family, was tested for its ability to inhibit the effect on neuronal differentiation of both wild type PrP^{C} and Δ32-134 PrP. PP3 was included as negative control. Immunofluorescence analyses showed that 5 μM of PP1 inhibited but not completely blocked the enhancing effects of PrP on the differentiation of PrP$^{0/0}$ ML cells under nonpermissive conditions (Fig.15). Altogether, these data indicate that PrP-mediated neurodifferentiation in PrP$^{0/0}$ ML cells implies the activation of the p59Fyn kinase signal pathways.
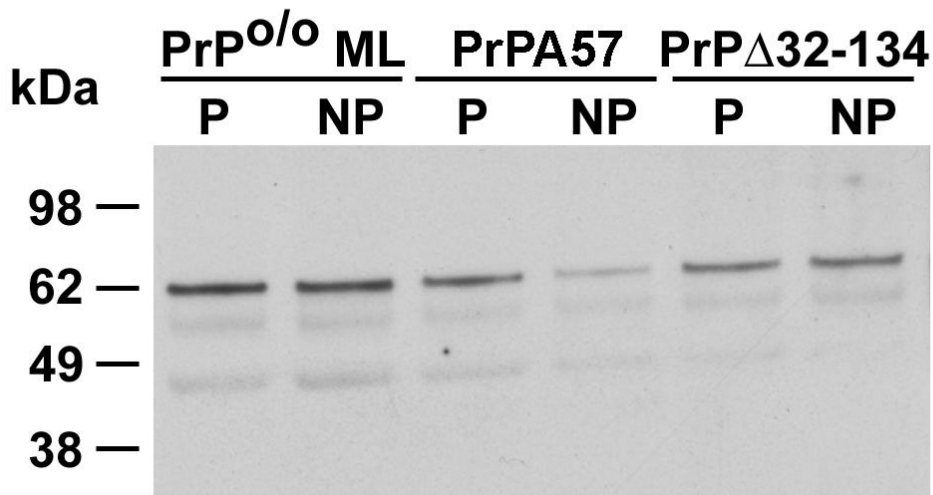
Figure 14. Temperature- and growth factor-dependent activation of the tyrosine kinase p59Fyn

Activation state of the p59Fyn kinase was determined in the PrP$^{0/0}$ ML, PrPA57 and PrPΔ32-134 cell lines cultivated under both permissive (P) and nonpermissive conditions (NP).The activation state of p59Fyn was determined by the specific detection of the p59Fyn inactive form after immunoprecipitation. A specific antibody recognizing the non-phosphorylated Tyr-416 was used. Under permissive conditions, a reduction in the levels of inactive p59Fyn was observed in both the PrPA57 and PrPΔ32-134 cell lines as compared to the PrP$^{0/0}$ ML cell line. When neuronal differentiation was induced by switching the growth conditions for 72 hours, a strong reduction in non-phosphorylated p59Fyn kinase levels was detected in the PrPA57 cells. On the other hand, no change was detected in the phosphorylation state of p59Fyn kinase in both PrP$^{0/0}$ ML and PrPΔ32-134 cells under nonpermissive conditions as compared to the activation state observed under permissive conditions.
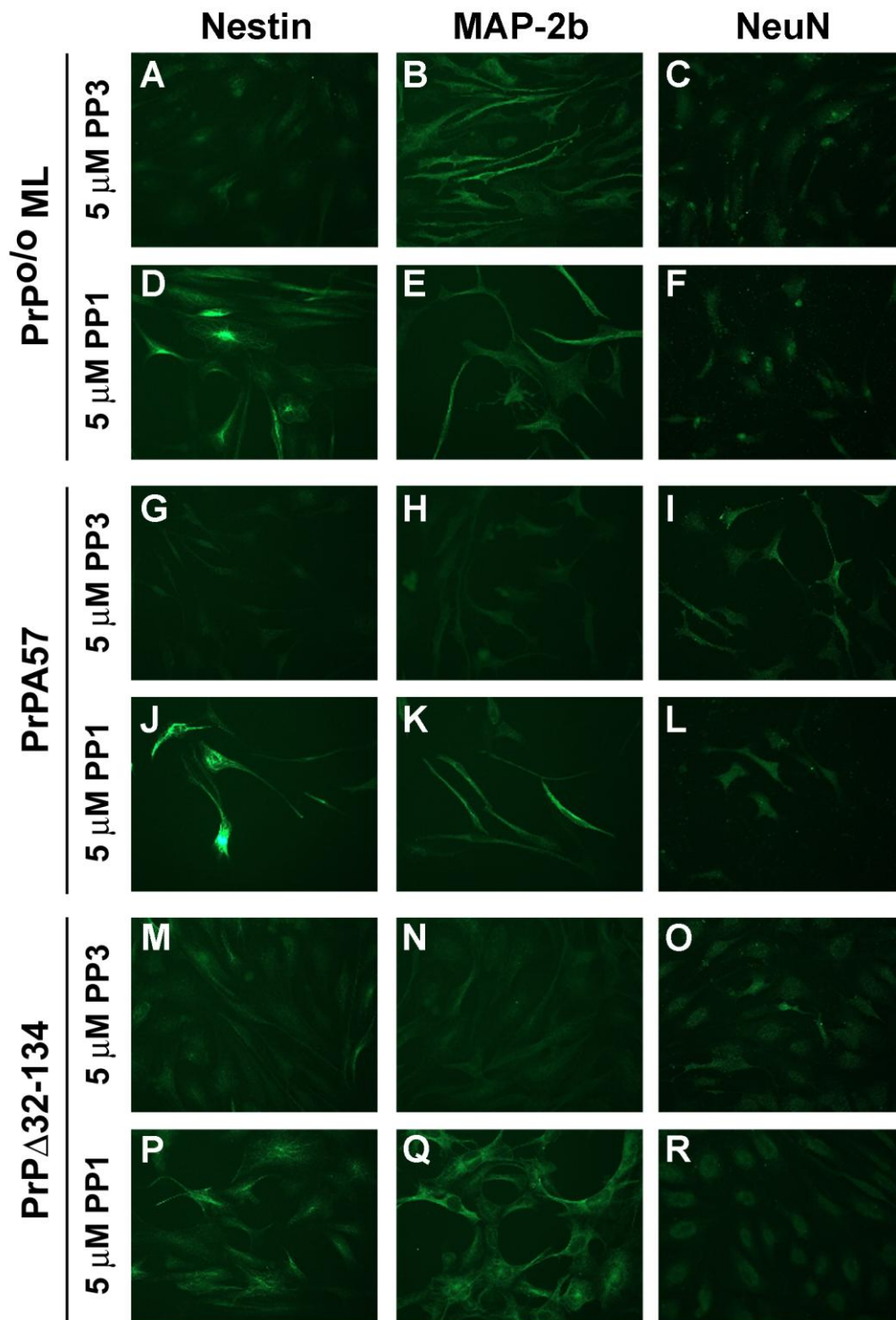
Figure 15. Partial inhibition of PrP^C-mediated neuronal differentiation by the p59Fyn kinase inhibitor PP1

Immunofluorescence analyses of cell specific markers in the PrP$^{0/0}$ ML, PrPA57 and PrPΔ32-134 cells cultivated under nonpermissive conditions for 48 hours (A-F, G-L and M-R, respectively). The addition of the inhibitor of the Src kinase family PP1 (5 μM) (D-F, J-L and P-R, respectively), but not of the control compound PP3 (A-C, G-I and M-O, respectively), inhibited but not completely blocked the enhancing effects of PrP on the differentiation of PrP$^{0/0}$ ML cells under nonpermissive conditions as shown by increased levels of the markers nestin (D, J and P, respectively) and MAP-2b (E, K and Q) and decreased levels of late neuronal marker NeuN (F, L and R). These data indicate that PrP^C-mediated neurodifferentiation in PrP$^{0/0}$ ML cells implies the activation of the p59Fyn kinase signal pathways.

4.1.7 Neural cell adhesion molecule (NCAM) and PrP-mediated neuronal differentiation

The neural cell adhesion molecule (NCAM) is a cell surface glycoprotein belonging to the cell adhesion molecules (CAMs) of the immunoglobulin (Ig) superfamily. NCAM is widely expressed during embryonic development, whereas it is found mainly in tissues of neuronal origin in the adult organism. NCAM exists in several isoforms, due to alternative splicing of a single gene, consisting of at least 26 exons. Three major isoforms are expressed: two transmembrane isoforms, NCAM 180 and NCAM 140, with apparent molecular weights of 180 kDa and 140 kDa, respectively, and a GPI-anchored isoform, NCAM 120, with an apparent molecular weight of 120 kDa (Barthels et al., 1992; Dickson et al., 1987; Jorgensen and Bock, 1974; Owens et al., 1987; Small et al., 1988; Thompson et al., 1989). In the vertebrate nervous system, NCAM is the dominant carrier of polysialic acid (PSA). Through its extracellular region, NCAM mediates both homophilic and heterophilic, cis and trans interactions. NCAM and its polysialylated form PSA-NCAM have been found to be involved in various aspects of neuronal and synaptic plasticity and play a major role during development of the nervous system. Due to its negative charge and large hydrated volume, PSA on NCAMs attenuates cell adhesion and is being studied for its ability to intervene in dynamic cellular changes (Theodosis et al., 1999). There is much evidence to support its role in the developing CNS in which PSA-NCAM has been shown to intervene in cell migration (Thiery et al., 1982), neurite outgrowth (Cremer et al., 1997; Fraser et al., 1984; Silver and Rutishauser, 1984; Thanos et al., 1984) and axonal fasciculation (Cremer et al., 1997; Thiery et al., 1982) (for reviews, Bruses and Rutishauser, 2001; Rougon and Hobert, 2003). The expression of PSA-NCAM markedly decreases during embryonic development and in the adult brain, PSA-NCAM is only expressed in areas which retain a high degree of plasticity (Chuong and Edelman, 1984; Rothbard et al., 1982).

Noteworthy, it has previously been shown that PrP^C and NCAM not only associate with each other at the surface of hippocampal neurons, but also directly interact with each other (Santuccione et al., 2005). Furthermore, PrP^C recruits to and stabilizes the transmembrane NCAM isoforms in lipid-rich microdomains to activate the p59Fyn kinase and promote NCAM-mediated neurite outgrowth by cis and trans interactions (Santuccione et al., 2005).

In order to assess whether PrP-mediated acceleration of neuronal differentiation and neuritogenesis in the PrP$^{0/0}$ ML cells involves the regulation of PSA-NCAM expression, the

PSA-NCAM expression patterns in the PrP$^{0/0}$ ML and PrPA57 cells under both the permissive and nonpermissive culture conditions for 48 and 72 hours were determined by FACS analyses. As shown in Figure 16, PSA-NCAM levels were higher in the PrP$^{0/0}$ ML cells as compared to the PrPA57 cells under permissive (Fig 16A) as well as under nonpermissive (Fig. 16 B) culture conditions. Interestingly, the switch to nonpermissive culture conditions increased the PSA-NCAM expression levels in the PrP$^{0/0}$ ML cells but not in the PrPA57 cells. As mentioned above (see 4.1.5), expression of PrP^{C} propels differentiation to the neuronal phenotype and neuritogenesis even under permissive conditions of culture. Therefore, the observed difference in PSA-NCAM levels between the PrP$^{0/0}$ ML and the PrPA57 cells could be ascribed to the neuronal differentiation-induced downregulation of PSA-NCAM expression. Being PSA-NCAM also a common marker used to identify immature neurons (Kempermann et al., 2004), these data further reinforce the finding that the PrP$^{0/0}$ ML cells are neuronal precursor cells (see 4.1.3).
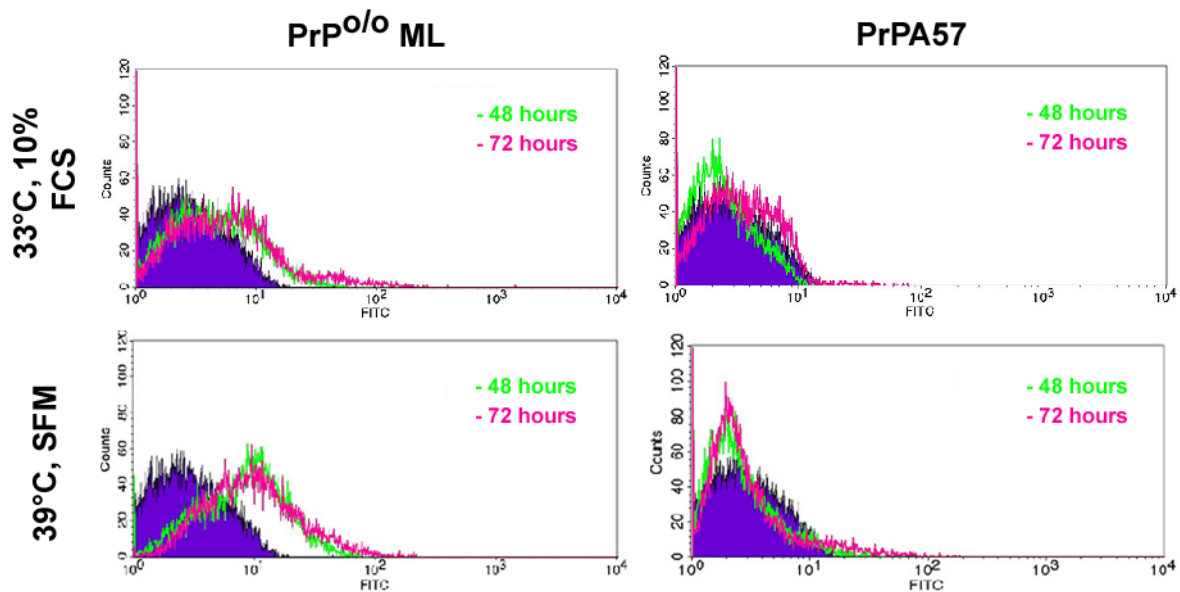
Figure 16. PSA-NCAM is expressed in PrP$^{0/0}$ ML and PrPA57 cells

FACS analyses for PSA-NCAM expression in the PrP$^{0/0}$ ML and PrPA57 cells cultivated under permissive (upper panels) and nonpermissive (lower panels) conditions for 48 hours (green curve) or 72 hours (purple curve). PrP$^{0/0}$ ML cells expressed higher PSA-NCAM levels as compared to the PrPA57 cells under both the permissive and nonpermissive culture conditions. Isotype control (black /blue filled curve) was included as negative control.

4.2 The role of plasma membrane-released microvesicles in the paracrine diffusion of PrP^C and propagation of prion infectivity

Shedding of membrane-derived microvesicles (MVs) by various cell types is a physiological phenomenon. Evidence exists that MVs shedding provide vehicles to transfer molecules among cells, and that MVs are important modulators of cell-to-cell communication (Ratajczak et al., 2006). It has previously been shown that neuronal cells release exosomes containing numerous proteins and lipids similar to those present in the membranes of the cells from which they originate, including typical neuronal proteins such as the GluR2/3 subunits of glutamate receptors and the cell adhesion molecule L1 (Faure et al., 2006). Interestingly, the exosomes released by cortical neurons also harbor PrP^C (Faure et al., 2006). Furthermore, the release of PrP^C and infectious PrP^{Sc} by prion infected epithelial, neuroglial and neuronal cells in association with exosomes has recently been highlighted (Fevrier et al., 2004; Vella et al., 2007a), suggesting that PrP^{Sc}-bearing exosomes may provide a mechanism for intercellular transmission of infectious prions in addition to cell–cell contact.

4.2.1 Neuronal cells release membrane-derived microvesicles bearing PrP^C and other lipid rafts components

In order to determine whether neuronal cells may also release plasma membrane-derived microvesicles (MVs) in addition to exosomes and to investigate whether these MVs contain PrP^C and other lipid rafts components, the cell culture supernatants of four different neuronal cell lines, namely the Neuro-2a, the PrP$^{0/0}$ ML, the PrPA109 and the Neuro-2a PK1 cells, were analyzed for the presence of MVs. The isolation of MVs from cell culture supernatants was based on a sequential centrifugation steps protocol in which increasing centrifugal forces were used (see Materials and Methods). Plasma membrane-derived MVs were collected at 20000g, a centrifugal force not sufficient to enrich exosomes, which were later isolated from the Neuro-2a PK1 cells by ultracentrifugation.

Electron microscopy (EM) analyses of MVs preparations negatively stained with uranyl acetate from both PrPA109 and PrP$^{0/0}$ ML cells demonstrated the presence in the 20000g fractions of vesicles with a diameter ranging from 100 nm to 1 μm (Fig 17), a size compatible with that described for MVs purified from other cells (Ratajczak et al., 2006).

To exclude the presence of contaminating exosomes within the MV-enriched fractions, the levels of Tgs101 and Alix 1, both cytoplasmic proteins previously identified as specific

markers for exosomes (Geminard et al., 2004; Thery et al., 2001), were assessed by immunoblot analysis. As shown in Figure 18, the signals obtained in the MV-enriched fraction were lower than those observed in the exosomes-enriched fraction prepared from Neuro-2a PK1 cells. That indicates either a low level cross contamination of MVs with exosomes or that both proteins are also incorporated in membrane-released MVs during blebbing of the membrane surface.

PrPA109 MVs   PrP$^{0/0}$ ML MVs
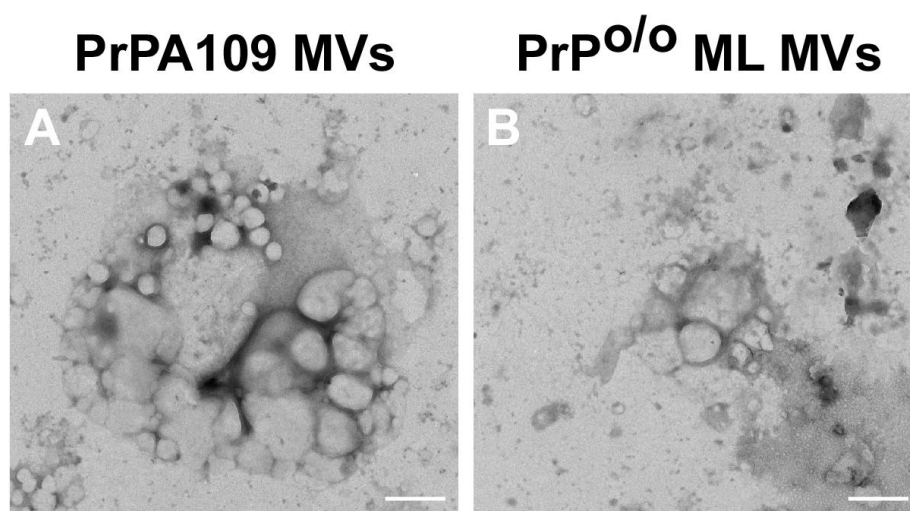
Figure 17. Neuronal cells release plasma membrane microvesicles

Electron microscopy analyses of MVs preparations from cell culture supernatants of the PrPA109 (A) and the PrP$^{0/0}$ ML (B) cells. Both cell lines released plasma membrane-derived MVs in the culture supernatants. MVs showed an average diameter ranging from 100 nm to 1 μm. MVs aggregates formed probably during the serial centrifugation steps used. Scale bar: 500 nm.
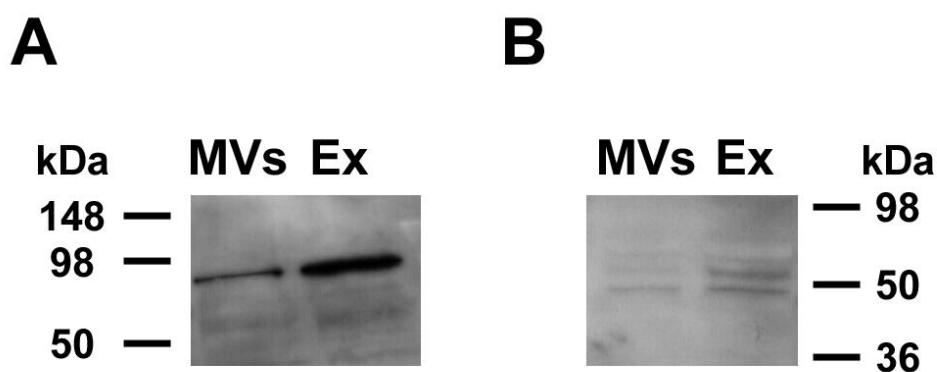
Figure 18. Detection of exosomal specific markers in the microvesicle-enriched fractions

Presence of the exosomal markers Alix 1 (95 kDa) (A) and Tsg101 (45 kDa) (B) were analyzed by immunoblot in both MV-enriched (MVs) and in exosome-enriched preparations (Ex). The cytosolic proteins were detected in both preparations. The lower levels of both exosomal markers present in the MVs indicated a possible exosomal contamination of these fractions. Positions of the molecular weight standards (in kDa) are indicated.

Like other GPI-anchored proteins, PrP^C as well as PrP^{Sc} are localized in glycosphingolipid-enriched microdomains (or lipid rafts) of neuronal and lymphocytic plasma membranes (Loberto et al., 2005; Mattei et al., 2002; Naslavsky et al., 1999; Vey et al., 1996), which are also enriched in several cytoplasmatic proteins, including tyrosine kinases (Masserini et al., 1999; Mouillet-Richard et al., 2000). In particular, in these specialized portions of cell plasma membrane PrP^C strictly associates with gangliosides and transducer proteins, such as p59Fyn kinase (Mouillet-Richard et al., 2000). Moreover, the association of PrP^C with rafts is required for its conversion into the protease-resistant isoform PrP^{Sc} (Baron et al., 2002; Masserini et al., 1999; Naslavsky et al., 1999).

The presence of PrP^C and other lipid rafts components such as Flotillin-2, gangliosides and the tyrosine kinase p59Fyn was therefore tested in MVs released by neuronal cells either by EM or by immunoblot analyses. As shown in Figure 19, immunoelectron microscopy (IEM) analysis revealed the presence of PrP^C on MVs isolated from the PrPA109 cells but not on MVs isolated from the $PrP^{0/0}$ ML cells, as expected. The binding of the monoclonal anti-PrP antibody 6H4 to PrP^C was visualized by an immunogold labeling (10 nm gold particles). The lack of immunogold labeling on $PrP^{0/0}$ ML-secreted MVs confirmed the specificity of the PrP^C-immunolabeling. Furthermore, IEM analysis of ultrathin sections of Neuro-2a PK1 cells revealed the unevenly distribution of PrP^C on the membrane of MVs as well as on the cell plasma membrane (Fig. 20). The presence of PrP^C in MVs released from the PrPA109 (Fig. 21), the Neuro-2a (Fig. 21) and the Neuro-2a PK1 cells (Fig. 23) was then confirmed by immunoblot analysis.
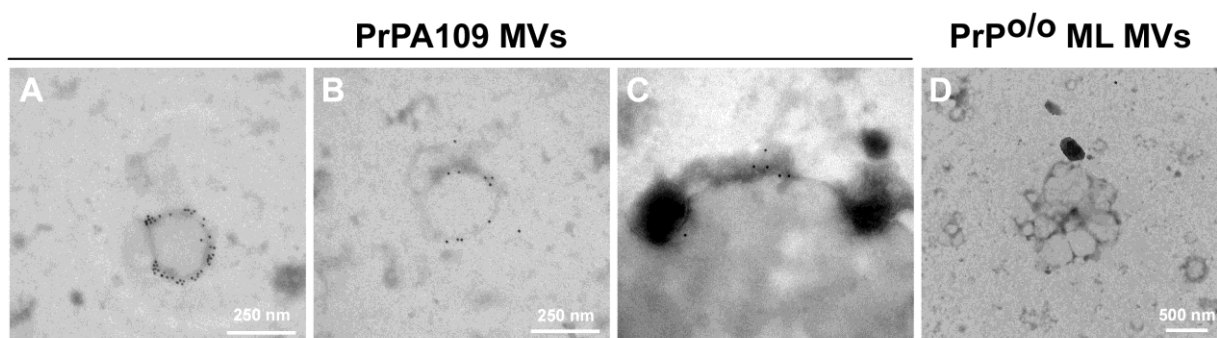
Figure 19. Neuronal cells shed PrP^C-bearing plasma membrane-derived microvesicles

Immunoelectron microscopy analyses of PrP^C expression on microvesicles released by the PrPA109 and the $PrP^{0/0}$ ML cell lines (A-C and D, respectively). PrP^C was specifically detected only in MVs released by the PrP^C-expressing (A-C) but not by the PrP^C-depleted cells (D). PrP^C was revealed by immunogold labelling (10 nm).
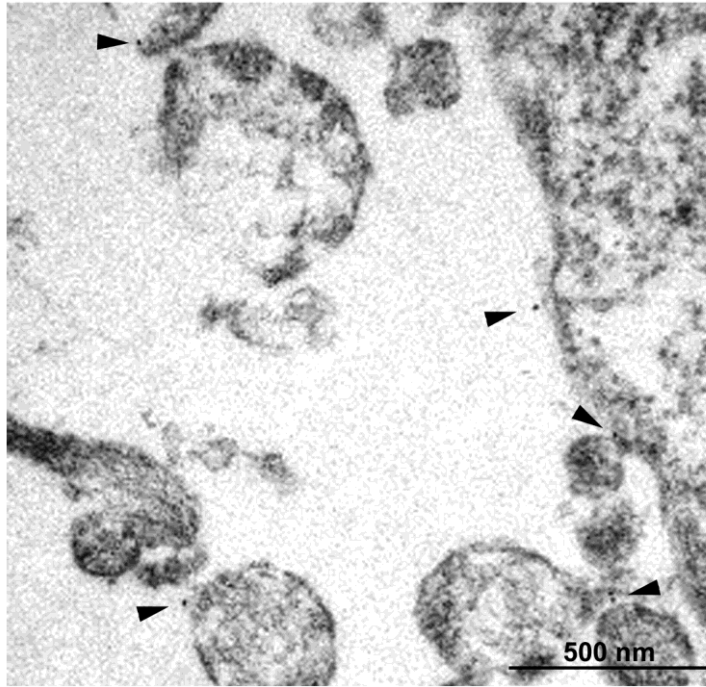
Figure 20. Unevenly distribution of PrP^C on the membrane of microvesicles as well as on the cell plasma membrane

Immunoelectron microscopy analyses of PrP^C expression in ultrathin sections of Neuro-2a PK1 cells. PrP^C was specifically detected both on the cellular plasma membrane (on the right) as well as on the membrane of MVs. PrP^C was revealed by immunogold labelling (10 nm) and indicated on the image by the black arrows.
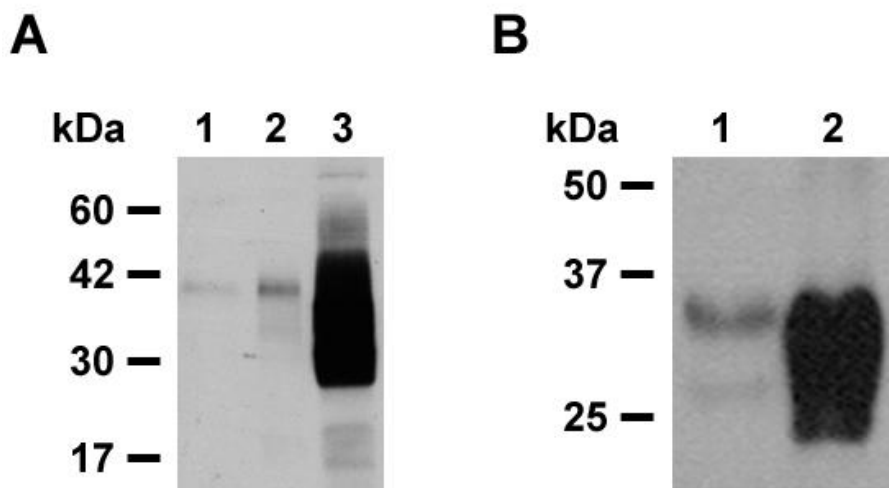
Figure 21. Presence of PrP^C in microvesicles released by different neuronal cell lines

Immunoblot analyses confirmed the presence of PrP^C in MVs derived from the PrPA57 (A, lane 2), PrPA109 (A, lane 3) and the Neuro-2a cells (B, lane 1). MVs isolated from PrP$^{0/0}$ ML cells were negative for PrP^C, as expected (A, lane 1). Crude brain homogenate from wild type mice was included as positive control (B, lane 2).

In order to assess the presence of gangliosides GM1 and GM2 in MVs released by the Neuro-2a cells, acidic glycosphingolipids extracted from MVs were immunostained by an anti-GM2 antibody (Kotani et al., 1992) and by HRP-CtxB, which stains GM1. The presence of GM2 but not that of GM1 was detected (Mattei, V.and Barenco, M.G., unpublished results).

Since p59Fyn as well as flotillin-2 have been indicated as raft components, which may be associated with PrP^C (Kramer et al., 1999; Mattei et al., 2004; Mouillet-Richard et al., 2000; Slaughter et al., 2003; Stuermer et al., 2004), the presence of both proteins in neuronal cells-derived MVs was investigated. Western blot analyses showed a 59 kDa band specifically recognized by the anti-Fyn antibody (Mattei, V., Barenco, M.G., unpublished results) and a 42 kDa band specifically recognized by the anti-flotillin-2 antibody (Mattei, V. and Barenco, M.G., unpublished results). Furthermore, the axonal membrane protein GAP-43, a neuronal protein which binds to rafts via its acylated N-termini, was also detected in MVs derived from the Neuro-2a cells (Mattei, V. and Barenco, M.G., unpublished results).

These findings suggest that raft components strictly associated with PrP^C on the cell plasma membrane, such as gangliosides, p59Fyn and flotillin-2, are recruited during the formation of MVs in neuronal cells.

4.2.2 Paracrine transfer of PrP^C by microvesicles

It has been shown that microvesicles are involved in paracrine and endocrine transport of cellular components such as membrane receptors, proteins, mRNA and organelles (e.g. mitochondria). Furthermore, MVs are also important modulators of cell-to-cell communication (Ratajczak et al., 2006). As MVs released by neuronal cells harbor PrP^C (see 4.2.1), they could be involved in the intercellular trafficking of PrP^C.

In order to experimentally approach this hypothesis, PrP^C-bearing (MVs^{A109}) and PrP^C-negative (MVs$^{0/0}$) MVs were first isolated accordingly to the established serial centrifugation step protocol (see Materials and Methods) from the PrPA109 and the PrP$^{0/0}$ ML cell line, respectively. The isolated MVs were then incubated for 2 hours with the PrP$^{0/0}$ ML cells to allow MVs uptake/fusion by the recipient cells. After intensive washing, cells were fixed and processed for immunofluorescence analysis to detect the presence of PrP^C. For PrP^C detection two different monoclonal antibodies, namely the 6H4 (Prionics AG, CH) and the 8G8 antibody (Cayman, USA), were used. The specificity of the staining obtained with the 6H4 antibody was clearly better than that obtained with the 8G8 antibody (data not shown).

As shown in Figure 22, a perinuclear and a cell membrane PrP^C specific staining was detected in the PrPA109 cells (Fig. 22C) but not in the $PrP^{0/0}$ ML cells (Fig. 22A), as expected. Negative was also the staining obtained with the isotype control antibody, namely the anti-TNP mouse $IgG_{1,k}$ monoclonal antibody (Fig. 22B and D). This was a further indication for the PrP^C specificity of the 6H4 antibody. Interestingly, a punctuate and, very rarely, a perinuclear PrP^C staining was detected in the $PrP^{0/0}$ ML cells incubated with the MVs^{A109} (Fig. 22H and I). The punctuate staining observed could be due to the binding and/or fusion of the PrP^C-bearing MVs to the plasma membrane of the recipient cells whereas the perinuclear staining could be due to the uptake and intracellular transport of PrP^C after MVs fusion. However, incubation of the $PrP^{0/0}$ ML cells with the $MVs^{0/0}$ led sometimes to similar observations, at least as for the punctuate staining (Fig. 22E). This could indicate an unspecific binding of the 6H4 antibody to MVs. Furthermore, in order to exclude a possible contamination of the MVs^{A109} preparations with PrPA109 donor cells, a contamination that could lead to false positive results, an additional experiment was performed. MVs^{A109} were isolated by centrifugations (see Materials and Methods) from PrPA109 cell supernatants that were first filtered through a 1,2 μm pore filter in order to remove detached and floating PrPA109 cells. The exclusion size of the filter allowed specific removal of cells but not that of the MVs (0,1-1 μm). PrP^C specific staining was not detected in the $PrP^{0/0}$ ML cells incubated with filtered MVs^{A109} anymore (Fig. 22J). No PrP^C signal was found in the control counterparts, i.e. the $PrP^{0/0}$ ML cells incubated with filtered $MVs^{0/0}$, as expected (Fig. 22G). These findings indicated that the detected PrP^C signals were most probably due to a contamination of the MVs^{A109} preparations with donor PrPA109 cells. This *in vitro* immunofluorescence-based assay was therefore considered as unsuitable for studying MV-mediated intercellular trafficking of PrP^C.
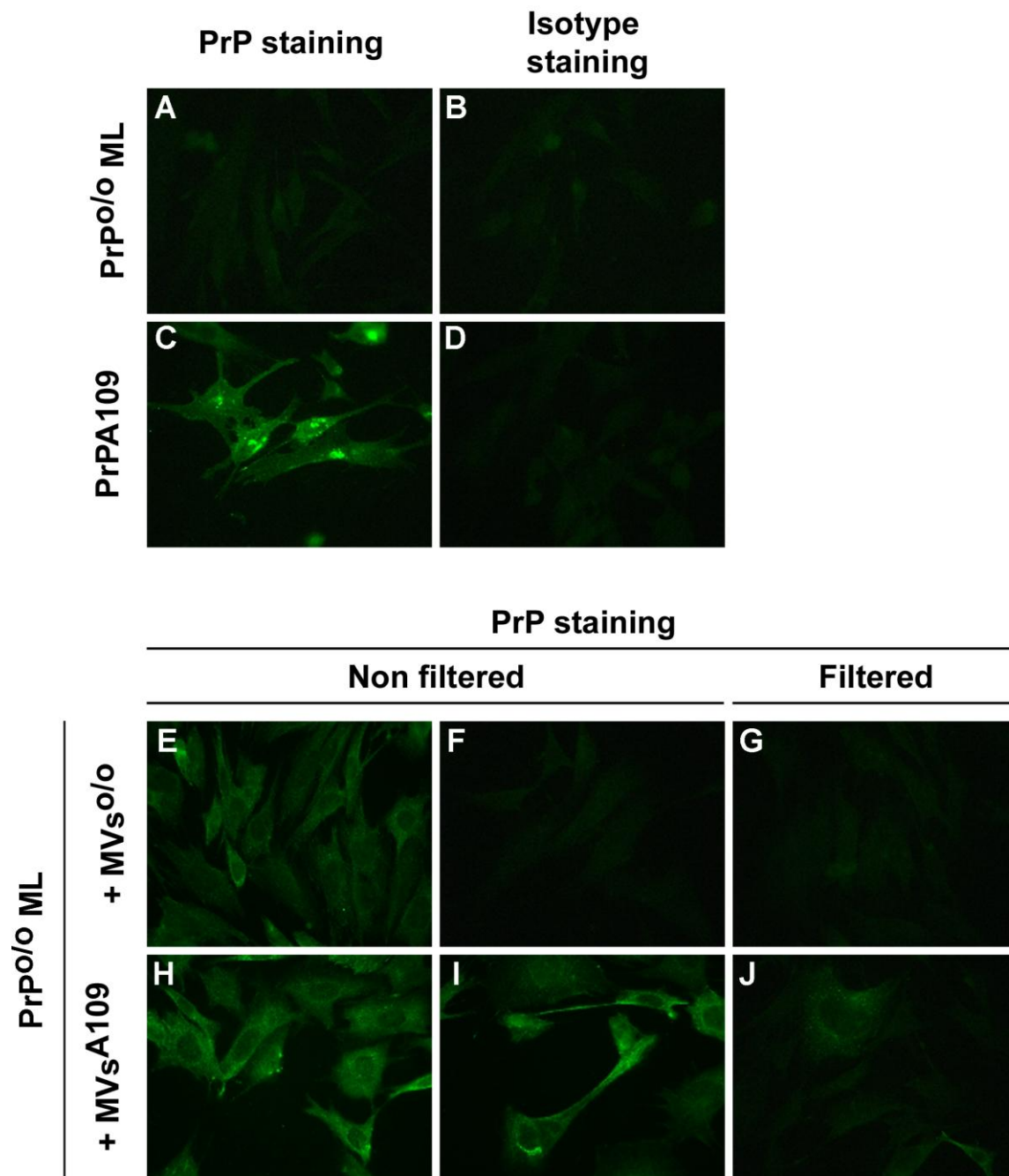
Figure 22. Paracrine diffusion of PrP^C

Upper panel: PrP immunostaining in PrP$^{0/0}$- (A-B) and PrPA109 (C-D) cell lines. A perinuclear and a cell membrane PrP^C specific staining was detected in the PrPA109 cells (C), but not in the PrP$^{0/0}$ ML cells (A), as expected. Isotype control antibody (B and D) was included as negative control.
Lower panel: PrP$^{0/0}$ cells were incubated with either MVs$^{0/0}$ (E-G) or MVs^{A109} (H-J). A PrP^C staining was detected in the PrP$^{0/0}$ ML cells incubated with the non-filtered MVs^{A109} (H-I), but not when the cells were incubated with the filtered MVs^{A109} (J).

4.2.3 Prion infected neuronal cells release membrane-derived microvesicles bearing the pathogenic isoform PrP^{Sc}

The release of PrP^C and infectious PrP^{Sc} by prion infected epithelial, neuroglial and neuronal cells in association with exosomes has recently been highlighted (Fevrier et al., 2004; Vella et al., 2007a), suggesting that PrP^{Sc}-bearing exosomes may provide a mechanism for intercellular transmission of infectious prions in addition to cell–cell contact. In order to assess whether prion infected neuronal cells may release membrane-derived MVs harboring PrP^{Sc} and prion infectivity, MVs were isolated from cell culture supernatants of Rocky Mountain Laboratory prion strain (RML)-infected and non-infected Neuro-2a PK1 cells. The presence of PrP^{Sc} was then assayed by immunoblot analysis after proteinase K (PK) digestion. The abnormally folded PrP^{Sc} is partially resistant against PK treatment, whereas the cellular isoform PrP^C is completely degraded by this protease (Prusiner, 1998). Different amounts of PK were used to digest 20 μg of proteins in MV- and cell-lysates, i.e. 0,1 μg or 0,3 μg or 1 μg PK. As shown in Figure 23, MVs derived from infected Neuro-2a PK1 cells harbored PK-resistant PrP characterized by the shift of the three bands, corresponding to the di-, mono- and
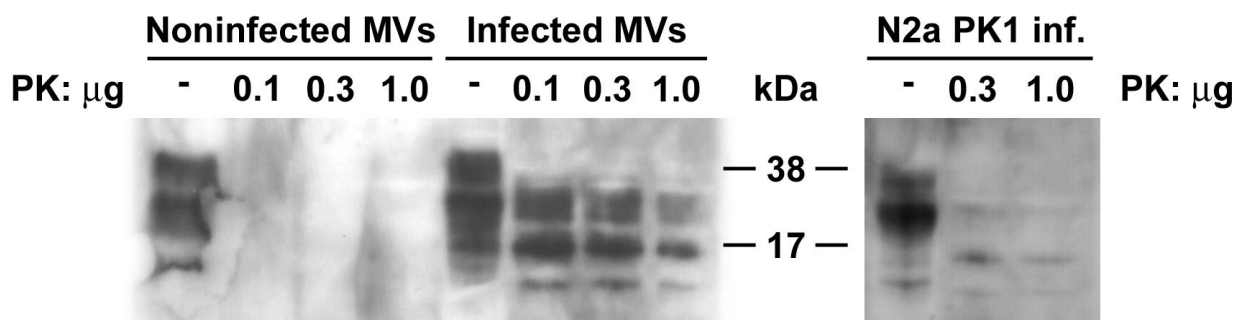
Figure 23. Prion infected neuronal cells shed PrP^{Sc} bearing microvesicles

Left panel: Detection of PrP^{Sc} in MVs isolated from culture supernatants of prion non-infected and infected Neuro-2a PK1 cells. Twenty μg total proteins of isolated MVs were PK digested. PrP^{Sc} was specifically detected in MVs released by the infected but not from the non-infected N2a PK1 cells.
Right panel: Detection of PrP^{Sc} in infected Neuro-2a PK1 cells. Two hundred and fifty μg total proteins of cell lysates were PK digested. The presence of PK-resistant PrP^{Sc} was assessed with increasing amounts of proteinase K and detected by the use of the 6H4 antibody. The positions of the molecular weight standards (in kilodaltons) are indicated.

unglycosylated forms of PrP, because of the PK-dependent removal of the first 90 aa (Fig. 23 left panel). This is a clear indication for the presence of PrP^{Sc}. Interestingly, the levels of PrP^{Sc} detected in the purified MVs were higher than those observed in the parental cell line (Fig. 23

right panel), suggesting that PrP^{Sc} is enriched on the membrane of MVs. PrP^{Sc} was not detected, as expected, in MVs secreted by non-infected Neuro-2a PK1 cells (Fig. 23 left panel), confirming that PrP^{Sc} is released specifically from prion infected neuronal cells. Thus, these findings clearly indicate that prion infected neuronal cells such as the Neuro-2a PK1 shed plasma membrane-derived MVs containing PrP^{Sc}.

4.2.4 *In vitro prion infectivity transmission by PrP^{Sc}-bearing microvesicles*

Having shown that RML-infected Neuro-2a PK1 release MVs harboring PrP^{Sc}, the hypothesis that MVs may act as carriers of prion infectivity was then experimentally challenged. The potential role of MVs in prion infectivity transmission was first tested in an *in vitro* infection model.

Noninfected Neuro-2a PK1 cells were incubated with MVs isolated from cell culture supernatants of either infected (MVs^{Inf}) or noninfected (MVs^{Noninf}) Neuro-2a PK1 cells. Brain homogenates (1% w/v) either from scrapie sick (Chandler) or from normal healthy (mock) mice were used as positive or negative inoculum controls, respectively. *De novo* amplification of PrP^{Sc} was then followed for longer than 3 months post infection by cell blot assay (Fig. 24). Conversion of the endogenous PrP^{C} into the prion disease-associated pathological isoform PrP^{Sc} was only detected in Neuro-2a PK1 cells treated with MVs derived from prion-infected but not from non-infected donor cells (Fig. 24). PK resistant PrP was detected in recipient Neuro-2a PK1 cells as early as at passage 1 after inoculation with MVs^{Inf} and Chandler but not after challenge with the negative control preparations (Fig. 24). As the amount of PrP^{Sc} at passage 2 was lower than at passage 1, the PK-resistant PrP detected early after infection was most probably due to the persistence of the PrP^{Sc}-positive inocula and not to prion replication. However, all along the first few weeks of cultivation after infection the amount of PrP^{Sc} increased constantly in the MVs^{Inf} and Chandler infected Neuro-2a PK1 cells. Prion replication was detected up to 30 passages after infection, indicating that MV-dependent prion transmission generated a persistent infection in the recipient neuroblastoma cells.
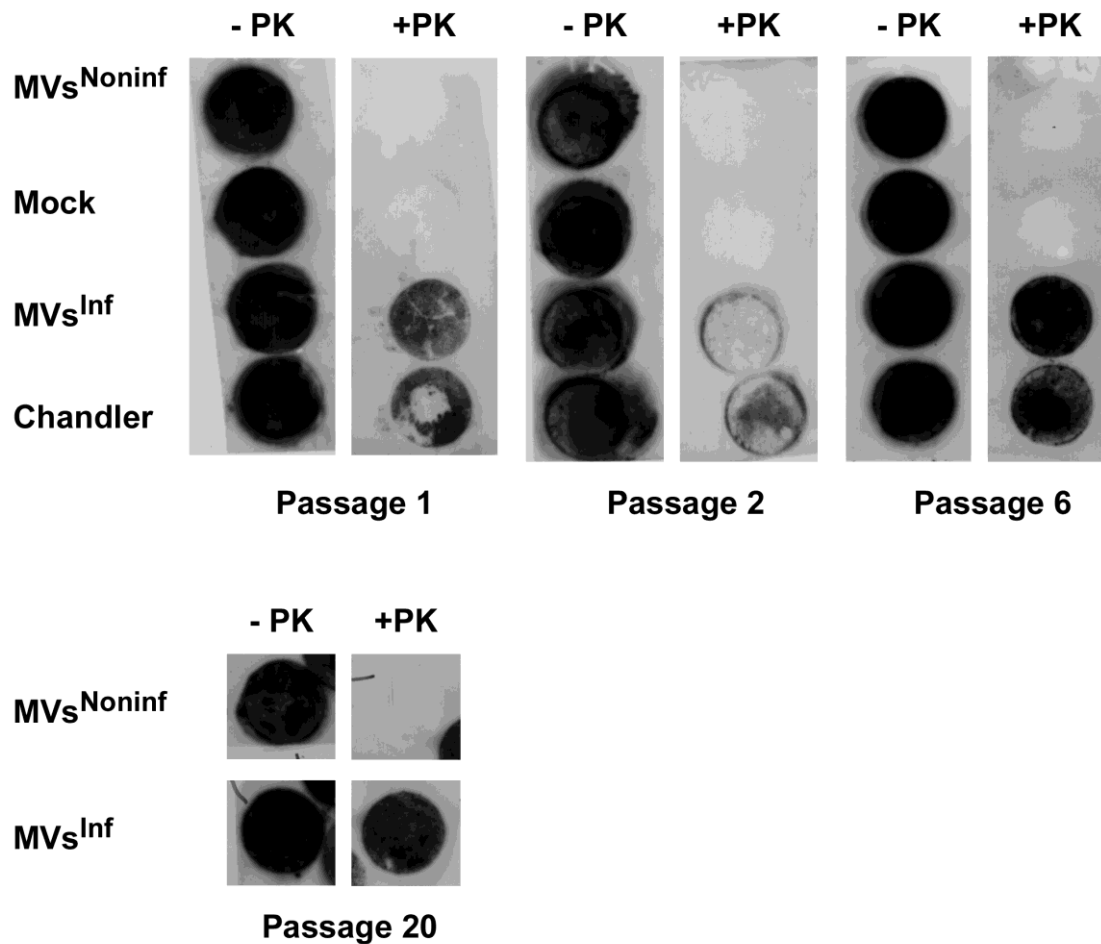
Figure 24. *In vitro* transmission of prion infectivity by PrP^{Sc} bearing microvesicles

Cell blot assay detecting *de novo* infected N2a PK1 cells at serial culture passages. N2a PK1 cells were incubated with MVs isolated from non-infected (MVs^{Noninf}) or from infected (MVs^{Inf}) N2a PK1 cells. Mock and Chandler infections were also included as negative and positive control, respectively. A time-dependent increase of PrP^{Sc} levels was detected after proteinase K treatment (+ PK).

4.2.5 In vivo prion infectivity transmission by PrP^{Sc}-bearing microvesicles

In order to further confirm the association of prion infectivity with membrane-derived MVs and to determine prion titres, *in vivo* biossay was performed in which purified MVs from infected (MVs^{Inf}) and non-infected (MVs^{Noninf}) Neuro-2a PK1 cells or cell lysates were intracerebrally inoculated into *tg*a*20* indicator mice (Fischer et al., 1996). Brain homogenates either from scrapie sick (RML) or from normal healthy (mock) mice were used as positive and negative inoculum controls, respectively. Incubation times until development of terminal scrapie were determined and infectious titres were calculated by comparing incubation times against a calibration curve (Table 3). Indicator mice developed clinical prion disease after

inoculation with Rocky Mountain Laboratory mouse-adapted prions (RML) at the three dilutions tested, but not when they were inoculated with mock control, as expected.

Interestingly, membrane-derived MVs from infected (MVs^{Inf}) but not those from non-infected (MVs^{Noninf}) Neuro-2a PK1 cells were capable of transmitting prion disease to *tga20* indicator mice, demonstrating that MVs^{Inf} are infectious not only *in vitro* but also *in vivo*. Furthermore, MV-associated prion titres were higher than those detected as cell-associated (Table 3), a finding confirming the observation that MVs^{Inf} contained more PrP^{Sc} then the cells from which they were derived (see 4.2.3). On the other hand, MVs^{Inf} seemed to be a little less infectious than crude brain homogenate: indeed mice challenged with $6x10^{-2}$ dilution of RML (i.e. corresponding to 10 µg of total brain proteins) developed clinical prion disease earlier (64 ± 2 days post infection) than those challenged with MVs (68 ± 2 days post infection).

Thus, release of microvesicles-associated PrP^{Sc} by infected cells, in addition to cell–cell contacts, could be considered as an acellular mechanism underlying the spread of prions.

Table 3. PrP^{Sc}-bearing microvesicles transmit disease to *Tga20* indicator mice

Inoculum	Indicator mice succumbing to scrapie	Scrapie death Days (mean +/-STDEV)	Estimated infectivity titres (log LD_{50}/ml)
Mock 10^{-4}	0/4	>150	<1.5
RML 10^{-4}	4/4	78 ± 2	4.6
RML 10^{-2}	4/4	68 ± 2	5.5
RML $6x10^{-2}$	4/4	64 ± 2	5.8
MVs^{Noninf}	0/4	>150	<1.5
MVs^{Inf}	4/4	68 ± 2	5.5
Neuro-2a PK1 cells	0/4	>150	<1.5
Neuro-2 a PK1 inf. cells	4/4	76 ± 6	4.8

Tga20 indicator mice were inoculated intracerebrally with either 30 µl (10 µg total proteins) of cell lysates or MVs or with 30 µl of different RML brain homogenate dilutions. Mock-inoculated animals were included as negative controls. Incubation time until development of terminal scrapie was determinated. The relationship $y =11.45-0.088\ x$, where y is $logLD_{50}$/ml homogenate and x is incubation time in days to terminal disease, was used to calculate infectivity titres (Prusiner et al., 1982)

4.2.6 *Establishment of a novel cell-based in vitro prion replication model*

Expression of PrP^C is necessary, although not sufficient, to confer capability of prion replication. As previously described (see 4.1.4), the neuronal precursor cell line PrP$^{0/0}$ ML was reconstituted for murine PrP^C expression. Several independent clones showing different PrP^C-levels were selected and further characterized. These clones could be used as novel models for studying *in vitro* prion replication and prion neurotoxicity given that they are susceptible to prion infection. Furthermore, once established that the PrP$^{0/0}$ ML cells are prion replication competent, one might reconstitute them for the expression of both human and bovine PrP in order to establish *in vitro* replication models for human and bovine prions. These models are nowadays still not available.

4.2.6.1 Susceptibility of the PrPA36 cell line to MV-mediated prion infection

The PrPA36 cells were derived by transfection of the neuronal precursor cell line PrP$^{0/0}$ ML with an expression cassette encoding for wild type PrP (see 4.1.4.2) and strongly overexpress PrP^C (data not shown). Having shown that MVs are efficient carriers of prion infectivity and can initiate efficiently prion infection both *in vitro* and *in vivo*, susceptibility of the PrPA36 cell line to prion infection was tested by challenging them with PrP^{Sc}-bearing MVs. Infectious MVs (MVs^{22L}) were isolated from Neuro-2a 58 cells stably infected with the 22L scrapie strain (Nishida et al., 2000). MVs (MVs^{Noninf}) isolated from noninfected Neuro-2a PK1 cells were included as negative control. *De novo* amplification of PrP^{Sc} was then followed by cell blot assay at each passage. As shown in Figure 25, weak PrP^{Sc} accumulation was detected in PrPA36 cells as early as at passage 2 after challenge with MVs^{22L} (Figure 25). Amounts of PrP^{Sc} increased constantly in the MVs^{22L}-infected PrPA36 cells upon time (Figure 25), a finding clearly indicating that in the PrPA36 cells a persistent prion infection was successfully established. No PrP^{Sc} was detected all along the infection period in MVs^{Noninf}-treated cells, as expected. Thus, the PrPA36 cell line is prion replication competent.

Of interest, the observation that the Neuro-2a PK1 cells were not as susceptible to 22L prions as to the Chandler prions (Figure 26). Indeed, upon MVs^{22L}-mediated infection PrP^{Sc} was detected in the Neuro-2a PK1 cells all along the infection period but at very low levels. Furthermore, the highest PrP^{Sc} levels were found at the earlier passages, as for example in passage 5, and at later passages no increase of PrP^{Sc} was observed. The different susceptibility of a cell line to different prion strains is a well established biological phenomenon.
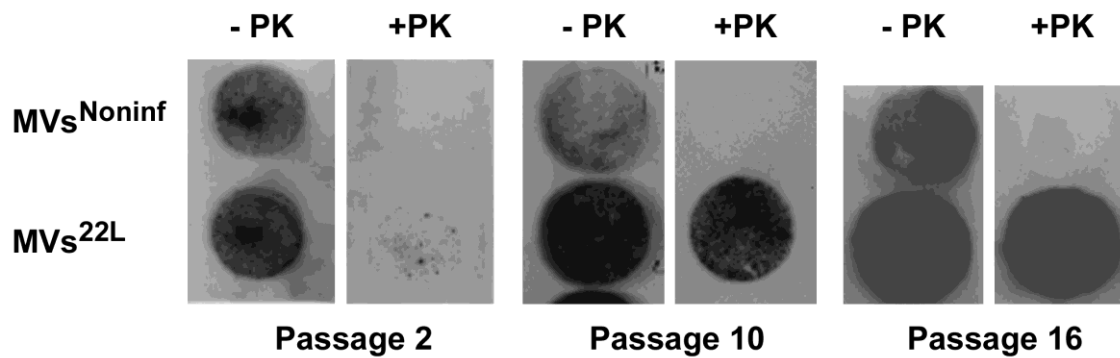
Figure 25. Establishment of a novel *in vitro* replication model for murine prions

The novel PrP^C-reconstituted neuronal progenitor cell line PrPA36 was infected by PrP^{Sc}-harboring MVs enriched from the N2a58 cells stably infected with the murine prion strain 22L. Cell blot assay detecting *de novo* infected PrPA36 cells at serial culture passages. PrPA36 cells were incubated with MVs isolated from non-infected (MVs^{Noninf}) Neuro-2a PK1 or from infected (MVs^{22L}) N2a58 cells. A time-dependent increase of PrP^{Sc} levels was detected after proteinase K treatment (+ PK).
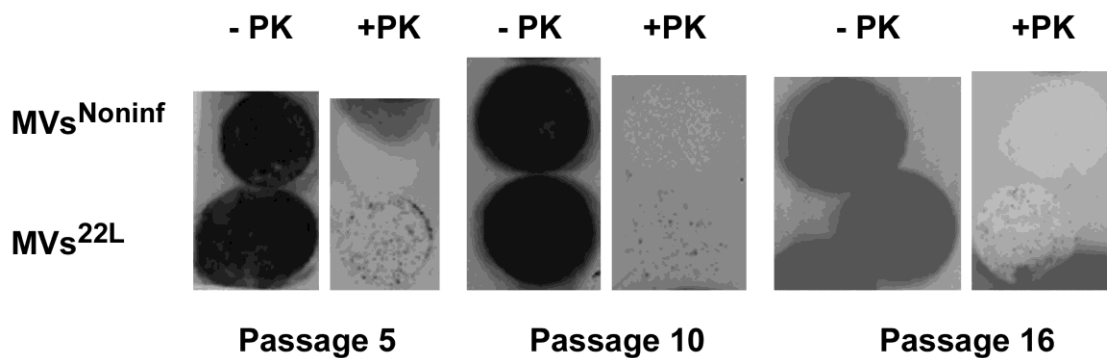
Figure 26. Prion strain specific susceptibility of the Neuro-2a PK1 cells

Neuro-2a PK1 cells were infected by PrP^{Sc}-harboring MVs enriched from the N2a58 cells stably infected with the murine prion strain 22L. Low levels of prion replication and PrP^{Sc} accumulation were detected by cell blot assay, indicating a reduced susceptibility of the Neuro-2a PK1 cells to the murine 22L prion strain as compared to the Chandler strain. PrP^{Sc} levels were detected after proteinase K treatment (+ PK).

4.2.6.2 Susceptibility of the PrPA36 cell line to prion infection: a coculture approach

As it has previously been shown that cell-to-cell contacts contribute to intercellular spread of prions, a co-culture approach was chosen to confirm prion replication capability by the PrPA36 cell line. Noninfected PrPA36 cells were co-cultured with the infected N2a58/22L cells in a ratio of 2:1. Cells were let grow to confluence and then passaged first at 1:2 dilution and then at 1:5 dilution. PrPA36 cells were positively selected by addition of 2 µg/ml Puromycin, a concentration sufficient to kill all N2a58/22L cells upon time. At passage 10, only PrPA36 cells were left, as determined by morphological examination. Cell blot assay performed at passage 16 (Fig. 27) showed a strong PrP^{Sc} accumulation, a finding indicating that the PrPA36 were infected. Thus, prion infectivity was efficiently transmitted from the infected N2a58/22L cells to the recipient PrPA36 cells and a persistent prion infection was then established in the PrPA36 cells.

The PrPA36 cell line is therefore a prion replication competent cell line, at least for the 22L prion strain as shown by two different infection approaches. This cell line could be used as an *in vitro* model for studying prion replication-dependent cellular pathways.
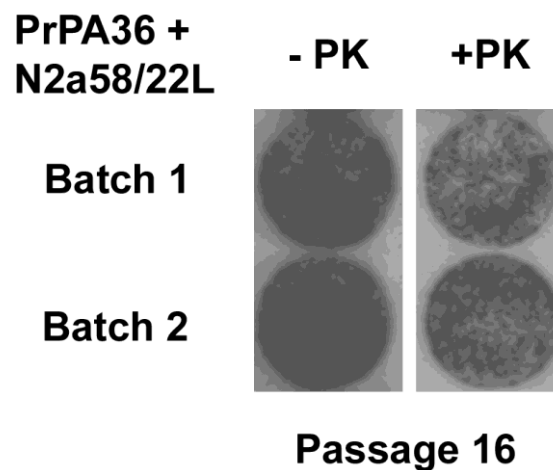
Figure 27. Establishment of a novel *in vitro* replication model for murine prions: an alternative infection approach

Noninfected PrPA36 cells were co-cultured with the infected N2a58/22L cells in a ratio of 2:1. PrPA36 cells were positively selected by addition of 2 µg/ml Puromycin. Cell blot assay performed at passage 16 showed a strong PrP^{Sc} accumulation, a finding indicating that the PrPA36 were infected. PrP^{Sc} levels were detected after proteinase K treatment (+ PK)

5 DISCUSSION

5.1 The cellular prion protein and neuronal differentiation

The cellular prion protein (PrP^C) is a highly conserved glycoprotein of unknown biological function. To gain insight into the physiological role of PrP^C, a novel PrP knockout cell line was generated by immortalization of neuroepithelial precursor cells derived from the cerebellum of PrP-knockout mice. The differentiation potential of this cell line was then monitored by evaluating structural parameters, including i) the expression of specific markers for mature neurons and ii) the development of a typical neuronal morphology.

5.1.1 Establishment and characterization of a novel PrP-knockout cell line

In the present study a novel PrP knockout cell line, $PrP^{0/0}$ ML, was successfully established by transducing cerebellar neuroepithelial precursors from the PrP knockout Zurich I mice (Bueler et al., 1992) with a retroviral vector that carries an expression cassette coding for a temperature-sensitive form of the SV40 large T antigen (Jat and Sharp, 1989). Overall the $PrP^{0/0}$ ML cell line presents some improvements when compared to the presently available PrP-depleted HpL 3-4, F14, Zpl and NpL2 lines (Holme et al., 2003; Kim et al., 2005; Kuwahara et al., 1999; Nishimura et al., 2007). The advantages are three-fold: i) the $PrP^{0/0}$ ML cells maintain the C57BL/6J x 129sv(ev) mixed genetic background of the original $Prnp^{0/0}$ Zurich I mouse line (Bueler et al., 1992), ii) the $PrP^{0/0}$ ML cells express neither PrP^C nor the prion protein homologue Dpl, which is neurotoxic when expressed in a PrP-knockout background (Anderson et al., 2004; Genoud et al., 2004; Moore et al., 2001; Rossi et al., 2001), and iii) immortalization by the tsSV40 large T conditional oncogene allows the control of the proliferative potential of the $PrP^{0/0}$ ML cells in a temperature-dependent manner (Frederiksen et al., 1988; Jat and Sharp, 1989), making the cells suitable for studying mechanisms of cell differentiation. In fact, the hippocampal neuronal HpL 3-4 cell line (Kuwahara et al., 1999) has been made from PrP-deficient animals of the Nagasaki-type and, therefore, expresses the neurotoxic prion protein homologue Dpl (Anderson et al., 2004; Genoud et al., 2004; Li et al., 2000; Moore et al., 1999; Moore et al., 2001; Rossi et al., 2001). The recently established F14, Zpl and NpL2 cell lines (Holme et al., 2003; Kim et al., 2005; Nishimura et al., 2007) have been derived from neuronal primary cells of Zurich I-type mice (Bueler et al., 1992; Manson et al., 1994). These cells express neither PrP nor Dpl but, being

already differentiated into neurons, might not be suitable for studying the possible role of PrP^C in processes of cell differentiation and neurite outgrowth.

Here it is shown that the PrP$^{0/0}$ ML cells are unipotent neuronal precursors which can specifically differentiate into neurons under nonpermissive conditions of culture in a time-dependent manner, as indicated by the progressive downregulation of nestin, a neuroepithelial stem cell marker, concomitantly to the upregulation of the neuronal antigens NeuN, MAP-2b and NF-H in association with an increase in the proportion of cells with neurites.

5.1.2 The role of the cellular prion protein in the process of neuronal fate specification

Several lines of evidence indicate that the cellular form of the prion protein is implicated in processes of neuronal differentiation, neurite outgrowth and synaptogenesis (Chen et al., 2003; Graner et al., 2000a; Kanaani et al., 2005; Lopes et al., 2005; Santuccione et al., 2005; Steele et al., 2006). PrP^C has been proposed to be a cell surface adhesion protein (Mange et al., 2002; Martins et al., 2002) and its early developmental distribution resembles that of adhesion molecules. Previous studies showed that PrP^C interacts with the laminin receptor precursor and binds laminin as well as glycosaminoglycans expressed on the surface of other cells (Gauczynski et al., 2001; Graner et al., 2000a; Hundt et al., 2001; Pan et al., 2002; Rieger et al., 1997; Shyng et al., 1995). It has to be noticed that during brain development, both laminin and glycosaminoglycans are developmentally regulated and contribute to axon growth and fiber tract formation (for review, Reichardt and Tomaselli, 1991). Indeed it has been showed that specific interaction between laminin and PrP^C induced neuritogenesis in mouse hippocampal neurons (Graner et al., 2000a) and mediated neurite adhesion and maintainance (Graner et al., 2000b). Moreover, PrP^C was localized on elongating axons suggesting a role for the protein in axon growth (Sales et al., 2002).

To determine the impact of PrP^C on these events, the expression of both the wild-type PrP^C and the N-terminally truncated PrPΔ32-134, a form of PrP shown to cause severe ataxia and neuronal death limited to the granular layer of the cerebellum and to Purkinje cells when expressed *in vivo* on a PrP-knockout background, was reconstituted in the PrP$^{0/0}$ ML cell line. Cell lines stably expressing low (PrPA117), average (PrPA57), and high (PrPA109, data not shown) PrP^C levels and one line expressing PrPΔ32-134 at high levels were established. Cell lines stably expressing the two proteins were then analyzed for their differentiation potential and were compared with the PrP-depleted cells. This work demonstrates that the presence of

PrP^C induces differentiation of the PrP$^{0/0}$ ML cells to the neuronal phenotype even under permissive conditions of culture as indicated by the reduced expression of nestin and by the concomitant occurrence of changes in cellular morphology. Switching the temperature to 39°C in low serum concentration further incremented this process as shown by the earlier appearance of antigens specific for mature neurons (e.g. NeuN), the increment of the proportion of cells expressing neuron-specific antigens and the increase in the proportion of cells with neurites. It is interesting to note that PrP$^{0/0}$ ML cells stably transfected with an empty vector differentiated into mature neurons under nonpermissive conditions with the same kinetic as the parenteral cells. Furthermore, a direct correlation between the levels of PrP^C expression and the proportion of cells showing the expression of neuronal markers was observed, providing definitive evidence that the effect described above is due to the presence of PrP^C rather than to the clonal selection of fast-differentiating cells. On the basis of these data, one can conclude that the prion protein facilitates the process of neuronal differentiation and neuritogenesis in PrP knockout stem/precursor cells. While no difference between wild-type and PrP$^{0/0}$ primary cerebellar neurons on total neurite length per cell was described by others (Chen et al., 2003; Santuccione et al., 2005), here a correlation was established between PrP^C expression and an increment in both the mean length of the longest neurite and the total length of neurites (data not shown) per cell. Therefore, we postulate that PrP^C plays a role in the process of neurite elongation during development but not on mature neurons, i.e. when differentiation is complete.

Contrary to the experiments in transgenic mice (Flechsig et al., 2003; Shmerling et al., 1998), the expression of the truncated PrPΔ32-134 induced no cytotoxic effects on the PrP$^{0/0}$ ML cells in both permissive and nonpermissive conditions of growth for up to 48 hours in culture. This could be explained by the fact that the amino-terminally deleted form of PrP^C is selectively toxic only in fully differentiated cerebellar neurons, such as Purkinje or granule cells, whilst the PrP$^{0/0}$ ML cells do not differentiate into specific neuronal phenotypes in the experimental conditions used. Similarly to the full-length PrP^C, it was found that high expression levels of PrPΔ32-134 mediate neuronal differentiation and neuritogenesis, though to a lesser extent than the wild-type protein. This last finding is not in agreement with the recently published results showing that full-length recombinant PrP^C, but not its C-terminal globular domain (SHaPrP90-231), dramatically enhances the development of neuronal polarity in cultured embryonic rat hippocampal neurons (Kanaani et al., 2005). One possible explanation for this discrepancy is that, in the cell model described in this thesis, PrP^C could interact with a putative binding partner both in *cis* and in *trans* whereas in the system used by

Kanaani and co-workers recombinant PrP induced neuritogenesis in *trans* (Kanaani et al., 2005).

Altogether, the data generated in this work add to the growing body of evidence that PrP^C is involved in mechanisms underlying cell differentiation and neuritogenesis. In addition, it is reported here for the first time that the N-terminal domain of the cellular prion protein is not crucial for PrP^C–mediated induction/acceleration of neuronal differentiation.

The PrP$^{0/0}$ ML and PrP-reconstituted cell lines represent novel *in vitro* models to further investigate the cellular physiopathological roles of both the non-pathogenic and the scrapie prion proteins and their signal transduction pathways. The future use of genome wide screening procedure at both the transcriptome and proteome level on such models may allow to gain further information on the still mysterious role of PrP^C in health and disease.

5.1.3 *Expression of PrP in PrP$^{0/0}$ ML cells accelerates neuronal differentiation via p59Fyn kinase activation*

PrP^C activation by antibody cross-linking induces a signalling pathway involving the nonreceptor Src-tyrosine kinase family member p59Fyn in a cell line capable of neuronal-like differentiation (Mouillet-Richard et al., 2000). Furthermore, p59Fyn signal transduction pathway has also been implicated in neurite outgrowth and neuronal survival elicited by PrP^C in cell culture of primary neurons (Chen et al., 2003). Recently it has been shown that PrP^C interacts directly with the neural cell adhesion molecule (NCAM) at the neuronal cell surface leading to NCAM recruitment and stabilization in lipid rafts. NCAM accumulation in lipid rafts activates then the p59Fyn kinase pathway thereby inducing NCAM-mediated neuritogenesis (Niethammer et al., 2002; Santuccione et al., 2005).

Since PrP^C expression in the novel cellular system described in this thesis enhanced neuronal differentiation and neurite outgrowth, it was assessed whether p59fyn kinase signal pathway was involved in this PrP^C-mediated process. It was found that the amount of inactive p59kinase was reduced in both wild type- and PrPΔ32-134-expressing cell lines by specifically detecting non-phosphorylated Tyr-416 under nonpermissive culture conditions. Furthermore, the inhibition of the Src-kinases signalling by the use of a specific inhibitor reduced the enhancing effect of PrP^C on neuronal differentiation of PrP$^{0/0}$ ML cell line. These data therefore further support the hypothesis that one of the signal pathways activated during PrP-mediated induction of neuronal differentiation and neurite outgrowth is the p59Fyn-

dependent one (Chen et al., 2003; Kanaani et al., 2005; Mouillet-Richard et al., 2000; Santuccione et al., 2005).

5.2 The role of plasma membrane-released microvesicles in the paracrine diffusion of PrP^C and propagation of prion infectivity

Prion diseases are typically initiated by infection of peripheral sites, as in the case of BSE, vCJD, Kuru, and most cases of iatrogenic CJD. The mechanisms by which prions spread from the site of peripheral exposure, such as the gastrointestinal tract, to the lymphoreticular system where a first replication phase occurs and subsequently to and within the central nervous system are still not completely elucidated (for reviews, Aguzzi and Heikenwalder, 2006; Aguzzi et al., 2008; Aguzzi et al., 2007; Mabbott and Turner, 2005; Mabbott and Macpherson, 2006). Although different cell types of the immune system, such as B lymphocytes (Brandner et al., 1999; Frigg et al., 1999; Klein et al., 1997; Klein et al., 1998; Montrasio et al., 2001; Raeber et al., 1999), follicular dendritic cells (Brown et al., 2000; Mabbott et al., 2003; McBride et al., 1992; Montrasio et al., 2000; Prinz et al., 2003; Prinz et al., 2002), macrophages (Beringue et al., 2000; Carp and Callahan, 1981; Maignien et al., 2005; Prinz et al., 2002) and dendritic cells (Aucouturier et al., 2001; Glaysher and Mabbott, 2007; Huang et al., 2002; Levavasseur et al., 2007; Luhr et al., 2004; Mohan et al., 2005; Raymond et al., 2007; Raymond and Mabbott, 2007; Sethi et al., 2007), and the peripheral nervous system (Favereaux et al., 2004; Glatzel and Aguzzi, 2000b; Glatzel et al., 2001; Groschup et al., 1999; Ishida et al., 2005; Kimberlin et al., 1983; Lee et al., 2005) have been recognized as key players in the process of prion neuroinvasion, relatively little information is available about the mechanism(s) underlying intercellular prion transfer.

Microvesicles (MVs) are submicron (0,03-1 μm) (Joop et al., 2001; Sabatier et al., 2002), membrane-bounded vesicles which are released both from the plasma membrane or the endosomal membrane compartment after fusion of secretory granules with the plasma membrane (exosomes) of a variety of cells undergoing activation or apoptosis (Combes et al., 1999). Although the molecular basis of protein sorting during MVs formation is not fully understood, they result from an exocytotic budding process involving lipids and proteins metabolism. The segregation of specific proteins is followed by blebbing of the membrane surface, leading to the formation of MVs and their release in the extracellular environment. Some evidence shows that MVs components mainly arise from lipid rafts, which therefore

might be involved in setting up sorting platforms to concentrate specific proteins within MVs, which could be destined to extracellular secretion.

The functional role of MVs is still largely unknown, however recent evidence suggests that MVs are important modulators of cell-to-cell communication and play an important pleiotropic role in many biological processes. Indeed, MVs may possibly act as paracrine vectors of transcellular exchange of messages between circulating and endothelial cells (Martinez et al., 2005), participate in a variety of intracellular adhesion processes, and induce cellular response(s) (Combes et al., 1999; Del Conde et al., 2005). Moreover, MVs may "hijack" infectious particles such as the immuno deficiency virus (HIV) from the cytoplasm of the releasing cells (Pelchen-Matthews et al., 2004; Rozmyslowicz et al., 2003) and possibly even whole intact organelles such as the mitochondria (Spees et al., 2006).

The release of PrP^C and infectious PrP^{Sc} by prion infected epithelial, neuroglial and neuronal cells in association with exosomes has recently been highlighted (Fevrier et al., 2004; Vella et al., 2007a), suggesting that PrP^{Sc}-bearing exosomes may provide a mechanism for intercellular transmission of infectious prions in addition to cell–cell contact. It has furthermore been shown that endogenous PrP^C is associated with exosomes released by blood platelets (Robertson et al., 2006), and a number of studies have demonstrated that prions are present in blood and blood components, buffy coats, plasma, and platelets in animal models (Bons et al., 2002; Brown et al., 1999b; Brown et al., 1998; Cervenakova et al., 2003; Holada et al., 2002). It has also been demonstrated that blood as well as plasma of animals experimentally infected with TSEs can efficiently transmit prion infection by transfusion (Cervenakova et al., 2003; Houston et al., 2000; Hunter et al., 2002; Ludlam and Turner, 2006; Taylor et al., 2000). Of concern, is the finding that vCJD is most probably efficiently transmitted between human patients by blood transfusion (Bishop et al., 2006; Fagge et al., 2005; Hewitt, 2006; Ironside, 2006; Llewelyn et al., 2004; Wroe et al., 2006). This infection process, in absence of sensitive vCJD blood tests for screening of blood and blood components donations, could lead to a horizontal spread of vCJD within the human population.

The aims of this study were to evaluate the potential role of plasma membrane-derived MVs in the mechanism(s) of PrP^C diffusion and prion infectivity transmission *in vitro* and *in vivo* and to characterize the interactions of PrP^C with lipid raft components in MVs.

5.2.1 Microvesicles released from neuronal cells harbor PrP^C and other lipid rafts components

It has previously been shown that neuronal cells release exosomes containing numerous proteins and lipids similar to those present in the membranes of the cells from which they originate, including typical neuronal proteins such as the GluR2/3 subunits of glutamate receptors, the cell adhesion molecule L1 and, interestingly, PrP^C (Faure et al., 2006). The current thesis reports for the first time that neuronal cells also release plasma-derived microvesicles (MVs). Indeed, MVs were isolated from the cell culture supernatants of four independent murine neuronal cell lines, namely the Neuro-2a, the PrP$^{0/0}$ ML, the PrPA109 and the Neuro-2a PK1 cells, by sequential centrifugation steps protocol in which increasing centrifugal forces were used. Electron microscopy (EM) analyses of MVs preparations negatively stained with uranyl acetate from both PrPA109 and PrP$^{0/0}$ ML cells demonstrated the presence of vesicles with a diameter ranging from 100 nm to 1 μm, a size compatible with that described for MVs purified from other cells (Ratajczak et al., 2006). However, MV-enriched fractions were most probably contaminated with exosomes, as shown by the detection of Tgs101 and Alix 1, both cytoplasmic proteins previously identified as specific markers for exosomes (Geminard et al., 2004; Thery et al., 2001). In fact, both proteins, which interact together (Strack et al., 2003; von Schwedler et al., 2003), are part of the molecular machinery allowing vesicles budding inside the intralumenal membranes of multivesicular bodies (MVBs) of the endocytotic pathway (Katzmann et al., 2002; Raiborg et al., 2003) and are enriched in exosomes released by dendritic cells (Thery et al., 2001). Since both the density and size of exosomes may partially overlap those of MVs, it could be that at 20000g also a portion of exosomes or exosomes aggregates sediment together with membrane-derived MVs. The development of density centrifugation protocols either on a continuous or a discontinuous sucrose gradient for the specific enrichment of MVs may in future be envisaged in order to minimize exosomal contaminations.

Like other GPI-anchored proteins, PrP^C as well as PrP^{Sc} are localized in glycosphingolipid-enriched microdomains, so called lipid rafts, of neural and lymphocytic plasma membranes (Loberto et al., 2005; Mattei et al., 2002; Naslavsky et al., 1999; Vey et al., 1996). In particular, in these specialized portions of cell plasma membrane PrP^C strictly associates with gangliosides (Mattei et al., 2002) and transducer proteins, such as p59Fyn kinase (Mouillet-Richard et al., 2000) and NCAM (Niethammer et al., 2002; Santuccione et al., 2005).

Moreover, the association of PrP^C with rafts is required for its conversion into the infectious and protease-resistant isoform PrP^{Sc} (Baron et al., 2002; Bate et al., 2004; Masserini et al., 1999; Naslavsky et al., 1999). Lipid raft-associated protein sorting in the process of exosomes shedding has been described (de Gassart et al., 2003; Faure et al., 2006; Fevrier et al., 2004) emphasizing the raft-like nature of the exosomal membrane environment and therefore suggesting that raft domains may represent weak points on the cell plasma membrane prone to outward shedding.

In the present work, the association of PrP^C with MVs shed by murine neuronal cells was demonstrated. Immunoelectron microscopy (IEM) clearly showed that PrP^C is present on the surface of MVs isolated from the PrPA109 cells, a cell line that strongly overexpress PrP^C, but not on MVs isolated from the PrP^C-depleted PrP$^{0/0}$ ML cells. Furthermore, IEM analysis of ultrathin sections of Neuro-2a PK1 cells, a murine neuroblastoma cell line endogenously expressing PrP^C, revealed the unevenly distribution of PrP^C on the membrane of MVs as well as on the cell plasma membrane. The presence of PrP^C in MVs released from three different neuronal cell lines, namely the PrPA109, the Neuro-2a and the Neuro-2a PK1 cells, was then confirmed by immunoblot analysis. Heterogeneity of PrP^C molecules is attributed mainly to various degrees of N-glycosylation on asparagine residues (DeArmond et al., 1997; Endo et al., 1989; Haraguchi et al., 1998; Somerville and Ritchie, 1990). Interestingly, MV-associated PrP^C displayed an electrophoretic mobility pattern on SDS-polyacrylamide gels resembling if not overlapping the one detected for cell-derived PrP^C, suggesting that in MVs fully processed and mature PrP^C molecules are incorporated during membrane shedding. However, in a recently published work, a novel processing pathway that involves the N-terminal modification of PrP^C and the selection of distinct PrP^C glycoforms for incorporation into exosomes has been described (Vella et al., 2007b). Thus, a more accurate and detailed analysis of MV-associated PrP^C should in future be performed in order to verify whether a similar processing pathway also occurs during incorporation of PrP^C into plasma-derived microvesicles. The presence of lipid rafts components such as the p59Fyn kinase, flotillin-2 and the ganglioside GM2 was furthermore detected in MVs released by the murine Neuro-2a cell line, suggesting that raft components strictly associated with PrP^C on the cell plasma membrane are recruited during the formation of MVs in neuronal cells. The neuronal origin of the analyzed MVs was confirmed by the detection of the axonal membrane protein GAP-43, a neuronal protein which binds to rafts via its acylated N-termini.

5.2.2 Paracrine transfer of PrP^C by microvesicles

It has been shown that MVs are involved in paracrine and endocrine transport of cellular components such as membrane receptors, proteins, mRNA and organelles (e.g. mitochondria) (for a review, Ratajczak et al., 2006). The phenomenon of receptor transfer between cells by MVs has been studied in platelet-derived MVs, which transfer platelet-expressed adhesion molecules to hematopoietic cells, increasing their adhesion to fibrinogen or endothelium (Baj-Krzyworzeka et al., 2002; Janowska-Wieczorek et al., 2001). Furthermore, it has recently been shown that MVs derived from monocytes may transfer the HIV co-receptor CCR5 between cells and are responsible for rendering these cells susceptible to R5 HIV infection (Mack et al., 2000). Until now only a few evidence suggests a role for MVs in cell-to-cell mechanisms of PrP^C diffusion (Faure et al., 2006; Robertson et al., 2006; Simak et al., 2002). In this work, the potential role of neuronal cell-derived MVs in the intercellular trafficking of PrP^C was challenged by a co-culture assay in which PrP-depleted cells ($PrP^{0/0}$ ML cells) were cultured in the presence of PrP^C-bearing microvesicles (MVs^{A109}). Immunoflorescence-based detection of PrP was used for monitoring the transfer of PrP^C from the MVs to the recipient cells. Unfortunately, this *in vitro* immunofluorescence-based assay was unsuitable for studying MV-mediated intercellular trafficking of PrP^C. Two major limitations of this *in vitro* assay were identified: i) the MV-enriched preparations were probably contaminated with some of the MV-producing PrP^C-expressing cells and ii) the sensitivity of the detection system was probably not sufficient for detecting the transfer of a limited number of PrP^C molecules by MVs. As experimentally shown, the implementation of a filtration step efficiently removed contaminating PrP^C-expressing cells from the MV-enriched preparations. In order to increase detection sensitivity, one might generate a murine neuronal cell line transgenically expressing high levels of an EGFP-PrP fusion protein, allowing incorporation of self-fluorescent PrP^C molecules into the released MVs. The use of self-fluorescent PrP^C moieties would avoid problems both with the sensitivity and the background that could arise by the use of an indirect immunolabelling detection system.

5.2.3 Prion infected neuronal cells release PrP^{Sc}- and prion infectivity-bearing microvesicles

The release of PrP^C and infectious PrP^{Sc} by prion infected epithelial (Rov cells), neuroglial (Mov cells) and neuronal cells (GT1-7 cells) in association with exosomes has recently been

highlighted (Fevrier et al., 2004; Vella et al., 2007a), suggesting that PrP^{Sc}-bearing exosomes may provide a mechanism for intercellular transmission of infectious prions in addition to cell-to-cell contact. Indeed it was found that both PrP^{Sc} and PrP^{C} are present in cell culture supernatants in a secreted, exosome-associated form and that exosomes bearing PrP^{Sc} were infectious both *in vitro* and *in vivo* (Fevrier et al., 2004; Vella et al., 2007a).

This thesis further extends these findings showing for the first time that stably infected neuronal cells endogenously expressing PrP^{C} shed plasma-derived MVs, which are carriers both of the pathological isoform PrP^{Sc} and of prion infectivity. Noteworthy, MVs isolated from cell culture supernatants of Rocky Mountain Laboratory prion strain (RML)-infected Neuro-2a PK1 harbored PK-resistant PrP, an indication for the presence of PrP^{Sc}. In fact, the abnormally folded PrP^{Sc} is partially resistant against PK treatment, whereas the cellular isoform PrP^{C} is completely degraded by this protease (Prusiner, 1998). Interestingly, the levels of PrP^{Sc} detected in the purified MVs were higher than those observed in the parental cell line, suggesting that PrP^{Sc} is enriched on the membrane of MVs. PrP^{C} and PrP^{Sc} are GPI-anchored proteins known to partition into lipid rafts (Gorodinsky and Harris, 1995; Harris, 1999a; Naslavsky et al., 1997; Sarnataro et al., 2002; Taraboulos et al., 1995; Vey et al., 1996) and the lipid raft-like nature of MVs membranes argues for an efficient insertion of PrP^{Sc} in lipid raft-rich domains of the plasma membrane involved in MVs formation. Furthermore, the evidence that lipid rafts play a role in the formation of PrP^{Sc} in scrapie-infected culture cells (Baron et al., 2002; Naslavsky et al., 1999) supports the hypothesis of a preferential distribution of PrP^{Sc} within lipid rafts.

On the other hand, PrP^{C} but not PrP^{Sc} was detected in MVs secreted by non-infected Neuro-2a PK1 cells, confirming that PrP^{Sc} is released specifically from prion infected neuronal cells. Thus, these findings clearly indicate that prion infected neuronal cells such as the Neuro-2a PK1 shed plasma membrane-derived MVs containing PrP^{Sc}.

The potential role of plasma membrane-derived MVs in prion infectivity transmission was tested both *in vitro* and *in vivo* infection models. This study demonstrates that PrP^{Sc}-bearing MVs can transmit prion infectivity both *in vitro* and *in vivo,* indicating that MVs contribute both to the intercellular mechanism(s) of PrP^{C} diffusion and to the process of infectious prions intercellular trafficking. Indeed, MVs isolated from cell culture supernatants of infected but not from noninfected Neuro-2a PK1 cells were capable of initiating *de novo* amplification of prions in the recipient cells. Interestigly, prion replication was detected up to 30 passages after infection, indicating that MV-dependent prion transmission generated a persistent infection in the recipient neuroblastoma cells. Hypothetically, PrP^{C} conversion may be initiated as a

consequence of the binding of PrP^{Sc}-bearing MVs to acceptor cells. In this context, it is noteworthy that the topology of exosomal and, most probably, of MVs membranes is identical to that of the plasma membrane (Mattei et al, personal communication and Stoorvogel et al., 2002). Alternatively, but not mutually exclusive, MVs captured by the target cells could fuse with the cell surface or be internalized by an unclear mode of entry to induce conformational change of PrP^{C} at the cell surface and/or in endocytic compartments, respectively. Moreover, *in vivo* biossay in transgenic mice that overexpress murine PrP^{C} and are highly susceptible to murine prions (Fischer et al., 1996) clearly demonstrated that prion infectivity is associated with PrP^{Sc}-harboring MVs shed by prion infected murine neuronal cells. In fact, membrane-derived MVs from infected but not those from non-infected Neuro-2a PK1 cells were capable of transmitting prion disease to *tga20* indicator mice.

These new findings suggest that plasma membrane-derived MVs shed by prion infected cells could actively participate in the intercellular trafficking of PrP^{Sc} and contribute to the transmission of infectious prions. One may therefore postulate that MVs act as carriers of prion infectivity during prion neuroinvasion and play a pivotal role in the propagation of prion infectivity from neuron to neuron, in this way contributing to the pathogenesis of the disease.

MVs are normal constituents of blood plasma and are secreted by leukocytes, endothelium, platelets and erythrocytes. Interestingly, only one-third of the PrP^{C} in human blood is cell associated, and the remaining two-third is present in plasma (MacGregor et al., 1999). In this context, it has previously been shown that PrP^{C} is released on exosomes from activated blood platelets (Robertson et al., 2006). Furthermore, a number of studies have demonstrated that prions are present in blood and blood components, such as buffy coats, plasma, and platelets in animal models (Bons et al., 2002; Brown et al., 1999b; Brown et al., 1998; Cervenakova et al., 2003; Holada et al., 2002). Noteworthy, it has also been demonstrated that blood as well as plasma of animals naturally or experimentally infected with TSEs can efficiently transmit prion infection by transfusion (Cervenakova et al., 2003; Houston et al., 2000; Hunter et al., 2002; Ludlam and Turner, 2006; Taylor et al., 2000). In the frame of a collaborative work, we identified PrP^{C} in association with the lipid raft components p59Fyn, flotillin-2, GM1 and GM3 in MVs from blood plasma of healthy human donors (Mattei, V. and Barenco, M.G., unpublished results). Accordingly to the findings reported above indicating that MVs released by infected neuronal cells are infectious, one can postulate that MVs may also contribute to blood transfusion-mediated transmission of prion diseases. However, this working hypothesis has to be experimentally challenged by isolating blood-derived MVs from infected animal

models. Association of both PrP^{Sc} and prion infectivity with blood-derived MVs has then to be demonstrated by biochemical and *in vivo* assays.

5.2.4 Establishment of a novel cell-based in vitro prion replication model

Expression of PrP^C is necessary, although not sufficient, to confer capability of prion replication. The neuronal precursor cell line PrP$^{0/0}$ ML was reconstituted for murine PrP^C expression by the use of an expression cassette encoding for wild type PrP. The clonal cell lines PrPA36 was chosen for establishing a novel model for studying *in vitro* prion replication and prion neurotoxicity. By the use of two independent prion infection approaches, a persistent prion infection of the PrPA36 cells was successfully established. Thus, the PrPA36 cell line is prion replication competent. This finding is in line with the recent publications in which it was reported that murine neuronal stem cells are susceptible to prion infections (Giri et al., 2006; Milhavet et al., 2006; Vilette, 2008). Therefore, prion replication capability is not restricted to fully mature neurons. Noteworthy, the reconstitution of homologous or heterologous prion protein expression in PrP-depleted cell lines is a powerful approach to develop novel *in vitro* prion replication models (this work and Maas et al., 2007). Nowadays, no *in vitro* models for human and bovine prion replication are available. Reconstitution of the PrP$^{0/0}$ ML cell line for the expression of either human or bovine PrP could be envisaged in order to establish *in vitro* replication models for human and bovine prions.

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7 ABBREVIATIONS

aa	amino acid
Arg	arginines
Asn	asparagines
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
bp	base pair
BSA	bovine serum albumin
BSE	bovine spongiform encephalopathy
CJD	Creutzfeldt-Jakob disease
CMF-PBS	calcium- and magnesium-free PBS
CMV	cytomegalovirus
CNS	central nervous system
DCs	dendritic cells
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylene di-amine tetra-acetate
EEG	electroencephalogram
EM	electron microscopy
FACS	fluorescent activated cell sorting
FCS	Fetal calf serum
FDCs	follicular dendritics cells
FFI	fatal familial insomnia
Fig.	Figure
FITC	fluorescence isothiocyanate
$GABA_A$	gamma-aminobutyric acid type A
GFP	green fluorescence protein
GSS	Gerstmann-Sträussler-Scheinker disease
GPI	glycosylphosphatidylinositol
HPTLC	high-performance-thin-layer-chromatography
HRP	horseradish-peroxidase
Hsp	heat shock protein

i.c.	intracerebrally
IEM	immuno electron microscopy
IF	immunofluorescence
Ig	immunoglobulin
IP	immunoprecipitation
kb	kilobasepairs
kDa	kilodalton
LDH	lactate dehydrogenase
LRS	local lymphoreticular system
Lys	lysines
MALT	mucosa-associated lymphoid tissue system
M cells	membranous epithelial cells
mRNA	messanger ribonucleic acid
MVB	multivesicular bodies
MVs	microvesicles
NB	Neurobasal Medium
NCAM	neuronal cell adhesion molecule
NMR	nuclear magnetic resonance
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PK	proteinase K
p.i.	post-infection
PMSF	phenylmethylsulfonylfluoride
PNS	peripheral nervous system
Prnp	mouse prion protein gene
PrP^C	cellular prion protein
$PrP^{0/0}$	PrP^C knockout (Zurich I)
PrP^{Sc}	pathogenic isoform of the prion protein
PrP*	infectious subspecies of the prion protein
PrP^U	unfolded intermediate of the prion protein
PSA	polysialyc acid
RML	Rocky Mountain Laboratory prion strain

RPE	R-phycoerythrin
RT	room temperature
SAF32	anti-PrP antibody binding the N-terminal region
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamid gel electrophoresis
SFM	serum-free medium
SOD1	superoxide dismutase
STI1	stress-inducible protein 1
SV40	simian virus 40
6H4	anti-PrP antibody binding epitope aa144-152
TAE	Tris-acetate-EDTA
Tris	Tris-(hydroxymethyl)-aminomethane
ts	temperature sensitive
TSE	transmissible spongiform encephalopathies
UV	ultraviolet
vCJD	new variant CJD
WB	Western blot
wt	wild-type

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