Probing reaction conditions and cofactors of conformational prion protein changes underlying the autocatalytic self-propagation of different prion strains

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Summary

Prions are the causative agent of transmissible spongiform encephalopathies in animals and humans such as scrapie, bovine spongiform encephalopathy (BSE) and Creutzfeldt-Jakob disease (CJD). Prions are thought to be composed essentially of a misfolded and aberrantly aggregated isoform of the cellular prion protein (PrP) and to replicate by seeded PrP polymerization. Prions replicate without a nucleic acid genome. However, prions derived from the same cellular prion protein may exist in the form of distinct strains that differ in their phenotypic characteristics. It is thought that prions encode strain specific information in distinct protein conformations and/or by interaction with cofactor molecules. Against this background the present study examined the effects of different cofactor molecules on the proteinaceous seeding activity of four hamster adapted prion agents (263K, BSE-H, ME7-H and 22A-H) using serial protein misfolding cyclic amplification (PMCA) as *in vitro* assay for PrP misfolding and aggregation. The study revealed strain dependent differences of PMCA conditions and cofactors required for efficient *in vitro* replication. PMCA had to be specifically adapted for each prion strain. The EDTA concentration and incubation times per PMCA cycle were found to be of great significance for efficient amplification. In turn, addition of selected metal ions basically inhibited amplification. All prion strains showed a general requirement for nucleic acids during PMCA. However, under nucleic acid deficiency the prion strains exhibited different abilities to use certain polyanions for the reconstitution of PrP conversion in PMCA assays. The impact of cofactors was assessed by comparative analyses of selected biological, biochemical and biophysical properties of PMCA products (PrPres) and native prion seeds (PrP^{Sc}). The biological seeding activity as monitored in a primary hamster glial cell assay, and biochemical properties such as electrophoretic migration in SDS-gels, were affected differently by different cofactors. FT-IR spectroscopy was used in an analytical pilot study to structurally characterize PrPres from PMCA products in order to investigate if varying cofactor conditions during PMCA caused detectable alterations in protein conformations. Largely preliminary data revealed spectral differences between parent and progeny PrPres for all prion strains, but no variations due to different cofactor conditions. In this study a novel PMCA derived BSE-H isolate that replicated efficiently in the absence of nucleic acids (and without the addition of other cofactors) was generated. Infectivity of this novel isolate in the cell assay suggested that it may be infectious in animals. Furthermore, metal ions were applied for the first time in a long-term assay (approx. 80 days) to glial cells to investigate metal effects on prion propagation. Metal ions selectively inhibited the seeding activity of 263K prions in PMCA as well as in the cell assay. Finally, a novel approach for the assessment of prion infectivity in unknown samples by jointly performed PMCA- and cell assay titrations without the need of bioassays in laboratory rodents was presented in this work. This will contribute to a reduction and replacement of animal bioassays in prion research.

Zusammenfassung

Prionen sind das infektiöse Agens transmissibler spongiformer Enzephalopathien von Tieren und Menschen, wie beispielsweise der Scrapie, bovinen spongiformen Enzephalopathie (BSE) und Creutzfeldt-Jakob-Krankheit (CJD). Prionen bestehen hauptsächlich, wenn nicht ausschließlich, aus einer abnormal gefalteten und aggregierten Isoform des zellulären Prionproteins (PrP). Die Replikation von Prionen findet mutmaßlich durch keiminduzierte Polymerisation („seeded polymerization“) des Prionproteins statt. Prionen replizieren ohne genomische Nukleinsäuren, jedoch ist die Existenz verschiedener Prionstämme bekannt, die unterschiedliche stammartige Eigenschaften aufweisen, aber vom selben zellulären Prionprotein abstammen können. Es wird vermutet, dass stammspezifische Informationen durch die jeweilige Konformation des Proteins und/oder durch Interaktion mit Kofaktoren definiert werden. In dieser Arbeit wurde die Auswirkung von verschiedenen Kofaktoren auf die Keimaktivität („seeding activity”) von vier Hamster-adaptierten Prionstämmen (263K, BSE-H, ME7-H und 22A-H) *in vitro* mittels der Methode der „Protein Misfolding Cyclic Amplification“ (PMCA) untersucht. Dabei wurden stammabhängige Unterschiede bezüglich der Anforderungen an die *in vitro* Replikationsbedingungen in der PMCA, sowie Kofaktor-Selektivitäten festgestellt. Die PMCA-Bedingungen wurden für jeden Prionstamm angepasst. Um eine effiziente Amplifikation zu erreichen, waren die EDTA Konzentration und die Inkubationszeit pro PMCA-Zyklus von entscheidender Bedeutung. Im Gegenzug führte der Zusatz von bestimmten Metallionen im Wesentlichen zu einer Hemmung der Amplifikation. Alle Stämme zeigten eine generelle Abhängigkeit von Nukleinsäuren in der PMCA. Jedoch wurden unter Bedingungen von Nukleinsäuremangel Stammunterschiede im Hinblick auf die Rekonstitution der Amplifikation mit bestimmten polyanionischen Kofaktoren beobachtet. Der Einfluss von Kofaktoren wurde durch den Vergleich ausgewählter biologischer, biochemischer und biophysikalischer Eigenschaften von *in vitro* erzeugten PMCA Produkten (PrPres) mit denen nativer Prionkeime (PrP^{Sc}) untersucht. Dabei wurde beobachtet, dass Kofaktoren Stammeigenschaften, wie die biologische Keimaktivität in primären Hamster-Gliazellkulturen und biochemische Eigenschaften, wie die Migration in SDS-Gelen, beeinflussen können. Um festzustellen, ob unterschiedliche Kofaktorbedingungen während der PMCA messbare Veränderungen der Proteinkonformation hervorrufen, wurde PrPres von PMCA Produkten mittels FT-IR Spektroskopie in einer Pilotstudie charakterisiert. Erste Untersuchungen zeigten spektrale Unterschiede zwischen den Proteinkeimen und deren PMCA Produkten bei allen Stämmen. Konformationelle Änderungen bedingt durch unterschiedliche Kofaktoren konnten zunächst nicht festgestellt werden. Außerdem wurde in dieser Arbeit mittels PMCA ein neues BSE-H Agens, welches vollkommen unabhängig von Nukleinsäuren replizierte, erzeugt. Dieses Agens war im Zellassay infektiös, was darauf hindeutet, dass es auch in Tieren infektiös sein könnte. Weiterhin wurden in dieser

Arbeit erstmals Metallionen in einem Langzeitversuch in einem Gliazell-Assay für Prionen eingesetzt. Metallionen inhibierten die Keimaktivität von 263K Prionen sowohl in der PMCA als auch im Zell-Assay selektiv. Schließlich, konnte ein neuer Ansatz zur Bestimmung von Prioninfektiosität in unbekannten Proben durch parallel durchgeführte PMCA- und Zell-Assays unter Verzicht auf Tierversuche aufgezeigt werden. Dies liefert einen Beitrag zur Reduktion und zum Ersatz von Tierversuchen in der Prionforschung.

Content

1. Introduction

Transmissible spongiform encephalopathies (TSEs), or prion diseases, are a group of fatal and infectious neurodegenerative disorders caused by prions that affect many mammalian species [Prusiner, 1982; Prusiner, 1998]. They include Creutzfeldt-Jakob disease (CJD) and its variant (vCJD), Fatal Familial Insomnia (FFI), Gerstmann-Sträussler-Scheinker syndrome (GSS) and kuru in humans, scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, and chronic wasting disease (CWD) in deer and elk. Prion diseases occur in sporadic, hereditary and acquired forms. TSEs are characterized, in general, by cognitive impairments, extensive brain damage and neuronal dysfunction which lead to the loss of motor control, paralysis, and dementia. A spongiform degeneration (vacuolation) is accompanied by gliosis, loss of neurons as well as pathological deposits of prion protein (PrP) as found by microscopic examination of the CNS. The key event in TSE development is the conformational transition of the cellular prion protein (PrP^{C}) into an abnormal misfolded and aggregated isoform (PrP^{Sc}).

The first cases of scrapie, a TSE that affects sheep and goats, were observed in the United Kingdom (UK) in the 18^{th} century. The name scrapie is derived from the observation that affected animals show compulsive scraping due to an itching sensation. It has been recognized early, around 1759, that scrapie is infectious. However, its experimental transmissibility has been shown not before 1936. Other clinical signs include excessive lip smacking, abnormal gait, tremors, weight loss and behavioral changes such as aggression or apprehension, sensitivity to noise and movement [Hörnlimann *et al.*, 2007].

The prion disease kuru among the Fore people in Papua New Guinea was observed in the 1950s. The Fore people did practice a ritualistic cannibalism, that resulted in transmission of the disease and killed a large number of the local population [Gajdusek and Zigas, 1957]. Creutzfeldt-Jakob disease was first reported in 1920 [Creutzfeldt, 1920; Jakob, 1921]. A connection between scrapie, kuru and CJD was made in 1959. In 1966, the transmissibility of kuru was established by passaging the disease to intracerebrally inoculated chimpanzees and two years later, the transmission of CJD to chimpanzees after intracerebral inoculation was reported (for review see: [Prusiner, 1998]).

Human prion diseases can arise as sporadic, hereditary or acquired disease, with the latter being caused by infection. The forms of CJD are briefly explained in the following paragraphs.

Most cases of CJD are sporadic forms (sCJD). Sporadic forms are assumed to be of an endogenous origin, which is however unknown. This origin could be a somatic mutation of the PrP gene (PRNP), or a spontaneous conversion of PrP^{C} into PrP^{Sc}. sCJD occurs usually at advanced age (≥ 60 years).

Familial CJD (fCJD) is an inherited prion disease. Different mutations in the human PrP gene have been linked to this disease form and are believed causative.

Iatrogenic forms of CJD occurred due to improperly sterilized medical instruments or grafts from infected and undetected donors. Young adults were reported to have developed CJD after treatment with human growth hormone or gonadotropin derived from infected and undetected donors.

A new form of human prion disease, termed variant CJD (vCJD), appeared in the UK approximately 10 years after the outbreak of the BSE epidemic. First patients were observed in Britain in 1994. In contrast to sCJD which occurs usually at a median age of 66 years, vCJD patients are on average 28 years old when symptoms occur. The occurrence of vCJD was ascribed to the consumption of BSE infected meat products. The BSE epidemic was caused by meat and bone meal that was fed primarily to dairy cows. The meat and bone meal was prepared from the offal of sheep, cattle, pigs and chickens. In the late 1970s, altered production methods for meat and bone meal used lower temperatures and shorter incubation times than before, and omitted fat extraction as done before. Since scrapie had occurred in the UK before, it is thought that these changes in the production process allowed scrapie prions from sheep to "survive" and to be transmitted into cattle. However, the real origin of BSE in cattle has not been definite. Alternatively, bovine prions (presumably of sporadic origin) might have been present and would be distributed by the feeding practices of meat and bone meal. Due to regulations, e.g. the ban to feed meat and bone meal to sheep and cattle since 1988, the BSE epidemic declined since 1993. In 2013, there have been only two BSE cases in the UK (October 2013), whereas over 37 000 cases were reported in 1992 [OIE, 2013]. Due to the restricted geographical occurrence and chronology of BSE and vCJD, as well as experimental evidence as to the similarity of the causative agents, it is assumed that BSE is the origin of human vCJD [Hörnlimann *et al.*, 2007; Prusiner, 1998]. vCJD has been reported with 177 cases in the UK so far, among which none patient is still alive (October 2013 [NCJDRSU, 2013]). There has not been a German case of vCJD so far. Furthermore, 3 secondary cases of vCJD in the UK were reported. These patients had probably become infected by blood transfusions from individuals who developed vCJD after the blood donation. The experimental transmission of BSE by blood among primates was reported in 2002 [Bons *et al.*, 2002; Zou *et al.*, 2008].

Since long incubation times were observed in TSEs, it was originally believed that the causative agent was a ''slow virus'' [Sigurdsson, 1954]. However, the agent related to

TSEs was highly resistant to inactivation by treatments that affect nucleic acids, such as ionizing radiation and ultraviolet light as well as inactivation by chemicals and heat treatments. Such treatments would effectively inactivate conventional pathogens, such as viruses and bacteria. Attempts to identify nucleic acids as part of the infectious agent were not successful, and it was suggested that a protein alone could possibly transmit TSEs and replicate in infected individuals in the absence of nucleic acids [Alper *et al.*, 1967; Griffith, 1967; Hunter *et al.*, 1976]. In the 1980s, the neuroscientist Stanley Prusiner characterized the infectious agent and demonstrated that infectivity of scrapie was reduced by procedures that modify proteins. He introduced the concept of the "prion" and coined the term derived from "**pro**teinaceous **in**fectious particle" to distinguish the TSE agent from conventional pathogens, such as viruses and bacteria. According to the "protein only" hypothesis the infectious agent of TSEs is composed mainly, if not entirely, of PrP^{Sc}, an aggregated misfolded conformer of the normal host glycoprotein PrP^{C} [Prusiner, 1982]. Interaction of PrP^{Sc} with PrP^{C} induces a structural transition in the latter which is refolded into the β-sheet enriched, aggregated PrP^{Sc} conformer. In this way, the pathogenic PrP^{Sc} replicates and accumulates without a nucleic acid genome.

1.1 Structure of PrP^{C} and PrP^{Sc}

The cellular prion protein PrP^{C} is ubiquitary expressed in mammals. PrP^{C} is encoded by the *Prnp* (mice) / *PRNP* (humans) gene, which is localized on chromosome 20 in humans and highly conserved among mammals. Human PrP consists of 253 amino acids. It is synthesized on the rough endoplasmic reticulum and transits through the Golgi apparatus to the cell surface. During protein processing the signal peptide comprising amino acids 1-22 is cleaved off. A glycosyl-phosphatidyl-inositol (GPI) anchor at the carboxy-terminus of the protein attaches it to the outer surface of the cell membrane. In addition to the GPI-anchored form of PrP also transmembrane forms were reported, and an incorporation into lipid rafts which are regions of the membrane enriched in cholesterol and sphingolipids was shown [Linden *et al.*, 2008; Stewart and Harris, 2003]. A disulfide bond exists between Csy179 and Cys214. Further posttranslational modifications include the glycosylation of Asn181 and Asn197 with complex glycans resulting in non-, mono- or diglycosylated PrP forms. The mature protein has a molecular mass of about 33-35 kDa.

PrP^{C} is expressed in many tissues of the central and peripheral nervous system, the immune system, lymphoreticular and intestinal system [Dodelet and Cashman, 1998; Linden *et al.*, 2008; Shi *et al.*, 2009b]. PrP^{C} is predominantly expressed in neurons pre- and post-synaptically and is also found in glial cells [Linden *et al.*, 2008].

The amino-terminus of PrP^C contains a flexible random coil sequence whereas a globular domain is found carboxy-terminally [Riek *et al.*, 1997]. The unstructured amino-terminus comprises a segment of five repeats of an eight amino acid sequence (PHGGSWGQ), the octapeptide repeat region. The globular domain of human PrP^C is arranged in three α-helices and a β-sheet formed by two antiparallel β-strands [Riek *et al.*, 1996]. The disulfide bond interconnects helices H2 and H3. The structure of human cellular PrP was obtained by nuclear magnetic resonance (NMR) [Zahn *et al.*, 2000] and is depicted in Figure 1-1.

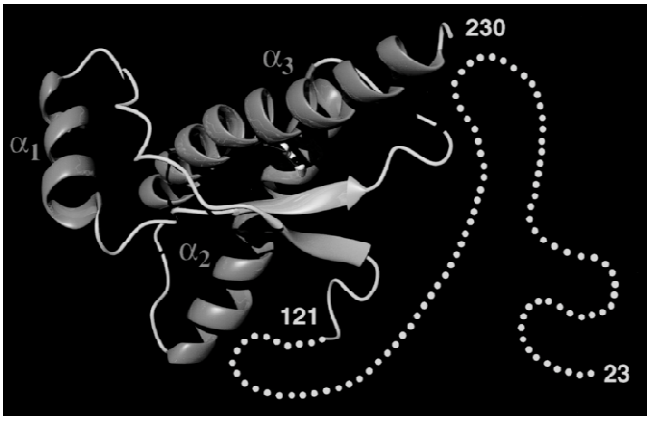

Figure 1-1: Structure of human PrP^C [Zahn et al., 2000]. The amino-terminus contains a flexible sequence that comprises the octarepeat region whereas the globular domain comprises three α-helices and an antiparallel β-sheet. Helix 2 and helix 3 are interconnected by a disulfide bond.

PrP^C and PrP^{Sc}, the pathological isoform, possess identical primary amino acid sequences and share the same posttranslational modifications. However, both forms of the protein have different biochemical and biophysical properties, such as solubility and susceptibility to proteinase K (PK). These characteristics arise from different secondary, tertiary and aggregate structures and are utilized to distinguish both isoforms [Hörnlimann *et al.*, 2007]. PrP^C is a monomeric protein and soluble in non-denaturing detergents. In contrast, PrP^{Sc} is oligo- or polymeric and insoluble in detergent. While PrP^C is rapidly degraded by PK, PrP^{Sc} is partially resistant to proteolysis and is therefore, in a biochemical sense, also referred to as PrPres[1]. PK digestion of PrP^{Sc} results in formation of a protease resistant core fragment, designated as PrP 27-30

[1] PrP27-30 is used to designate the PK-resistant core of PrP^{Sc} originating from TSE-infected animals or humans; PrPres designates, in a biochemical sense, PK-resistant forms of PrP; thus PrPres includes both PrP27-30 as well as PK-resistant PMCA (protein misfolding cyclic amplification, see chapter 1.4) products. While PrP27-30 is per se associated with infectivity, PrPres may or may not be associated with infectivity.

according to its approximate molecular mass of 27-30 kDa, which is generated by amino-terminal truncation around amino acid 90 [Prusiner *et al.*, 1983]. PrP 27-30 conveys prion infectivity. However, under specific conditions PrP^{Sc} was also found to be sensitive to PK [Safar *et al.*, 2005].

As PrP^{Sc} forms aggregates, is insoluble and not amenable to crystallization, its three-dimensional structure could not be resolved so far by high-resolution techniques such as X-ray diffraction and NMR. Fourier-transform infrared (FT-IR) spectroscopy and circular dichroism (CD) studies showed that PrP^{C} has a high content of α-helices (42 %) and only a minor β-sheet content (3 %) [Pan *et al.*, 1993]. However upon the conversion from PrP^{C} to PrP^{Sc} the content of β-sheet increases dramatically to about 43 % while the content of α-helices decreases to about 30 % [Caughey *et al.*, 1991; Safar *et al.*, 1993]. Electron crystallographic analysis of 2D crystals of PrP 27-30 allowed a modeling of PrP^{Sc} structure. Govaerts *et al*. [Govaerts *et al.*, 2004] proposed that monomers attain a left-handed, β-helical fold with two remaining α-helices. The left-handed β-helices readily form trimers that assemble in a disc structure. Stacking of those discs leads to assembly into prion fibrils (Figure 1-2).

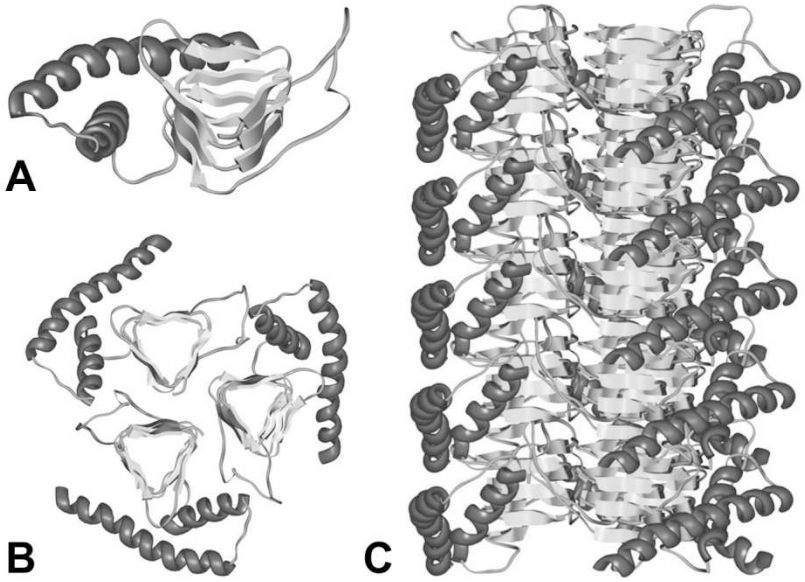

Figure 1-2: Model of PrP^{Sc} structure [Govaerts et al., 2004]. (A) Model of a PrP 27-30 monomer. (B) Trimeric model of PrP 27-30. (C) Model of a PrP 27-30 fiber built up by assembly of five trimeric discs.

1.2 Physiological functions of PrP

Despite many efforts to elucidate the physiological role of PrP^C a precise function of normal PrP has not been determined so far. PrP^C is highly expressed in the CNS of mammals and its sequence is highly conserved throughout different mammalian species which implies an important functional role. However, PrP knockout mice basically showed a normal development and no striking pathology. Older mice showed demyelination in the peripheral nervous system without clinical symptoms [Bueler *et al.*, 1992; Manson *et al.*, 1994]. The behavior was normal except for alterations in circadian rhythm and sleep pattern. PrP deficient mice were resistant against infection with Scrapie prions [Bueler *et al.*, 1993; Weissmann and Flechsig, 2003], which shows that PrP^C must be present in order to develop a TSE. Many different molecules are known to interact with PrP^C and could be involved in its function. Many proposed functions of PrP are attributed to its property as a metal binding protein. The protein was speculated to have a function as metal transporter, since metal levels in brains of knockout mice, prion infected mice and wild type mice were different. Copper was found to modulate the expression of PrP^C. A function in the uptake of copper and iron and thus a role in cellular copper and iron metabolism was suggested. A loss of function due to aggregation of PrP^C has been thought to disturb cellular iron and copper homeostasis, resulting in neurotoxicity [Hodak *et al.*, 2009]. As PrP^C is present at pre- and post-synaptic sites of neurons it was suggested to function in the binding and reuptake of copper into cells after the release of copper into the synaptic cleft upon depolarization [Linden *et al.*, 2008]. Additionally, since PrP^C is localized to the lipid membrane, a role as membrane receptor and involvement in signal transduction has been hypothesized. Furthermore, PrP^C can protect against oxidative damage and a function as an antioxidant has been discussed. However, a function as cell adhesion molecule, a role in cell communication and as signal transducer were discussed as well. Moreover, the protein has been discussed to be involved in cell proliferation and differentiation, regulation of cellular homeostasis, growth and function of synapses (reviewed by [Choi *et al.*, 2006; Linden *et al.*, 2008]).

1.3 Models for the conversion of PrP^C to PrP^{Sc}

The precise mechanism for the propagation and spread of prions *in vivo* has not been determined so far. However, the conformational change from cellular PrP to its pathological isoform PrP^{Sc} is considered as an essential event in prion replication. In the following the two mainly discussed models for prion replication are introduced.

According to Stanley Prusiner and colleagues [Prusiner, 1991] prions may multiply by template-directed refolding. This concept is referred to as heterodimer model (Figure 1-3A). According to this model, the spontaneous conversion from PrP^C into PrP^{Sc} in the absence of PrP^{Sc} is a very rare event and prevented by a high energy barrier. However, when PrP^{Sc} is present a heterodimer with PrP^C may be formed. The rearrangement of the structure of PrP^C is then induced and thus the heterodimer becomes a homodimer. Formation of homodimers from PrP^C has been observed, but a heterodimer could not be detected experimentally [Hörnlimann *et al.*, 2007].

Alternatively, the model of nucleation-dependent, or seeded, PrP polymerization has been proposed [Jarrett and Lansbury, 1993]. According to this concept, PrP^C and PrP^{Sc} exist in their monomeric forms in an equilibrium that is however shifted towards PrP^C (Figure 1-3B). An initial oligomerization of PrP^{Sc} occurs very slowly. However, highly ordered oligomeric structures stabilize the PrP^{Sc} conformation. If such an oligomer is present, further PrP^{Sc} monomers are recruited that become incorporated into the growing aggregates and thus promote the further conversion of PrP^C into PrP^{Sc}. This eventually leads to the formation of amyloid fibrils. PrP^{Sc} aggregates that are capable to promote PrP^C conversion into PrP^{Sc} are referred to as seeds or nuclei. According to this hypothesis monomeric PrP^{Sc} is not infectious, only its highly ordered aggregates bear infectivity. Fragmentation of aggregates generates novel nuclei that can recruit more PrP^{Sc} monomers and thus propagate PrP^{Sc} aggregation. The generation of novel seeds from pre-existing aggregates by fragmentation is termed secondary fragmentation.

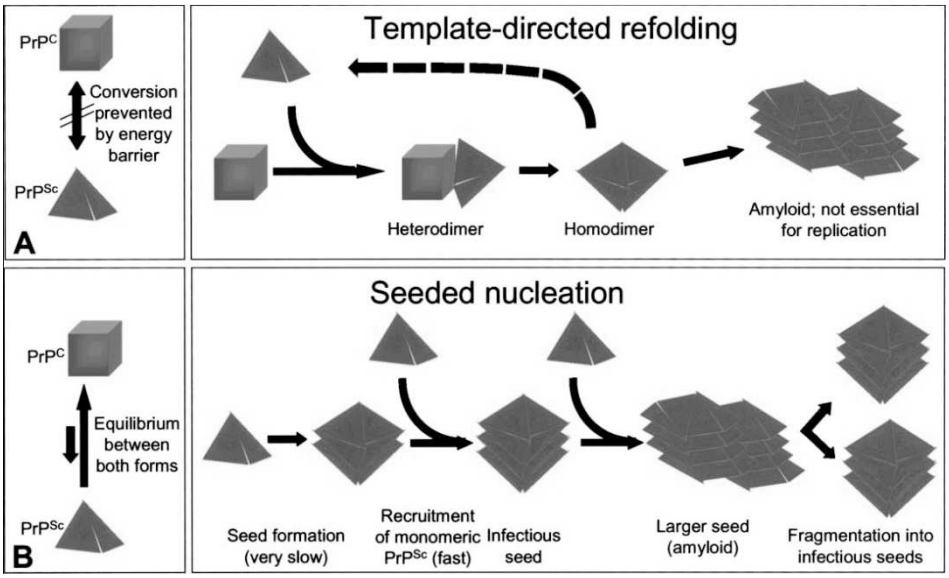

Figure 1-3: Models for conversion of PrP^C into PrP^{Sc} [Aguzzi and Polymenidou, 2004]. (A) The conversion from PrP^C into PrP^{Sc} is induced by template-directed refolding when a heterodimer between PrP^C and PrP^{Sc} is formed. Spontaneous conversion from PrP^C to PrP^{Sc} in the absence of PrP^{Sc} is a very rare event and occurs very slow. (B) Seeded nucleation model. PrP^C and PrP^{Sc} exist in an equilibrium that is shifted towards the side of PrP^C. The formation of highly ordered PrP^{Sc} aggregates leads to recruitment, stabilization and incorporation of monomeric PrP^{Sc} and aggregate growth.

1.4 *In vitro* amplification of prions by protein misfolding cyclic amplification (PMCA)

In 2001 Claudio Soto and colleagues presented a method to amplify misfolded prion protein *in vitro* by a technique called protein misfolding cyclic amplification (PMCA) [Saborio *et al.*, 2001]. In PMCA minute amounts of brain PrP^{Sc} are incubated in an excess of PrP^C. The method utilized brain homogenate (BH) from healthy, uninfected hamsters and terminally diseased animals (PrP^{Sc}) as PrP^C and seed sources, respectively, and is thus a cell-free system. In repetitive cycles of incubation and sonication PK resistant aggregates are formed. According to the concept of "seeded polymerization" PK resistant aggregates are being produced by the recruitment of PrP^C molecules and their incorporation into growing PrP^{Sc} aggregates.

Incubation was performed at 37 °C (body temperature) for 30 minutes. During this phase PrP^{Sc}-seeds become elongated (Figure 1-4). As instable monomeric PrP^{Sc} or an intermediate form of partially misfolded PrP^C may be recruited and incorporated into PrP^{Sc}-oligomers the pathological conformation is stabilized. Polymerization occurs at the ends of PrP^{Sc}-seeds. The subsequent sonication brakes down large aggregates into smaller oligomers or polymers and thus multiplies the number of existing PrP^{Sc}-seeds by secondary nucleation. Novel PrP^{Sc}-seeds in turn promote further polymerization and

PrP conversion. In this manner, under optimal PMCA conditions, an exponential multiplication of conversion active seeds occurs. Cycles of incubation and sonication can be repeated as often as required in an automated manner using programmable ultrasonic devices [Castilla *et al.*, 2006]. By this way of accelerated prion replication with PMCA even minute amounts of PrP^{Sc} seeds can be amplified to become detectable, in the form of PrPres, by Western blotting.

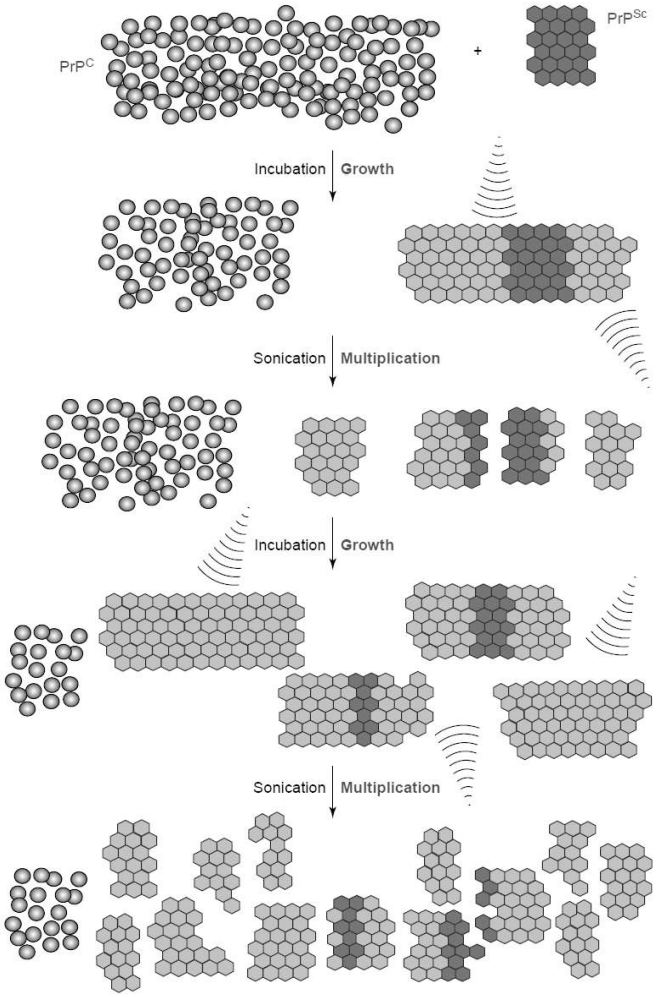

Figure 1-4: Protein misfolding cyclic amplification [Soto et al., 2002]. Minute amounts of PrP^{Sc} seeds are incubated at 37 °C in an excess of PrP^{C}. During incubation PrP^{Sc}-aggregates recruit PrP^{C} molecules which become incorporated, in a converted misfolded conformation, into the oligomer/polymer leading to growing aggregates. Ultrasound brakes down large aggregates into smaller ones and thus multiplies the number of PrP^{Sc}-seeds. Cycles of incubation and sonication are repeated several times. In this manner minute quantities of PrP^{Sc} seeds become amplified to detectable levels by Western blot.

A substantial improvement of the PMCA technique was the serial PMCA (sPMCA) [Bieschke *et al.*, 2004] that was subsequently automated by Castilla *et al*. (2005a). After several amplification cycles an aliquot of the reaction mixture is diluted into fresh

normal brain homogenate and subjected to a new round of alternating amplification cycles. By performing sequential passages, the initial seeding material is diluted with every passage step. It was shown that PrP^{Sc} can be propagated in principle infinitely in serial PMCA. Which demonstrates that the newly generated PK resistant PrP amplificate, that can be detected in the Western blot as PrPres, is able to autocatalytically promote PrP misfolding [Bieschke *et al.*, 2004; Castilla *et al.*, 2005a]. *In vitro* generated PrPres was shown to have similar properties as PrP 27-30 from infectious brain homogenate. Such properties were assessed by biochemical methods which include electrophoretic mobility, glycosylation profile, resistance to PK and resistance to denaturation with guanidine hydrochloride. Furthermore, it was demonstrated that PMCA PrPres is infectious to wildtype laboratory rodents, which developed disease upon experimental transmission. By analysis of behavior, neurological and biochemical properties, the disease was not distinguishable from infections with prion brain homogenate [Castilla *et al.*, 2008b; Weber *et al.*, 2006]. It was reported that infectious PrP^{Sc} particles of a minimal size of approximately 26 molecules are required to induce a specific and reproducible amplification in PMCA to enable detection [Saa *et al.*, 2006].

Since PrP^{Sc} is the only validated surrogate marker for prion diseases their diagnosis is often based on the detection of PrP^{Sc} in tissue samples. In this regard PMCA has been successfully applied to detect the presence of PrP^{Sc} in blood, urine and feces of scrapie infected hamsters [Castilla *et al.*, 2005b; Gonzalez-Romero *et al.*, 2008; Kruger *et al.*, 2009]. Furthermore, PMCA could detect infections in pre-symptomatic animals [Soto *et al.*, 2005]. The detection of prions in milk from sheep infected with scrapie before clinical onset of disease by PMCA was also reported [Maddison *et al.*, 2009]. PMCA is a promising tool not only for the diagnosis of TSEs but also for the identification and evaluation of inhibitors for prion replication in regard to novel therapeutic targets. PMCA has been also shown to be a valuable tool to study prions in regard to achieve a better understanding of the molecular nature of the infectious prion agent. PMCA has been used to study the strength and molecular determinants of transmission barriers between different species [Barria *et al.*, 2011; Castilla *et al.*, 2008a]. Using purified PrP^{C} and PrP^{Sc} from the brains of healthy and diseased animals, respectively, Supattapone and co-workers could generate infectious PrPres in serial PMCA in the presence of synthetic poly(A) RNA anions [Deleault *et al.*, 2007]. PMCA has therefore been applied to study conversion factors that may be involved in prion propagation. Earlier, the same group had applied a modified PMCA assay that used BH and continuous shaking instead of ultrasound to study the effect of putative PrP conversion factors [Deleault *et al.*, 2003]. In other experiments, it was demonstrated that also bacterially expressed recombinant PrP (rPrP) could be used as substrate for PMCA [Atarashi *et al.*, 2007]. However, those PMCA amplificates were not or only at a very

low level infectious. Furthermore, it has been reported that reduced nicotinamide adenine dinucleotide phosphate (NADPH) enhanced the propagation of PrP^{Sc} in BH in PMCA [Shi *et al.*, 2009a]. Heparin was shown to enhance the *in vitro* propagation of vCJD-associated PrPres in PMCA which could have implications for the development of highly sensitive *in vitro* screening tests that are able to detect minute amounts of PrP^{Sc} in blood or other body fluids [Yokoyama *et al.*, 2011].

1.5 Cofactor molecules of PrP conversion

Since the conformational transition from PrP^{C} into PrP^{Sc} in the absence of PrP^{Sc} aggregates is hindered by a high energy barrier (see Figure 1-3), it has been suggested that other molecules are crucial for spontaneous (or genetically triggered) prion formation which may function as adjuvants that lower the energy barrier and thus catalyze or facilitate the PrP conversion reaction [Gomes *et al.*, 2012; Silva *et al.*, 2010]. Accordingly, such putative cofactors may also accelerate the conversion of PrP by PrP^{Sc} seeds, i.e. prion replication. The *in vitro* conversion of PrP in a cell-free system that used only purified proteins [Kocisko *et al.*, 1994] was less efficient than sPMCA that employed crude brain homogenates [Castilla *et al.*, 2005a]. This indicated that additional factors were required for efficient conversion and that brain homogenate is likely to contain such factors. Furthermore, *in vitro* produced protease-resistant prion protein generated from recombinant prion protein carries none or only very low infectivity [Hill *et al.*, 1999; Makarava *et al.*, 2010]. As an explanation, PrPres composed of rPrP may just not have an infectious conformation, however, cofactors may also be required for conveying infectivity [Klingeborn *et al.*, 2011].

1.5.1 Non-metal cofactors for prion conversion

Many potential ligands for PrP^{C} have been identified, of which cellular adhesion molecules, extracellular matrix molecules, glycosaminoglycans (GAGs), and nucleic acids have emerged as candidates for conversion factors. Recently, polyanions, such as nucleic acids and GAGs, and furthermore lipids have been found to influence prion propagation and infectivity.

Proteoglycans and GAGs have been implicated in the pathogenesis of prion diseases [Ben-Zaken *et al.*, 2003; Caughey and Raymond, 1993; Shaked *et al.*, 2001; Wong *et al.*, 2001b] and have been detected in amyloid plaques of prion diseases [McBride *et al.*, 1998; Snow *et al.*, 1989; Snow *et al.*, 1990]. Proteoglycans are glycoproteins that consist of a core protein which is covalently linked to one or more glycosaminoglycan chains. GAGs are linear polyanionic polysaccharides composed of a repetitive

disaccharide unit that can be sulfated. They include heparin, heparan sulfate, chondroitin sulfate or pentosan sulfate. GAGs are involved in numerous biological activities and functions, including cell-cell and cell-matrix interactions, regulation of cell growth and proliferation, roles in brain development and wound repair [Capila and Linhardt, 2002; Silva *et al.*, 2010].

Various studies have demonstrated that prion conversion did not occur when purified PrP^{C} and PrP^{Sc} were mixed and incubated *in vitro* [Abid *et al.*, 2010; Deleault *et al.*, 2010; Saborio *et al.*, 1999]. Conversion was only observed when other cellular components were present. Supattapone and colleagues have shown that various polyanions, such as different kinds of nucleic acids (mammalian and invertebrate RNA, synthetic RNA and DNAs) and proteoglycans (heparan sulfate proteoglycan) can function as cellular conversion factors to enable $PrP^{C} \rightarrow PrP^{Sc}$ conversion by PMCA of infectious material [Deleault *et al.*, 2005; Deleault *et al.*, 2007; Deleault *et al.*, 2003; Geoghegan *et al.*, 2007]. It has been shown that treatment of PrP^{C} substrates (e.g. normal brain homogenate) with various enzymes that degrade nucleic acids abolish PrP^{Sc} amplification. It was furthermore demonstrated that the addition of RNA molecules isolated from hamster brain reconstituted the amplification of PrPres in PMCA samples which were pre-treated with nucleases. Another study showed that heparan sulfate and pentosan sulfate stimulated the cell-free conversion of PrP [Wong *et al.*, 2001b]. Cofactor molecules were proposed to act as scaffolds or surfaces that facilitate interaction between PrP^{C} and PrP^{Sc} molecules and in this way accelerate the conversion process [Wong *et al.*, 2001b]. Later it was hypothesized that polyanionic compounds were able to stimulate PrPres amplification *in vitro* by mimicking negatively charged surfaces of specific accessory proteins and that such proteins would facilitate prion propagation [Deleault *et al.*, 2005]. Wang *et al*. succeeded in generating infectious synthetic prions from recombinant PrP by PMCA when lipids and mouse-extracted RNA molecules were present as cofactors [Wang *et al.*, 2010].

Lipids are another type of cofactors that were shown to promote prion propagation in cell-free conversion assays [Baron and Caughey, 2003]. Lipids, particularly sphingomyelin, galactosylceramide and cholesterol, were detected in scrapie hamster prions purified from infected hamster brain [Klein *et al.*, 1998; Riesner, 2003]. However, preparations with high infectivity titers contained fewer lipid molecules than low titer samples. That phospholipids may be crucial for replication of infectious mouse scrapie-associated PrPres by PMCA was indicated by several different recent reports [Deleault *et al.*, 2012a; Deleault *et al.*, 2012b; Wang *et al.*, 2010].

The precise mechanism by which cofactors influence prion formation, propagation and infectivity is still unclear. Cofactors could be involved in several steps of PrP conversion. As cofactor binding to PrP^{C} can alter its conformation in terms of an

increased β-sheet content [Sanghera *et al.*, 2009; Wang *et al.*, 2007], it is conceivable that PrP^C may be rendered susceptible to conversion upon cofactor binding. As mentioned above, cofactors may also facilitate PrP conversion by concentrating both PrP^{Sc} and PrP^C in close proximity on the surface of membranes or single polyanion molecules. Furthermore, cofactors could stabilize infectious PrP conformations by forming a complex with PrP^{Sc} or being a part of the infectious particle [Ma, 2012]. Possible roles by which cofactors might participate in prion replication are depicted in Figure 1-5.

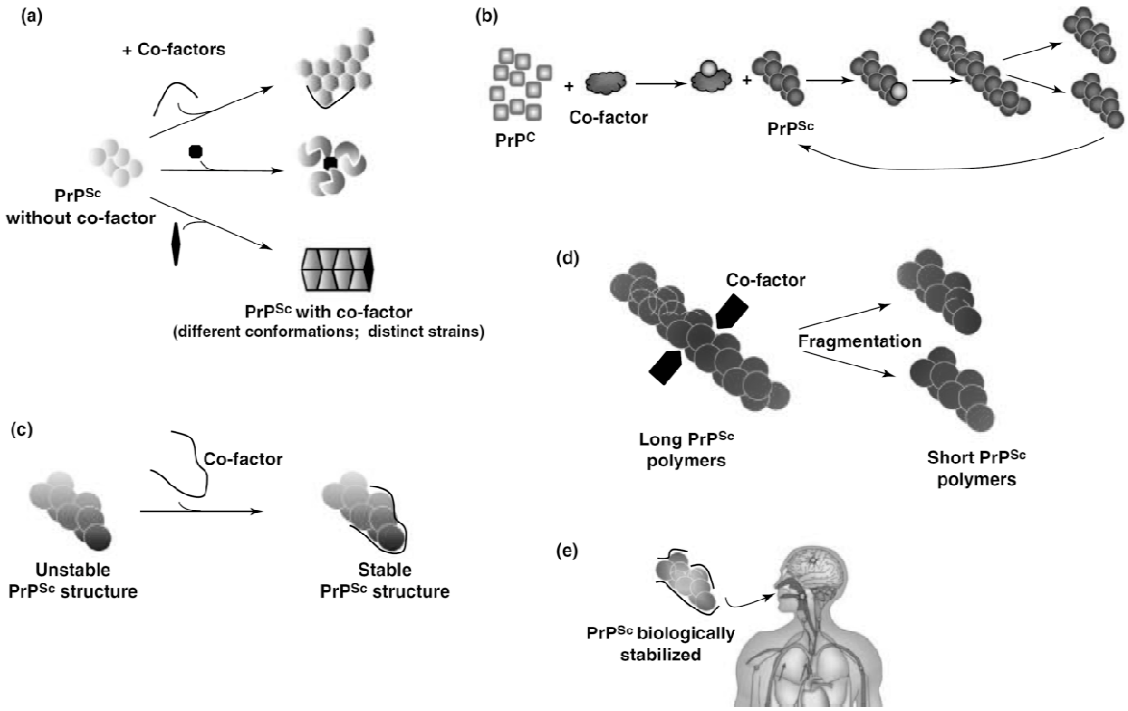

Figure 1-5: Possible roles by which cofactors might participate in prion replication [Soto, 2011]. (a) Cofactors could become incorporated into the infectious agent, and could thus determine the folding characteristics of prions. (b) Cofactors could act as essential catalysts for prion replication or (c) cofactors could stabilize the conformation of PrP^{Sc}. (d) Cofactors might participate in the process of PrP^{Sc} polymer-fragmentation and thus help to multiply the number of seeds that catalyze prion replication. (e) Cofactors might also increase the biological stability of prions, thereby reducing their in vivo clearance and increasing its chances to reach target organs.

1.5.2 Metal ions as cofactors for prion conversion

In the search for modulators and causes of prion diseases metal ions increasingly became a target in research. The prion protein is known to bind divalent metal ions such as copper, zinc and manganese and thus may function as a metal binding protein or metal transporter. Metals are bound preferentially to the octapeptide repeat sequence (PHGGSWGQ) towards the N-terminus of PrP. Binding affinities increase from manganese to zinc to copper [Jackson *et al.*, 2001]. Diseased individuals were found to have an altered brain metal content with differences in iron, lower copper and higher manganese levels [Wong *et al.*, 2001a]. An increase of total iron level and a change in the Fe^{2+}/Fe^{3+} ratio in favor of Fe^{3+} was observed in scrapie infected mice [Kim *et al.*, 2000]. Higher levels of Mn^{2+} were observed in whole brain, mitochondria and scrapie associated fibril (SAF) enriched fractions of diseased animals [Kim *et al.*, 2005]. It was suggested that Mn^{2+} may participate in the PrP conversion mechanism *in vivo* and in the pathogenesis of prion diseases. It was shown that PrP^{C} normally binds Cu^{2+} ions *in vivo*, whereas PrP^{Sc} becomes depleted of Cu^{2+} and enriched in Mn^{2+} and to a lesser extent of Zn^{2+} [Brown *et al.*, 1997; Thackray *et al.*, 2002; Wong *et al.*, 2001a]. A loss of function due to aggregation of PrP^{C} possibly disturbs cellular iron and copper homeostasis and may be neurotoxic [Hodak *et al.*, 2009].

Metal ions are usually strictly regulated and bound to proteins. However, as described in the literature about 300 µM zinc [Assaf and Chung, 1984; Frederickson and Bush, 2001; Howell *et al.*, 1984] are transiently present as free ions in the synaptic cleft in the brain, and low concentrations of free copper are also found in the extracellular space [Rana *et al.*, 2009; Schlief *et al.*, 2005]. Metal concentrations in the brain are in a range of 70 µM Cu^{2+}, 350 µM Zn^{2+} and 340 µM Fe^{2+} [Bush and Tanzi, 2008; Rana *et al.*, 2009]. It was however reported that Cu^{2+} concentrations of 15 µM are released within the synaptic cleft during synaptic vesicle release and higher concentrations between 100 and 300 µM are achieved during neuronal depolarization [Vassallo and Herms, 2003]. As PrP^{C} is known to be partly localized near neuronal synapses and was suggested to be involved in the regulation of Zn^{2+} and Cu^{2+} homeostasis a disruption of this function may lead to neuronal damage [Choi *et al.*, 2006; Rana *et al.*, 2009].

Many studies focused on the effects of metal ions on the induction of PK resistance, PrP refolding, β-sheet- and fibril formation. The results are often contradictory, which implies a complex role of metal ions in the pathogenesis of prion disease, and so far it could not be determined whether the observed metal imbalances in prion diseased brains are a cause or a result of PrP conversion and aggregation (reviewed by [Choi *et al.*, 2006; Rana *et al.*, 2009; Singh *et al.*, 2010]). Earlier studies reported that the application of copper chelators resulted in a spongiform-like degeneration in the brain of laboratory animals [Blakemore, 1972; Pattison and Jebbett, 1971], and later it was found that oral administration of copper prolonged scrapie incubation times in hamsters

[Hijazi *et al.*, 2003]. In contrast to that, others reported a delay in the onset of the disease when chelating copper [Sigurdsson *et al.*, 2003]. Using *in vitro* PMCA studies, Kim *et al*. showed that conversion of PrP^{C} from normal hamster brain homogenate into PrPres could be induced by Mn^{2+}, Cu^{2+} and Fe^{2+} [Kim *et al.*, 2005]. Further results have also shown that manganese could induce a strong pro-aggregatory effect on PrP [Giese *et al.*, 2004]. Moreover, besides copper also zinc was reported to enhance the aggregation of a PrP derived peptide [Jobling *et al.*, 2001]. However, an inhibition of PrPres amplification and PrP conversion into amyloid fibrils by copper and zinc was shown as well [Bocharova *et al.*, 2005; Orem *et al.*, 2006]. Another very recent publication reported that an internalization, accumulation and conversion of exogenous recombinant PrP into PK resistant PrPres was induced in cells by Fe^{3+} but not Fe^{2+} [Choi *et al.*, 2013].

1.6 Prion strains

Prions exist as multiple strains in a single mammalian species with identical amino acid sequences of PrP. Strains are associated with distinct characteristics, or phenotypes, by which they can be distinguished. For example, two prion strains (named Hyper and Drowsy) from mink have been isolated and serially passaged in outbred Golden Syrian hamsters. They produced a clinical disease with distinct phenotypes. While animals infected with Hyper showed hyperexcitability, hamsters infected with Drowsy exhibited progressive lethargy. Prion strains can vary in incubation times, clinical manifestations, neuropathological characteristics e.g. patterns of PrP^{Sc} deposition and vacuolation profiles in the brain. They can also differ in terms of their biochemical characteristics such as electrophoretic mobility and glycosylation of PrP^{Sc}, or its resistance to detergents and proteases (Figure 1-6). Strain properties are usually maintained upon serial passage in the same host species. Conventional pathogens encipher strain information in the nucleic acid genome. In the context of the prion hypothesis it was proposed that strain diversity can be enciphered in different protein conformations or aggregation states that can faithfully replicate at the expense of host PrP^{C} [Bessen and Marsh, 1992; Collinge, 2010; Safar *et al.*, 1998; Telling *et al.*, 1996]. However the molecular principles of the strain phenomenon have not yet been completely elucidated. It has been also proposed that PrP^{Sc} molecules might bind to other specific host molecules to generate strain diversity. Such molecules could be polyanions or lipids [Geoghegan *et al.*, 2007]. Furthermore, it is not clear whether posttranslational PrP modifications (such as glycosylation) are a determinant of strain properties [Cancellotti *et al.*, 2010; Piro *et al.*, 2009; Tuzi *et al.*, 2008].

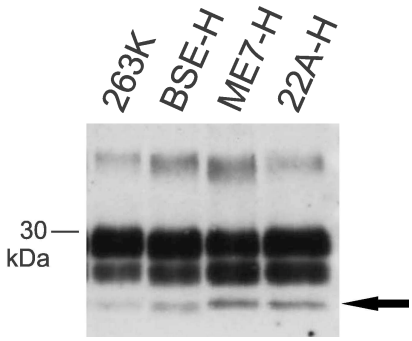

Figure 1-6: Biochemical differences of PrP 27-30 from Syrian hamsters that were infected with 263K scrapie, BSE-H, ME7-H scrapie or 22A-H scrapie. BSE-H shows a different electrophoretic mobility of the unglycosylated band (arrow) due to different molecular masses of approx. 20 kDa for 263K, ME7-H and 22A-H and 19 kDa for BSE-H. Furthermore, ME7-H shows in this blot a different glycosylation profile from 263K. While the diglycosylated band is predominant in 263K, the monoglycosylated band is predominant in ME7-H.

1.7 Objectives

The mechanism of prion propagation and the involvement of other molecules than PrP in the replication process are still poorly understood.

In this research project the recently introduced method of PMCA which allows an *in vitro* replication of prions should be used to study the role of putative cofactor molecules (e.g. nucleic acids) and the mechanisms involved in the propagation of misfolded and aggregated prion proteins. In order to provide a better understanding of the molecular basis of the "prion strain phenomenon", i.e. the existence of distinct prion strains that differ in their phenotypic characteristics although they are derived from the same cellular prion protein, the role of cofactor molecules in the propagation of four different hamster adapted prion agents were to be examined.

To assess the impact of cofactor molecules on *in vitro* generated PrPres, the biological, biochemical and biophysical properties of PMCA derived PrPres should be investigated and compared to the native prion seeds. As a measure for the biological infectivity, the biological seeding activity of native prion seeds and their progeny PrPres should be monitored *in vitro* by testing the ability to infect glial cell cultures. In order to define the impact of putative cofactors on the molecular conversion of PrP in more detail, changes in the spatial structure associated with different cofactor molecule conditions during amplification of PrPres in PMCA should be monitored by Fourier transform-infrared (FT-IR) spectroscopic analysis.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals and reagents

Table 2-1: Chemicals and reagents.

Reagent	*Manufacturer*
2-Mercaptoethanol	Sigma-Aldrich (Steinheim, D)
Acetic acid	Sigma-Aldrich (Steinheim, D)
Acrylamide/Bisacrylamide (Rotiphorese® Gel 40)	Roth (Karlsruhe, D)
Ammonium hydroxide (NH_4OH)	Merck (Darmstadt, D)
APS (Ammonium persulfate)	Roth (Karlsruhe, D)
Bromphenol blue	Sigma-Aldrich (Steinheim, D)
C16 Galactosyl(ß) Ceramide (d18:1/16:0)	Avanti Polar Lipids, Inc. (Alabaster, USA)
Chondroitin sulfate A	Sigma-Aldrich (Steinheim, D)
Citric acid	Sigma-Aldrich (Steinheim, D)
Copper(II) chloride ($CuCl_2$)	Sigma-Aldrich (Steinheim, D)
D-Glucose	Sigma-Aldrich (Steinheim, D)
EDTA (Ethylenediaminetetraacetic acid)	Sigma-Aldrich (Steinheim, D)
Ethanol	Roth (Karlsruhe, D)
Formaldehyde (37 %)	Roth (Karlsruhe, D)
Formic acid	Roth (Karlsruhe, D)
Glutaraldehyde (25 %)	Merck (Darmstadt, D)
Glycerol	Serva (Heidelberg, D)
Guanidinium thiocyanate	Merck (Darmstadt, D)
Iron(II) chloride ($FeCl_2$)	Sigma-Aldrich (Steinheim, D)
Iron(III) chloride ($FeCl_3$)	Merck (Darmstadt, D)
Magnesium chloride	Roth (Karlsruhe, D)
Manganese(II) chloride ($MnCl_2$)	Sigma-Aldrich (Steinheim, D)
Methanol	Roth (Karlsruhe, D)
Monosodium phosphate (NaH_2PO_4)	Sigma-Aldrich (Steinheim, D)
N-Lauroylsarcosine Na-salt (Sarkosyl)	Serva (Heidelberg, D)
PMSF (Phenylmethylsulfonyl fluoride)	Sigma-Aldrich (Steinheim, D)
Poly(A) RNA	Sigma-Aldrich (Steinheim, D)
Poly-L-glutamate	Sigma-Aldrich (Steinheim, D)
Potassium chloride (KCl)	Merck (Darmstadt, D)
SDS (Sodium dodecyl sulfate)	Serva (Heidelberg, D)
Silver nitrate ($AgNO_3$)	Merck (Darmstadt, D)
Skimmed milk powder	Sucofin

Sodium bicarbonate ($NaHCO_3$)	Merck (Darmstadt, D)
Sodium chloride (NaCl)	Roth (Karlsruhe, D)
Sodium hydroxide (NaOH)	Merck (Darmstadt, D)
Sphingomyelin	Sigma-Aldrich (Steinheim, D)
Sucrose	Sigma-Aldrich (Steinheim, D)
TEMED (N,N,N,N,-Tetramethylendiamin)	Roth (Karlsruhe, D)
Tris base (Trizma® base)	Sigma-Aldrich (Steinheim, D)
Triton X-100	Sigma-Aldrich (Steinheim, D)
Tween-20	Serva (Heidelberg, D)
Zinc chloride ($ZnCl_2$)	Sigma-Aldrich (Steinheim, D)
Zwitterionic detergent 3-14 (Z3.14)	Sigma-Aldrich (Steinheim, D)

2.1.2 Buffers and solutions

Table 2-2: Buffers and solutions.

General buffers and solutions	
PBS (phosphate buffered saline)	8 mM Na_2HPO_4 1.5 mM KH_2PO_4 137 mM NaCl 2.7 mM KCl pH 7.4 with HCl
SDS/NaOH for general disinfection	0.3 % (w/v) NaOH 0.2 % (w/v) SDS pH ≈ 13
TBS	50 mM Tris 150 mM NaCl pH 7.5 with HCl
PMCA	
Conversion buffer	4 mM EDTA 1 % (v/v) Triton X-100 1 Protease Inhibitor Cocktail Complete Mini (Roche) in 10 ml buffer PBS pH 7.4 with HCl
Protein purification for FT-IR	
Homogenization buffer	10 % Sarkosyl 8 mM NaH_2PO_4 in 0.2 mM Tris/HCl pH 7.5

SDS-PAGE	
Electrophoresis running buffer	25 mM Tris % (w/v) SDS 192 mM Glycine pH 8.3 – 8.7
Sample loading buffer	125 mM Tris 4 % (w/v) SDS 20 % (v/v) Glycine 0.05 % (w/v) Bromphenol blue 10 % (v/v) Mercaptoethanol pH 6.8 with HCl
Separating gel (12.5 %)	4.3 ml ddH_2O 2.5 ml Separating gel buffer (pH 8.8) 3.1 ml Acrylamid/Bisacrylamid 37.5:1 (40 %) 50 µl SDS (20 %) 30 µl APS (10 %) 10 µl TEMED
Separating gel (15 %)	3.65 ml ddH_2O 2.5 ml Separating gel buffer (pH 8.8) 3.75 ml Acrylamide/Bisacrylamide 37.5:1 (40 %) 50 µl SDS (20 %) 30 µl APS (10 %) 10 µl TEMED
Separating gel buffer	1.5 M Tris pH 8.8 with HCl
Stacking gel (5 %)	6.14 ml ddH_2O 2.5 ml Stacking gel buffer (pH 6.8) 1.25 ml Acrylamide/Bisacrylamide 37.5:1 (40 %) 50 µl SDS (20 %) 50 µl APS (10 %) 10 µl TEMED
Stacking gel buffer	500 mM Tris pH 6.8 with HCl
Western blotting and PrP detection	
Assay buffer	100 mM NaCl 100 mM Tris pH 9.5 with HCl
Blocking buffer	3 % (w/v) Skimmed milk powder 0.05 % (w/v) Tween 20 TBS

Blotting buffer	48 mM Tris 38 mM Glycine 0.037 % (w/v) SDS pH ~ 9,0 20 % (v/v) Methanol (added prior to use)
TBS-T	0.05 % (w/v) Tween 20 TBS

2.1.3 Cell culture equipment and media

Table 2-3: Cell culture equipment and media.

6-well plates (8.96 cm^2/well)	TPP (Trasadingen, CH)
Cell culture flasks (175 cm^2)	Nunc (Roskilde, DK)
Cell scraper	TPP (Trasadingen, CH)
Cell strainer, pore size 40 µm	BD Falcon (Bedfort, USA)
CO_2-Incubator Hera Cell 150i	Thermo Scientific (Langenselbold, D)
Dimethyl sulfoxide (DMSO)	Biochrom AG (Berlin, D)
Dulbecco`s modified eagle medium (DMEM) without $NaHCO_3$, with 4.5 g/l D-glucose	Biochrom AG (Berlin, D)
Fetal Bovine Serum (FBS-Superior)	Biochrom AG (Berlin, D)
Freezing container (Nalgene Mr. Frosty)	Thermo Scientific (Langenselbold, D)
Freezing medium	70 % (v/v) DMEM 20 % (v/v) FBS 10 % (v/v) DMSO
Glial cell isolation medium (GIM)	18.2 % (v/v) FBS 0.5 % (v/v) D-glucose 2.7 mM sodium bicarbonate 1.8 mM glutamine 90.1 U/ml / 90.1 µg/ml Penicillin/Streptomycin 0.5 µg/ml Partricin DMEM
Growth medium (GM)	10 % (v/v) FBS 2 mM glutamine 100 U/ml / 100 µg/ml Penicillin/Streptomycin 0.5 µg/ml Partricin DMEM
L-glutamine (200 mM)	Biochrom AG (Berlin, D)
Neubauer hemocytometer	Paul Marienfeld (Lauda-Königshofen, D)

Partricin (50 µg/ml)	Biochrom AG (Berlin, D)
PBS/glucose	0.6 % (v/v) D-glucose 100 U/ml /100 µg/ml Penicillin/Streptomycin 0.5 µg/ml Partricin PBS
Penicillin/Streptomycin (10.000 U/ml /10.000 µg/ml)	Biochrom AG (Berlin, D)
Serological pipettes	TPP (Trasadingen, CH)
Trypan blue 0.5 %	Biochrom AG (Berlin, D)
Trypsin/EDTA (0,05 %/0,02 %)	Biochrom AG (Berlin, D)

2.1.4 Antibodies, enzymes and kits

Table 2-4: Antibodies, enzymes and kits.

Product	*Manufacturer*
Anti-PrP antibody 3F4 monoclonal (mouse)	In-house cell culture [Kascsak *et al.*, 1987]
Benzonase	Merck (Darmstadt, D)
CDP-Star® Tropix	Applied Biosystems (Foster City, USA)
Goat Anti-Mouse IgG/AP	Dako (Hamburg, D)
PNGase F	New England Biolabs (Beverly, USA)
Proteinase K (*Tritachium album*)	Roche (Mannheim, D)
RNaseA	Qiagen (Hilden, D)

2.1.5 Consumables

Table 2-5: Consumables.

Product	*Manufacturer*
Blot membrane PVDF (Polyvinylidene fluoride), pore size 0.45 µm	Millipore (Neu-Isenburg, D)
Blotting filter paper	Schleicher Schuell (Kent, UK)
Disposable 20 gauge needles	Braun (Emmenbrücke, CH)
Disposable syringes Omnifix®-F, 1 ml	Braun (Bad Arolsen, D)
Glass beads, ø 0,75 – 1 mm	Roth (Karlsruhe, D)
Paraffin Roti® Plast	Roth (Karlsruhe, D)
Precast gels Roti® PAGE 12 %	Roth (Karlsruhe, D)
Protease Inhibitor Cocktail Complete Mini	Roche (Mannheim, D)

Protease Inhibitor Cocktail Complete Mini, EDTA-free	Roche (Mannheim, D)
Reaction tubes 1,5 ml tubes Safe Lock Ultracentrifuge tubes	 Brand (Wertheim, D) Eppendorf (Hamburg, D) Beckman Coulter (Fullerton, USA)
X-ray film Amersham Hyperfilm™ ECL	GE Healthcare Limited (Buckinghamshire, UK)

2.1.6 Laboratory devices

Table 2-6: Laboratory devices.

Device	*Manufacturer*
Centrifuges Heraeus Biofuge pico Heraeus Labofuge 400 Heraeus Sepatech Varifuge 3.0R Optima Max Ultracentrifuge	 Thermo Scientific (Langenselbold, D) Thermo Scientific (Langenselbold, D) Heraeus (Osterode, D) Beckman Coulter (Fullerton, USA)
Electrophoresis Power Supply Phero-Stab 0312	 Biotec Fischer (Reiskirchen, D)
Electrophoresis system Mini Protean 3 and Mini Protean Tetra Cell	 Bio-Rad (München, D)
Fast Blot	Biometra (Göttingen, D)
FT-IR spectrometer	Bruker Optics GmbH (Ettlingen, D)
Gyro Rocker SSL3	Bibby Scientific (Staffordshire, UK)
Inverted microscope	Leica Microsystems (Wetzlar, D)
pH meter pH 211 Microprocessor	 Hanna Instruments (Aachen, D)
PMCA devices Sonicator 3000-MPD, 4000-MPX and Q500 with microplate horn	 Misonix (Farmingdale, USA) / Qsonica, LLC. (Newtown, USA)
Potter Homogeniser	BBraun (Melsungen, D)
Probe sonicator Sonopuls HD 2070	 Bandelin (Berlin, D)
Scanner HP Scanjet G 4050	 Hewlett Packard (Palo Alto, CA, USA)
Thermomixer Compact	Eppendorf (Hamburg, D)
X-ray developer Curix 60	 Agfa (Düsseldorf, D)

2.1.7 Software

Table 2-7: Software.

Corel DRAW	Corel (Ottawa, Kanada)
HP Scan	Hewlett Packard (Palo Alto, CA, USA)

2.1.8 Animals

All studies that involved animals or the use of material from animals were performed with strict observance of the legal regulations (e. g. the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and the German Animal Welfare Act) and where necessary on the basis of formally permitted animal experimentation. Euthanasia of normal animals was voluntarily reported to the local animal protection authority.

Golden Syrian hamsters (*Mesocricetus auratus*) of both sexes were purchased from Charles River (Sulzfeld, Germany).

2.1.9 Prion agents

Hamster-adapted prion agents 263K scrapie, 22A-H scrapie, ME7-H scrapie and BSE-H were used for the experiments in this work. The TSE-infected brain tissue was obtained from terminally diseased Syrian hamsters that had been intracerebrally inoculated with hamster brain homogenates from clinically diseased donors [Thomzig *et al.*, 2004]. Scrapie strain 263K showed a mean incubation time of 83 days in Syrian hamsters after intracerebral inoculation of 50 µl of 10 % brain homogenate from a terminally diseased donor animal. Hamster-adapted strains ME7-H and 22A-H originated from mice and were passaged in Syrian hamsters [Kimberlin *et al.*, 1989] with a mean incubation time of 331 days and 206 days, respectively. BSE-H is a hamster-adapted BSE strain that had been isolated from a cow in Germany and was firstly transmitted to mice. Subsequently, the strain was passaged in Syrian hamsters with a mean incubation time of 287 days [Thomzig *et al.*, 2004].

These strains differ markedly in their incubation times. Whereas 263K scrapie and BSE-H could be distinguished from ME7-H and 22A-H by neurological and behavioral symptoms, neuropathological lesion profiles, pattern of cerebral PrP^{Sc} distribution, electrophoretic mobilities and glycosylation pattern of PrP^{Sc}, 22A-H and ME7-H were indistinguishable by these methods.

2.2 Methods

2.2.1 Preparation of normal hamster brains

Brains from normal Syrian hamsters aged 12-15 weeks were used as donors for PrP^C containing substrate for PMCA experiments. Adult hamsters were euthanized by exposure to CO_2 and neonatal hamsters were sacrificed by decapitation. To remove blood from the tissue, adult animals were transcardially perfused with approx. 40 ml of 5 mM EDTA/PBS (pH 7.4) after cardiac and breathing arrest. The brains were removed, cut into pieces with an exposable scalpel, immediately frozen in liquid nitrogen and stored at -70 °C until use.

2.2.2 Preparation of 10 % (w/v) brain homogenate from normal hamster brains

10 % (w/v) brain homogenate from normal hamsters (in the following referred to as NBH) in conversion buffer was used as PrP^C substrate for PMCA. For cell culture experiments tissue homogenizations were performed in PBS or TBS. To minimize protein degradation processes in crude brain homogenate due to active enzymes all work was performed quickly on ice. Brains were homogenized in cooled conversion buffer containing protease inhibitors. To prevent contamination of the homogenate only sterile filtertips were used. Cylinder and plunger of the potter homogenizer were treated 5 min with a solution of 0.2 % SDS/0.3 % NaOH for general disinfection prior to use. 5 ml conversion buffer was added immediately to one hamster brain. Subsequently, brains were homogenized with the potter homogenizer. To get a 10 % (w/v) brain homogenate, conversion buffer was added to a final volume of 10 ml per one hamster brain (which weighs approx. 1 g). The homogenate was subjected to centrifugation at 3000 rpm (Varifuge 3.OR Heraeus) and 4 °C for 5 min to remove cell debris. The clarified supernatant was pooled and stored in aliquots at -70 °C until use.

2.2.3 Preparation of 10 % (w/v) brain homogenate from TSE-infected hamster brains

Infected hamster tissue was processed in a biosafety cabinet only. Brain homogenate from TSE-infected tissue was used as source for PrP^{Sc}-seeds in PMCA and cell culture experiments. Brains were thawed quickly at room temperature and cut into small pieces using an exposable scalpel. 4 ml PBS were added and the material was homogenized by ultrasound using a probe sonicator. PBS was added to a final volume of 10 ml and aliquots were stored at -70 °C until use. The micro tip of the sonicator was treated with 2 M NaOH and rinsed with *aqua dest*. prior and after each use.

One brain (about 1 g) of a diseased Syrian hamster in the clinical stage of disease contains about 100 µg PrP^{Sc} [Beekes *et al.*, 1996]. Brain homogenates from 263K, ME7-H, 22A-H and BSE-H were digested with proteinase K (PK) and serial dilutions were subjected to SDS-PAGE and Western blotting. This revealed that all brain homogenates contained similar concentrations of PrPres.

2.2.4 Protein Misfolding Cyclic Amplification (PMCA)

The basic principle of the PMCA technology has been published previously [Bieschke *et al.*, 2004; Castilla *et al.*, 2006; Murayama *et al.*, 2006; Saborio *et al.*, 2001; Weber *et al.*, 2006]. Within the scope of this dissertation exclusively serial automated PMCAs (saPMCA) with a serial transmission of PMCA product into fresh brain homogenate from normal hamsters according to Bieschke *et al*. (2004) were performed. 10 % (w/v) NBH from Syrian hamsters was used as PrP^{C} containing substrate for PMCA experiments. A basic protocol that was recently published [Pritzkow *et al.*, 2011] was utilized for the propagation of 263K by PMCA. PMCA protocols for other prion strains 22A-H scrapie, ME7-H scrapie and BSE-H had to be adapted individually.

2.2.4.1 Basic protocol for 263K scrapie

To prevent cross contamination of samples during PMCA only sterile filtertips were used. PMCA experiments were performed in 0.5 ml Safe Lock Eppendorf tubes that are particularly leakproof and that had been subjected to steam sterilization at 121 °C and 3 bar for 20 min prior to use. Approximately 30 µl of glass beads (equivalent to 10 mg) were filled into each reaction tube. Glass beads had been washed five times with distilled water and subsequently had been also subjected to steam sterilization at 121 °C and 3 bar for 20 min prior to use. 140 µl of NBH were filled into each reaction tube. An aliquot of 10 % (w/v) 263K brain homogenate was quickly thawed and sonicated for 40 sec at about 200 W using the waterbath of the PMCA ultrasound device. 10 % (w/v) 263K brain homogenate was serially diluted in NBH to obtain the final dilutions which were used for PMCA seeding. 10 µl of the diluted 263K brain homogenate were added to the reaction tubes. The specific dilution of 263K brain homogenate that was used to seed each individual PMCA reaction is indicated for each experiment.

The caps of all reaction tubes were sealed with parafilm tape and liquefied paraffin wax. Thereafter, reaction tubes were disinfected in 0.2 % SDS/0.3 % NaOH for 5 min. Samples were transferred to a tube holder designed to fit in the reservoir of the microplate horn of the sonicator device. To prevent cross contamination during automated processing within the sonication apparatus samples were placed in a 4 M guanidinium thiocyanate filled reservoir. After an initial sonication step at approx.

200 W for 40 sec, samples were incubated at 37 °C for one hour followed by a next cycle of sonication and incubation. Unless stated otherwise one round of PMCA consisted of approx. 24 alternating cycles of sonication and incubation of samples at 37 °C. Upon completion of one PMCA round (approx. 24 h) samples were disinfected again in 0.2 % SDS/0.3 % NaOH for 5 min and spun down gently (Heraeus Labofuge 400). Subsequently, reaction mixtures were collected without the need to open the reaction tube. Disposable 20 gauge needles were used to puncture the cap of reaction tubes. For each reaction tube a new needle and syringe was used. Gloves were changed always before handling a new sample. For a next round of PMCA, aliquots of 30 µl harvested reaction mixtures were diluted with 120 µl fresh NBH (1:5 dilution) in a new reaction tube. Samples were subjected to PMCA as described above. Additionally 30 µl of the harvested reaction mixtures were collected for limited proteolysis with PK and subsequent analysis by Western blotting (chapter 2.2.5 and 2.2.7 ff.).

2.2.4.2 Adapted protocol for other prion strains

Amplification efficiency of all prion strains was found to be increased in the presence of 20 mM EDTA in NBH, instead of 5 mM. Therefore, also 263K was propagated in the presence of elevated EDTA concentrations, unless stated otherwise. Furthermore, a cyclic incubation time of 2 h instead of 1 h was beneficial for amplification of ME7-H and BSE-H. Due to this prolonged incubation time one round of PMCA (approx. 24 h) was reduced to 12 alternating cycles. Amplification of the 22A-H prion agent was performed with an incubation time of 4 h and 12 cycles, hence one PMCA round lasted approx. 48 h.

2.2.4.3 Substrate preparations for PMCA with putative cofactor molecules of PrP conversion

Nucleic acids in NBH used as substrate for PMCA assays were removed by enzymatic digestion with benzonase. Aliquots of the prion seeding material were also treated with benzonase for two hours prior to performing PMCA. Benzonase degrades all kinds of nucleic acids (single-stranded, double-stranded, linear, circular or supercoiled DNA and RNA). NBH was prepared in conversion buffer without EDTA. NBH was incubated for approx. 20 h with 2 mM $MgCl_2$ and 2.5 U/µl benzonase at 37 °C and 450-500 rpm (Thermomixer comfort, Eppendorf). Addition of 20 mM EDTA to the substrate inhibited benzonase enzyme activity. Substrate was stored in aliquots at -70 °C until use.

2.2.5 Limited proteolysis with proteinase K (PK)

For the analysis of PrP^{Sc} /PrPres by Western blotting treatment of samples with PK is used as a standard tool to distinguish PrP^{C} and misfolded, aggregated forms of PrP. The cellular form of the prion protein is readily degraded by PK whereas aggregated and misfolded forms of the protein (PrP^{Sc}) maintain a PK-resistant core (at least for longer periods of time) under the same conditions. Under limited proteolysis PrP^{Sc} as well as prion progeny seeds amplified by PMCA are processed to N-terminally truncated protease resistant core fragments (referred to as PrP27-30 or PrPres, respectively). PrP27-30 /PrPres can be detected in Western blot using anti-PrP antibodies. It is visible as a typical three band pattern (diglycosylated band: 27-30 kDa, monoglycosylated band: 24-26 kDa, unglycosylated band: 19-21 kDa). Samples were digested at a final concentration of 150 µg/ml PK, 1 % (v/v) sarkosyl and 0.06 % (w/v) SDS and incubated for 1 h at 55 °C and 450-500 rpm (Thermomixer comfort, Eppendorf). Subsequently, samples were centrifuged at 13000 rpm (Heraeus Biofuge pico) and ambient temperature for 1 min. 30 µl aliquots of the supernatant were mixed with an equal volume of sample loading buffer (Table 2-2) and incubated for 10 min at 100 °C. The protease inhibitor PMSF was added to a final concentration of 2.5 mM. Samples were stored at -20 °C until further processing. Aliquots of 10 µl were subjected to SDS-PAGE and Western blotting.

2.2.6 Deglycosylation with PNGase F

The endoglycosidase PNGase F (Kit Biolabs) cleaves the Asn-linked oligosaccharide chains from the prion protein. Subsequently, the protein shows a single band upon detection with an anti-PrP antibody in Western blots. The unglycosylated band has a molecular mass in the range of 19-21 kDa. Samples were digested with PK for 1 h as described above (chapter 2.2.5). Subsequently, 2.2 µl 10 x denaturing buffer was added to 20 µl sample material and incubated at 100 °C and 450 rpm for 10 min (Thermomixer comfort, Eppendorf). Thereafter, 3 µl 10 x G7-buffer, 3 µl NP-40 and 3 µl PNGase F were added and incubated at 37 °C for 2 h while shaking. Samples were mixed with an equal volume of sample loading buffer and boiled for 10 min under shaking. The protease inhibitor PMSF was added to a final concentration of 2.5 mM. Samples were stored at -20 °C until further processing. Aliquots of 10 µl were subjected to SDS-PAGE and Western blotting.

2.2.7 SDS-PAGE

Proteins were separated according to their electrophoretic mobility by discontinuous, reducing SDS-polyacrylamide gel electrophoresis [Laemmli, 1970]. The anionic detergent SDS attaches to the polypeptide chain and supports its linearization and also confers a negative charge to the protein. Generally the binding of SDS to proteins is in proportion to their mass and thus they become separated by their approximate size during electrophoresis. The electrophoresis systems Mini Protean® 3 and Mini Protean® Tetra Cell (Bio-Rad) were used. In general 10 µl samples were loaded on the SDS-polyacrylamide gel. The composition of stacking gel (5 %) and separating gel (12.5 %) is listed in Table 2-2. A Tris-glycine buffer was used as running buffer for electrophoresis (Table 2-2). In addition to handcast gels precast gels "Roti® PAGE 12 %" (Roth) were used. When separating PNGase F treated samples a 15 % separating gel was loaded. Electrophoresis was performed at 15 mA for 15-20 min and 20 mA for approx. 30-45 min.

2.2.8 Western blotting and immunodetection

The separated proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane using a semi-dry electroblotting procedure [Seidel *et al.*, 2007; Thomzig *et al.*, 2003]. PVDF membranes were soaked in methanol for a few seconds and kept in blotting buffer afterwards. Two blotting filter paper were soaked in blotting buffer and placed onto the graphite surface (anode) of the blotting device, followed by the PVDF membrane. The SDS-polyacrylamide gel was removed from between the glass plates. The separating gel was washed in blotting buffer and placed onto the PVDF membrane followed by two more filter papers. Air bubbles were removed by moving a roller carefully over the stack. The cathode surface of the blotting device was placed on top closing the device. Proteins were transferred from the gel onto the PVDF membrane by a current of 180 mA for 25 min.

Detection of PrP was performed as described by Thomzig *et al*. (2003) and Seidel *et al*. (2007). The blocking of non-specific binding sites on the blot membrane was achieved by incubation in blocking buffer that contained 3 % skimmed milk powder for 1 h under continuous rocking. The monoclonal anti-PrP antibody 3F4 (in-house cell culture, [Kascsak *et al.*, 1987]) was diluted 1:2000 in blocking buffer and incubated with the blot membrane overnight. The 3F4 antibody recognizes amino acids 109-112 (Met-Lys-His-Met) of the human and hamster prion protein [Bolton *et al.*, 1991; Lowenstein *et al.*, 1990; Rogers *et al.*, 1991]. The blot membrane was washed three times in TBS-T and three times in blocking buffer for 5 min. The blot membrane was incubated for 1.5 h with the secondary goat anti-mouse antibody that was diluted 1:5000 in blocking buffer. The secondary antibody was conjugated to the reporter enzyme alkaline

phosphatase. After three washing steps in TBS-T, the blot membrane was incubated in blocking buffer for 1 h. For about 30 min the blot membrane was incubated in assay buffer that resulted in a more alkaline pH that is required for the alkaline phosphatase to be active. The blot membrane was dried with a filter paper and incubated 5 min in CDP-Star diluted 1:100 in assay buffer. Subsequently the blot membrane was dried with a filter paper and transferred to a film cassette. The enzyme catalyzes the dephosphorylation of the CDP-Star which results in light emission. Light emission was recorded on X-ray films that were placed directly above the blot membrane after 15 min and were allowed to rest for a few minutes up to 1 h. Developing of X-ray films was carried out fully automated (Curix 60, AGFA). Subsequently films were digitalized.

2.2.9 Silver staining

Silver staining of gels was performed according to Oakley's procedure [Oakley *et al.*, 1980]. Gels were placed in a glass plate and incubated for 30 min in 50 % methanol and 7.5 % acetic acid, 30 min in 5 % methanol and 7.5 % acetic acid. After a 30-minute incubation in freshly prepared 5 % glutaraldehyde, gels were rinsed once in ddH_2O and incubated in ddH_2O overnight at 4 °C. The next day gels were incubated for 15 min in a freshly prepared solution of silver nitrate (0.4 g $AgNO_3$ was dissolved in 2 ml ddH_2O and added dropwise under stirring to a solution of 10.5 ml 0.36 % NaOH, 0.7 ml 25 % NH_4OH and 36.7 ml ddH_2O). Subsequent procedures were carried out each in a new glass plate. The gels were washed twice for 2 min in ddH_2O and developed for 1-5 min in a fresh solution of 100 µl 5 % citric acid and 100 µl 37 % formaldehyde in 100 ml ddH_2O. The reaction was stopped by incubation in 5 % acetic acid for 10 min. Subsequently gels were rinsed twice for 2 min in ddH_2O. Gels were shrinkwraped in a clear plastic film and immediately digitalized.

2.2.10 Cell culture techniques

2.2.10.1 Preparation of hamster glial cell cultures

Isolation and culture of glial cells is based on a procedure from Lima and colleagues [Lima *et al.*, 2007] and was carried out with modifications as described by Pritzkow *et al*. (2011) and Boerner *et al*. (2013). All equipment was sterile and aseptic techniques were used where possible. For the preparation of glial cell cultures, normal neonatal Syrian hamsters were used as donors of brain tissue. Animals were sacrificed 2-3 days after birth by decapitation. Brains were removed and kept in PBS/glucose on ice until further processing. The cerebellum and the meninges were removed using a disposable

scalpel and a stereo microscope, and the remaining brain tissue was cut into pieces and kept in PBS/glucose afterwards. The tissue was centrifuged for 3 min at 1000 rpm (Varifuge 3.0 R Heraeus) and ambient temperature. Brain tissue was washed twice with 9 ml of glial cell isolation medium (GIM) and was subsequently resuspended in 5 ml fresh GIM. Using serological pipettes (5 ml and 2 ml pipettes) the tissue pellet was sheared carefully to separate cells until a homogeneous single cell suspension was obtained. The solution was drained trough a cell strainer (pore size 40 µm) to remove cell debris and was subjected to centrifugation. The pellet was resuspended in 10 ml growth medium (GM) and density of cells was determined by vital staining with trypan blue. Cell count was determined using a Neubauer hemocytometer. $3x10^6$ cells per one plastic cell culture flask (175 cm^2, NUNC) were grown in 20 ml GM. All culture incubations were performed in a humidified 37 °C, 5 % CO_2 atmosphere incubator. Cells were allowed to adhere to the bottom of the culture flask over night. The next day, cells were washed with 10 ml PBS to remove unattached cells. Incubation was continued for further seven days in fresh GM. Subsequently, cells were washed again with PBS and enzymatically detached from the bottom of the culture flask. Cells were incubated with 2.5 ml trypsin for approx. 5 min until they were floating. 20 ml GM was added and the cell suspension was subjected to a subsequent centrifugation to clear from trypsin. The cell pellet was resuspended in 10 ml GM and homogenized to obtain a single cell suspension using serological pipettes and a cell strainer. For cryopreservation, $3x10^6$ cells were diluted in 1 ml freezing medium per cryovial. Vials were cooled down over night to -70 °C using a freezing container with a cooling rate of -1 °C/min. The next day, cryovials were transferred to liquid nitrogen for long-term storage.

2.2.10.2 Plate cultivation

Cryovials were thawed quickly in order to avoid toxic effects of DMSO on cells and were diluted with 9 ml GM and subjected to centrifugation for 3 min at 1000 rpm (Varifuge 3.0 R Heraeus) and ambient temperature. Cells were resuspended in GM, separated and the number of vital cells was determined by staining with trypan blue using a hemocytometer as described above. For cell infection assays, cells were cultivated in 6-well plates at a density of $1.5x10^4$ cells per well and 3 ml GM for 2-3 days prior to infection.

2.2.10.3 Infection of glial cell cultures

Cell cultures were exposed to the indicated amounts of infectious or control hamster brain tissues or PMCA products. Cultures for negative controls were exposed to normal hamster brain tissue. Cells were inoculated with hamster brain tissue that was homogenized in PBS. PMCA products contained Triton X-100 and thus were toxic to cells. They were subjected to ultracentrifugation at 45000 rpm (Optima Max Ultracentrifuge, Beckmann Coulter) and 4 °C for 2.5 h. The pellet was resuspended in 100 µl PBS in a ultrasound waterbath. Cells were inoculated with 10 µl suspension per culture well that was added to the culture medium. After three days post initial exposure (DPE), the inoculum was removed and the cells were washed once with PBS. Subsequently, cells were cultured further and harvested at the indicated time points. During cultivation the medium was renewed once a week. Cells were examined regularly for vitality and microbial contaminants with an inverted microscope.

Metal ion solutions for cell culture applications were prepared with sterilized ddH_2O. When cells were cultured in the presence of metal ion solutions this was done at final concentrations of metal ions of 20 µM or 50 µM.

2.2.10.4 Harvesting

Cell cultures were harvested at the indicated DPE as previously described [Boerner *et al.*, 2013]. Cells were washed with PBS and detached with a cell scraper and collected in 1 ml PBS. Cells were pelleted by quick spin. The supernatant was discarded and the pellets were stored at -70 °C until further processing.

2.2.10.5 Limited proteolysis with PK

Pellets from cells were resuspended in 50 µl PBS and were subjected to enzymatic digestion with proteinase K. However, in the course of infection experiments it was found that the processing of cell products by PK can be optimized by resuspending pellets in NBH instead of PBS. In this way, the sensitivity of the detection of PrPres from cell cultures infected with BSE-H associated PrPres could be increased. Pellets were resuspended using a vortex mixer and additionally by two sonication steps at approx. 250 W for 40 sec in an ultrasound waterbath. Caps of reaction tubes were sealed tight with parafilm tape to prevent contamination of samples with water from the waterbath. 15 µl of the cell suspension were mixed with 3 µl PK and 1.5 µl sarkosyl for a final concentration of 150 µg/ml PK and 1 % (v/v) sarkosyl, respectively. Samples were incubated for 1 h at 37 °C and 450-500 rpm and were subsequently mixed with an equal volume of sample loading buffer (Table 2-2) and incubated for 10 min at 100 °C. 1 µl PMSF was added to a final concentration of 2.5 mM. In this way samples could be

stored at -20 °C prior to further processing. Samples were subjected to SDS-PAGE (chapter 2.2.7) and Western blotting (chapter 2.2.8) as described above.

2.2.10.6 Deglycosylation with PNGase F

Cell pellets were subjected to limited proteolysis with PK and subsequently deglycosylated with PNGase F as described in chapter 2.2.6.

2.2.11 Extraction of PrPres from PMCA products for Fourier transform-infrared (FT-IR) micro-spectroscopic analysis

PrPres was purified from PMCA products and analyzed by FT-IR micro-spectroscopy according to a protocol by Daus *et al*. (2013). This protocol allowed the extraction of PrP from very small amounts of PrPres starting material (such as PMCA aliquots). By the novel approach, minute amounts (i.e. 25-75 ng per dried sample spot) of extracted PrPres are sufficient for analysis by FT-IR micro-spectroscopy.

Approx. 1 ml of PMCA product was used for the extraction of PrPres for FT-IR measurements. This amount of PMCA product was obtained from the reaction mixtures of ten single PMCA samples after six PMCA rounds. These ten PMCA samples had been produced in one PMCA experiment under equal conditions for each sample and samples were pooled after each round of PMCA to ensure homogeneity between the samples. The pooled PMCA product was centrifuged for 1 min at 5000 rpm and ambient temperature. The supernatant was repeatedly centrifuged two times. Supernatant was mixed with homogenization buffer (Table 2-2) to a final volume of 10 ml and was allowed to incubate for 30 min and ambient temperature. The suspension was subjected to ultracentrifugation at 16000 rpm (rotor TLA-55) at 20 °C for 8 min. The clarified supernatant was mixed with 0.8 ml 0.1 M EDTA (pH 7.6) and was transferred to centrifuge tubes that were underlaid with 40 µl 20 % (w/v) sucrose and subjected to centrifugation at 55000 rpm at 20 °C for 52 min. Supernatant was discarded. With the pipette tip, clear yellowish pellets were resuspended carefully (by gentle stirring and up and down pipetting) in 30 µl 0.1 % (w/v) Z3.14/20 mM Tris/HCl (pH 7.5) and were transferred to a 15 ml falcon tube. In a volume of 2 ml pellets were resuspended by applying ultrasound from a probe sonicator repeatedly for about 5 sec to obtain a homogenous suspension. Afterwards, the volume was adjusted to 10 ml with 0.1 % (w/v) Z3.14/20 mM Tris/HCl (pH 7.5). The suspension was transferred to centrifuge tubes that were underlaid with 60 µl 20 % (w/v) sucrose and subjected to centrifugation at 55000 rpm at 20 °C for 35 min. Subsequently, pellets were collected in 30 µl 0.2 mM Tris/HCl (pH 7.5) and were resuspended until being homogenous in a volume of 2 ml in a falcon tube by the application of ultrasound.

Afterwards, the volume was adjusted to 6 ml with 0.2 mM Tris/HCl (pH 7.5) and the suspension was transferred to centrifuge tubes that were underlaid with 60 µl 20 % (w/v) sucrose and subjected to centrifugation at 55000 rpm at 20 °C for 35 min. Pellets were resuspended in 100 µl 0.2 mM Tris/HCl (pH 7.5) and 200 µl 20 mM Tris/HCl (pH 7.5). Using ultrasonic treatment, the suspension was homogenized. 0.6 µl 0.5M $MgCl_2$, 0.5µl RNaseA and 0.5µl benzonase were added and allowed to incubate under agitation (500 rpm thermomixer) and ambient temperature over night. The next day, 2 µl PK was added (final concentration of 2 µg PK) and incubated for 1 h at ambient temperature. 80 µl 0.1 M EDTA (pH7.6) was added and incubated further for 15 min. Afterwards, the suspension was transferred to two centrifuge tubes, 100 µl 0.2 mM Tris/HCl (pH 7.5) was added to a final volume of 1 ml and tubes were underlaid with 40 µl 20 % (w/v) sucrose. Tubes were centrifuged for 66 min at 55000 rpm and 4 °C. Subsequently, pellets were resuspended in 200 µl 0.2 mM Tris/HCl (pH 7.5) and were treated repeatedly with ultrasound to obtain a homogenous solution. The suspension was divided in 50 µl aliquots, 0.2 mM Tris/HCl (pH 7.5) was added to a final volume of 1 ml and the tubes were centrifuged at 55000 rpm and 4 °C for 2 h. Pellets were washed three times with 1 ml ddH_2O for 2 h at 55000 rpm and 4 °C. Subsequently one pellet of each purified PMCA product was analyzed by Western blotting and silver staining. Remaining pellets were stored at -20 °C. As determined by Western blot detection with the anti-PrP antibody 3F4, some extracts still contained full-length PrP, i.e. not proteolytically processed PrP. In order to improve digestion with PK in these samples, all remaining extracted pellets of the same PMCA product were pooled and were again subjected to the purification protocol starting with the PK digestion step. This is a modification of the protocol by Daus *et al*. which was necessary because only extracts in which PrP^C was completely degraded could be subjected to FT-IR spectroscopic analysis. Samples that were repeatedly digested with PK are indicated in the results. For SDS-PAGE pellets were resuspended in 12.5 µl sample loading buffer (Table 2-2) and boiled for 5 min. PMSF was added to a final concentration of 2.5 mM. For analysis by Western blot 5 µl of the sample were loaded on the SDS-polyacrylamide gel. For silver staining 10 µl of the sample were loaded on the gel and were processed as described above (chapter 2.2.7 ff.).

2.2.12 FT-IR measurement of extracted PrPres

Pellets were resuspended in approx. 5 µl ddH_2O by applying ultrasound to reaction tubes in a waterbath (PMCA device). Tubes were treated three times with approx. 340 W for 30 sec. Reaction tubes were sealed with parafilm tape to prevent them from opening. Thereafter, 1-2 µl were applied on a clean CaF_2 microscope slide and left for drying over night. Infrared micro-spectroscopic measurements of dried protein samples

were carried out as described by Daus *et al*. (2013). The FT-IR spectrometer was equipped with a microscope. Background spectra were obtained before sample measurements at positions outside of the protein sample. For each sample spectrum 256 scans were averaged. Approx. 5 single point absorbance spectra were acquired for each dried protein sample. Second derivative spectra were calculated using the instrument software. Spectra were recorded between 1000 and 4000 cm^{-1}. However, the amide I region encompassing the region between 1600-1700 cm^{-1} gives information about secondary structure elements of proteins. The amide I band originates mainly from C=O stretching vibrations of amide groups of the protein backbone. The position of IR bands is influenced by a variety of factors, e.g. the strength of hydrogen bonds and the packing of β-strands [Beekes *et al.*, 2007].

3. Results

3.1 Establishment of the PMCA assay for hamster adapted prion strains other than 263K scrapie

In PMCA, prion associated seeding activity converts normal protease sensitive PrP into proteinase K resistant PrPres. This assay was already established in our laboratory for a sensitive and quantitative detection of 263K scrapie associated seeding activity [Pritzkow *et al.*, 2011]. Within the scope of this dissertation the application of the PMCA assay had to be expanded to other hamster adapted prion strains, namely ME7-H scrapie, 22A-H scrapie and BSE-H. These strains differ markedly in their incubation times. Whereas 263K scrapie and BSE-H can be distinguished between each other and from ME7-H and 22A-H by clinical, neuropathological and immunobiochemical diagnostic methods, a discrimination of 22A-H and ME7-H was only possible by their incubation times or structural Fourier transform-infrared spectroscopical analysis of PrP27-30 [Thomzig *et al.*, 2004]. Brain homogenates from all prion agents were digested with proteinase K (PK) and serial dilutions were subjected to SDS-PAGE and Western blotting. This revealed that all brain homogenates contained similar amounts of PrPres.

When the standard PMCA protocol was carried out with the prion strains ME7-H, 22A-H and BSE-H only a poor amplification was achieved (Figure 3-1). For PMCA 1 x 10^{-10} g 263K, 1.5 x 10^{-5} g ME7-H or BSE-H and 1.0 x 10^{-5} g 22A-H homogenized hamster brain tissue were mixed with 10 % normal hamster brain homogenate (NBH) in a final volume of 150 µl, respectively. Samples were subjected to cycles of sonication and 1 h incubation at 37 °C. Sonication was performed for 40 sec at 180-200 W. After approx. 24 h samples were harvested and aliquots were diluted in a 1:5 ratio in fresh NBH. Samples were then subjected to another round of 24 cycles of incubation and sonication. In total four of those PMCA rounds were carried out. Prior to or after each PMCA round aliquots were harvested and subjected to digestion with PK, followed by SDS-PAGE and Western blotting. Detection of PrPres was performed using the hamster specific anti-PrP-antibody 3F4. Under the applied conditions a very efficient propagation of PrPres was achieved in 263K seeded PMCA reactions, as can be seen by the strong increase of PrPres within four successive PMCA rounds. In contrast, for samples that were seeded with ME7-H tissue a decline of detectable PrPres was observed during the first PMCA rounds. With the third round PrPres formation increased slowly. In samples seeded with BSE-H no PrP converting activity was seen at all. The PrPres amount in these samples corresponds merely to the serial dilution of the original seeding material, hence no formation of new PrPres had occurred. The finding

was similar when samples were seeded with the 22A-H scrapie prion strain. However, the fact that PrPres was still detected after four PMCA rounds indicated that 22A-H possessed a very low efficacy of amplification. It has to be noted that PMCA samples with 22A-H shown in Figure 3-1 were incubated 2 h instead of 1 h between the sonication steps. Nevertheless a stable autocatalytic propagation of PrPres was not achieved with the utilized standard PMCA procedure. Controls contained pure NBH without a prion seed. Reaction mixtures were processed in the same way as the other samples. Without the addition of a PrP^{Sc} seed no formation of PrPres was detected.

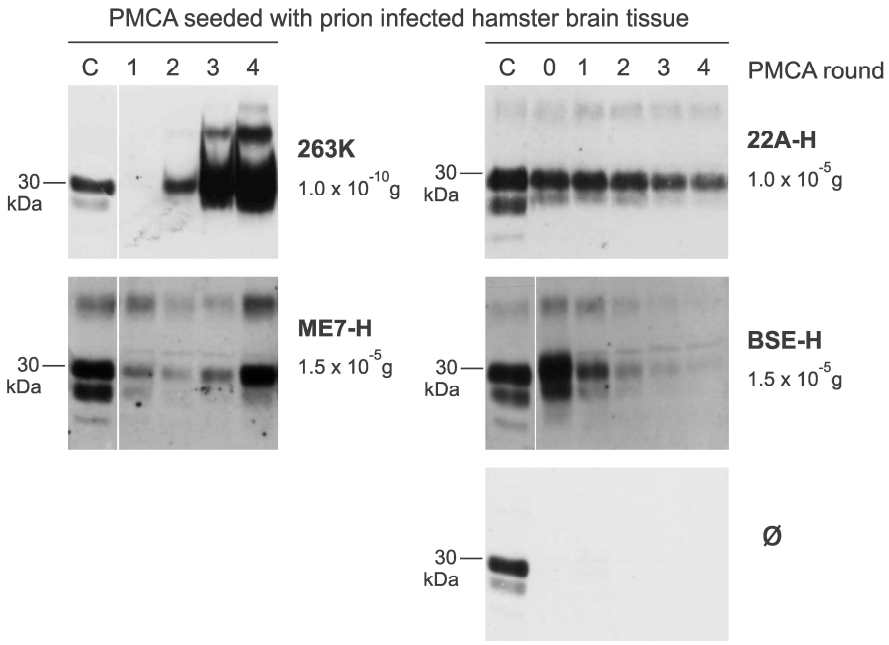

Figure 3-1: PMCA seeded with brain homogenate from hamsters infected with different prion strains. Amounts of prion seeding material are indicated as grams of homogenized brain tissue. PMCA procedure was carried out according to the standard conditions used for a very efficient propagation of 263K. Note: Samples seeded with 22A-H brain tissue were incubated for two hours instead of one hour. PMCA round 0 refers to the start sample, which was immediately frozen and not subjected to PMCA. ∅ – Control sample in which the prion seed was omitted. C – Internal Western blot control.

In order to optimize the amplification of ME7-H, 22A-H and BSE-H in PMCA several parameters could be adjusted to influence the efficiency of the seeding reaction. Parameters which were modified in this work and additional parameters which had been adjusted in previously published studies are listed below:

- Incubation time per PMCA cycle (1 h up to 4 h)
- Duration of individual PMCA rounds (24 h, 48 h or longer)

- Strength of sonication (watts) [Yamaguchi *et al.*, 2012]
- Length of sonication intervals [Fujihara *et al.*, 2009]
- Dilution ratio of serial passage steps between PMCA rounds (1:2 vs. 1:5) [Gonzalez-Montalban *et al.*, 2011]
- EDTA concentration (5-20 mM).

In the following, in extensive series of PMCA experiments the effect of changes of selected parameters on the seeding efficiency were explored for each single prion strain. Thereby it was found that the incubation time per PMCA cycle and the EDTA concentration are of great significance.

3.1.1 Parameters for optimized *in vitro* amplification of PrPres from different prion strains by PMCA: Dependence on EDTA

The presence of chelating agents in reaction mixtures was found to be of great importance for an accelerated PrPres amplification during PMCA. The addition of the chelator EDTA in a concentration up to 20 mM was in general beneficial for amplification of all hamster prion strains (Figure 3-2). EDTA forms strong complexes with divalent as well as trivalent metal ions [Harris, 2010]. The standard PMCA procedure did not include 20 mM EDTA, it was however recommended to include 5 mM EDTA in the conversion buffer, which is used to prepare the NBH [Barria *et al.*, 2012]. To assess the effect of EDTA on amplification of hamster prion strains in PMCA, EDTA was added to the NBH in final concentrations of 0, 1, 5, or 20 mM. To obtain NBH without EDTA, EDTA-free Protease Inhibitor Cocktail Complete Mini tablets were used for preparation of the conversion buffer. The accordingly prepared brain homogenates were subsequently seeded with 10^{-9} g 263K, 10^{-6} g BSE-H, 10^{-6} g 22A-H or 10^{-5} g ME7-H brain tissue, respectively, and were subjected to up to five PMCA rounds. For all samples an incubation time of 2 hours was chosen between the sonication steps. Figure 3-2 shows Western blot results of samples before (PMCA round 0) and after each PMCA round.

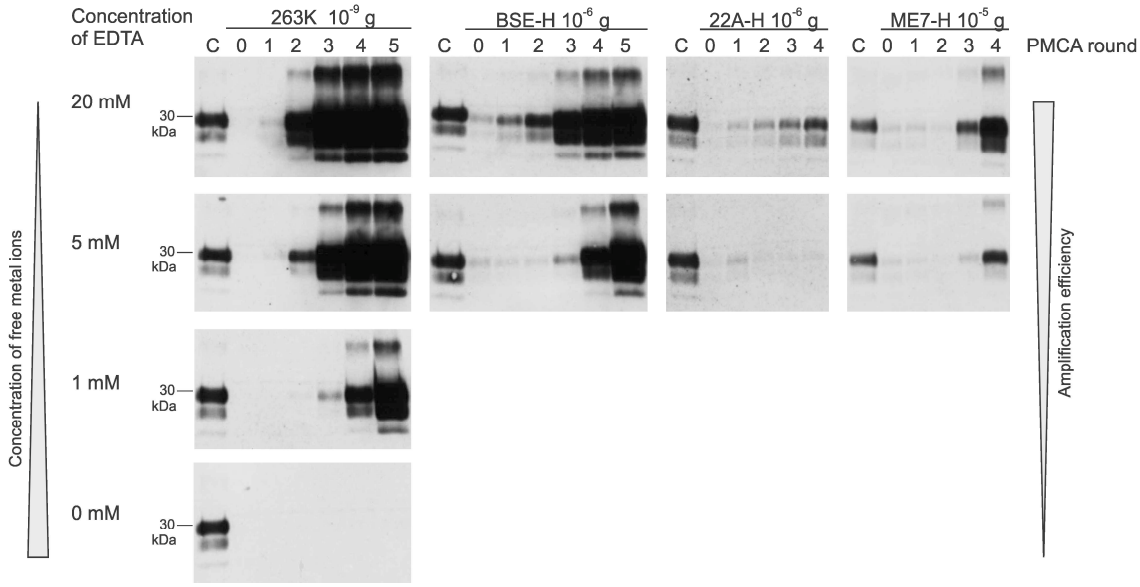

Figure 3-2: Impact of the EDTA concentration on the amplification efficiency of different hamster prion strains in the PMCA assay. Samples were seeded with the indicated amounts of 263K, BSE-H, 22A-H or ME7-H brain tissue, respectively. Up to five serial PMCA rounds were carried out in the presence of 0, 1, 5 or 20 mM EDTA with 2 h incubation time per PMCA cycle. PMCA round 0 refers to the start sample, which was immediately frozen and not subjected to PMCA. C – Internal Western blot control.

It is clearly visible that the presence of 20 mM EDTA in reaction mixtures accelerated the seeded conversion of PrP^C to PrPres as compared to the 5 mM EDTA generally used in PMCA assays. This acceleration is concentration dependent and appeared to be basically independent from the prion strains used in this study. Higher concentrations of EDTA above 20 mM were not examined. Because EDTA is a metal chelator and seeding is more efficient in preparations that contain higher levels of EDTA, the presence of free metal ions seems to reduce the amplification efficiency. PrPres formation was never observed in non-seeded PMCA control samples containing 20 mM EDTA (not shown).

3.1.2 Parameters for optimized *in vitro* amplification of PrPres from different prion strains by PMCA: Impact of incubation time

In addition to the concentration of the chelator EDTA, the length of incubation times in an individual PMCA cycle was identified as another important parameter that modified amplification efficiency of prion agents. It was found that variations of incubation time had particular strong effects on the seeding activity of 22A-H associated prions. Figure 3-3 shows results of samples that were seeded with 10^{-5} g or 10^{-6} g 22A-H brain tissue and were subjected to four rounds of PMCA. In set A, samples were incubated at cycles

of 2 hours incubation, whereas in set B the incubation time was raised to 4 hours. The number of 12 PMCA cycles per one PMCA round and the EDTA concentration of 5 mM was kept constant in both sets. Under the conditions of set A no amplification of PrPres was achieved. The amount of detectable PrPres was reduced after four PMCA rounds compared to the amount of prion seeding material that is visible in round 0. In contrast, the samples that were incubated 4 hours in set B showed a stable amplification of PrPres in successive PMCA rounds. A prolonged incubation time as well as an elevated EDTA concentration (Figure 3-2) independently boosted the amplification of 22A-H in PMCA. The additive or possibly synergistic effect of both of these parameters yielded a further increased amplification efficacy, as shown in Figure 3-3C. Here, PMCA was performed with 4 hours incubation and in the presence of 20 mM EDTA.

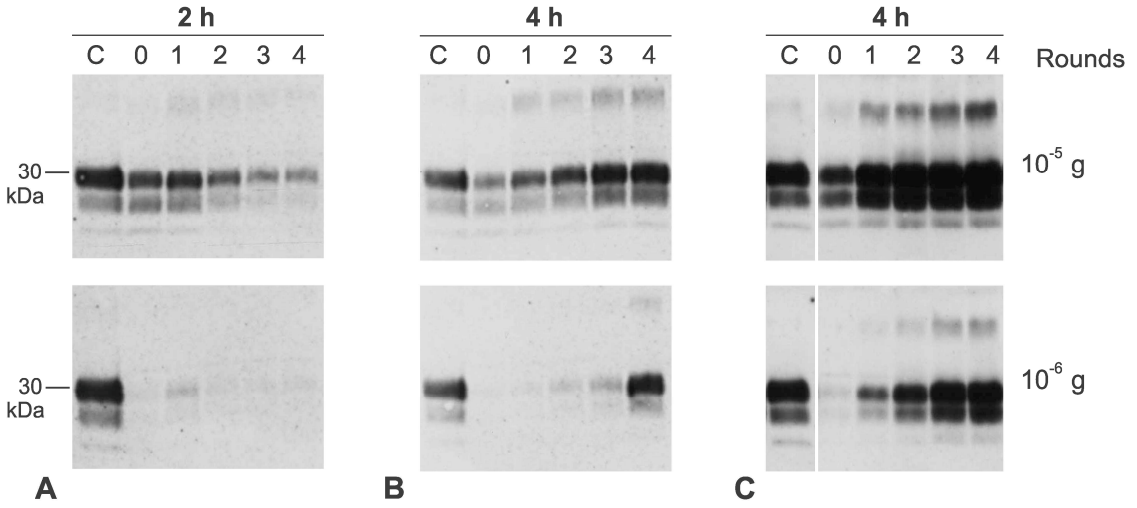

Figure 3-3: Effect of the incubation time on in vitro propagation of 22A-H associated PrPres in the PMCA assay. (A) PMCA was performed with a cyclic incubation time of 2 hours or (B) 4 hours and in the presence of 5 mM EDTA. (C) PMCA was performed with 4 hours incubation and 20 mM EDTA. C – Internal Western blot control.

For the other hamster prion strains ME7-H and BSE-H it was found that an incubation time of 2 h allowed a stable *in vitro* propagation of PrPres in PMCA reactions when 12 incubation cycles were carried out per one PMCA round. Due to the advantage of time saving (with respect to the overall processing time) when performing 12 PMCA cycles with 2 h incubations each vs. 12 cycles with 4 h incubations each, the 2 h-protocol was chosen for all following PMCA experiments with ME7-H and BSE-H. Furthermore if not stated otherwise, PMCAs were performed in the presence of 20 mM EDTA.

The findings demonstrate that the used hamster prion strains differ in their individual requirements for a stable and efficient PrPres propagation in PMCA assays.

3.2 Cofactors in PMCA

In order to examine, for different prion strains, the role of putative cofactor molecules in the process that converts PrP^C into misfolded and aggregated PrPres, three sets of experiments were carried out. Firstly, by simple addition of putative cofactors to the reaction mixtures an environment with an excess of the molecules of interest was created in PMCA. This was expected to provide information as to whether an excess of specific molecules act as accelerators or decelerators of PrP^C conversion. In a second approach nucleic acids were depleted in the PMCA substrate and the effect on PMCA was studied. Finally, in PMCA experiments with nucleic acid depleted NBH the ability of certain molecules to restore the amplification was assessed.

3.2.1 Non-metal cofactors in PMCA

In the following, individual experiments with simple addition of synthetic poly(A) RNA and the sphingolipids sphingomyelin and galactosylceramide were carried out. Increasing evidence suggest that lipids are involved in prion propagation and infectivity. It was demonstrated recently that phospholipids are required for replication of infectious mouse PrP^{Sc} in PMCA experiments [Deleault *et al.*, 2012a; Deleault *et al.*, 2012b; Wang *et al.*, 2010]. Since galactosylceramide and sphingomyelin were previously identified in scrapie hamster prions [Klein *et al.*, 1998] these sphingolipids were examined as putative cofactors in this study. Substances were added directly to the reaction mixtures (prior to performing PMCA) to achieve final concentrations of 25-400 µg/ml in a reaction volume of 150 µl as indicated in Table 3-1. To rule out misleading results due to a degradation of cofactor molecules during a PMCA round by the ultrasound or remaining enzyme activities in the crude brain homogenate substrate, cofactor molecules were added prior to every PMCA round at the indicated concentrations. After each PMCA round aliquots were digested with PK and subjected to SDS-PAGE and Western blotting. PrPres was detected with a specific anti-PrP antibody (3F4). Effects of cofactor molecules on PrPres propagation were assessed by comparison of the Western blot signals between individual PMCA rounds of samples with added cofactor molecules or reference samples that did not contain such cofactor molecules. Figure 3-4 shows the Western blot results for the addition of poly(A) RNA to individual PMCA runs with all four hamster prion strains.

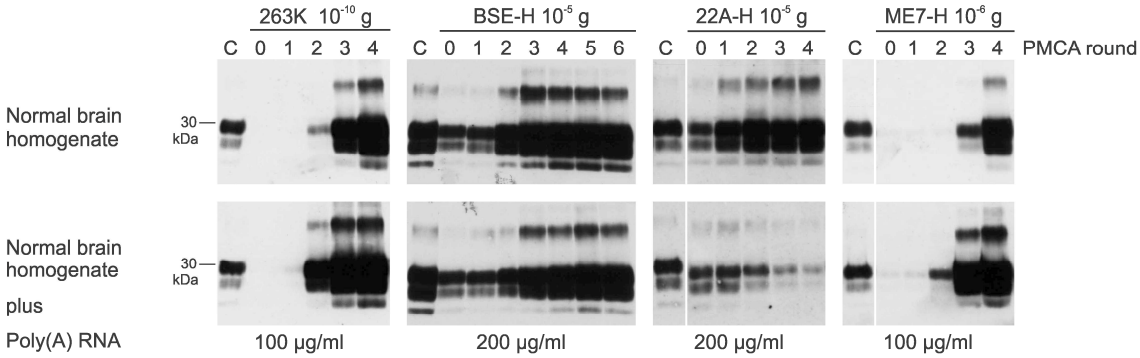

Figure 3-4: Addition of 100-200 µg/ml poly(A) RNA to PMCA samples that were seeded with the indicated amounts of brain tissue from hamsters that had been infected with different prion strains. Poly(A) RNA was added prior to every PMCA round and PMCA was performed with incubation times of 1 h, 2 h, 4 h and 2 h for 263K, BSE-H, 22A-H and ME7-H seeded samples, respectively. Reference samples are shown in the upper line. References did not contain exogenous cofactor molecules and were otherwise treated equally. C – Internal Western blot control.

RNA is generally considered to facilitate PrP conversion [Deleault *et al.*, 2007; Deleault *et al.*, 2003] and in accordance it was found in this thesis that the addition of synthetic RNA molecules was beneficial for the propagation of 263K and ME7-H associated prions. PrPres could be detected roughly one PMCA round earlier in samples with excess RNA molecules compared to reference samples. However, a contrary effect was observed for the propagation of 22A-H associated PrPres. Here, additional RNA molecules clearly inhibited the amplification of PrPres during four PMCA rounds. Addition of RNA did not show such major effects on the amplification of BSE-H prion seeds. Detection of BSE-H associated PrPres after six PMCA rounds in the presence of elevated RNA levels revealed no significant differences from reference samples. The sphingolipids sphingomyelin and galactosylceramide were added only to 263K seeded PMCA samples in concentrations up to 400 µg/ml. An effect on 263K propagation could not be observed. All results from PMCA experiments with addition of exogenous cofactor molecules are summarized in Table 3-1.

Table 3-1: Addition of putative cofactor molecules to the reaction mixtures during PMCA assays that were seeded with different hamster prion strains. Molecules were added to the indicated final concentrations. Effects on PrPres amplification efficacies were analyzed by Western blotting.

Putative cofactor	*263K*		*ME7-H*		*22A-H*		*BSE-H*	
Synthetic poly(A)-RNA	100 µg/ml	+	100-200 µg/ml	+	100-200 µg/ml	–	100-200 µg/ml	Ø to –
Sphingomyelin	25-400 µg/ml*	Ø	N/D		N/D		N/D	
Galactosylceramide	25-400 µg/ml*	Ø	N/D		N/D		N/D	

– Inhibition of PrPres formation
+ Increase of PrPres formation
Ø No effect on PrPres formation
* Sphingomyelin and Galactosylceramide were added to final concentrations of 25, 100 and 400 µg/ml
N/D no data

3.2.2 PMCA after depletion of under nucleic acids in the conversion substrate

Nucleic acids in normal brain homogenate used as PrP^C substrate for PMCA assays were depleted by enzymatic digestion with benzonase for approx. 20 h. Aliquots of the prion seeding material were also treated with benzonase prior to performing PMCA for two hours. Benzonase is known to degrade all kinds of nucleic acids: single-stranded, double-stranded, linear, circular or supercoiled DNA and RNA. Addition of 20 mM EDTA to the PMCA substrate inhibited benzonase enzyme activity. EDTA was also added to reference samples which were used to carry out PMCA with NBH that still contained endogenous nucleic acids. Figure 3-5 depicts the Western blot results from PMCA assays in which either NBH or the nucleic acid free normal brain homogenate was used as PrP^C substrate for the amplification of prion seeds. Reactions were seeded with 10^{-5} or 10^{-6} g hamster brain tissue, respectively. While the amplification of PrPres increased over the first PMCA rounds and finally reached a constant level in samples with endogenous nucleic acids, samples without nucleic acids in the substrate did not show an amplification of PrPres. The detection signal for PrPres constantly decreased over the PMCA rounds and eventually fell below the detection limit. This confirmed recent findings indicating that RNA is required for efficient prion propagation [Deleault *et al.*, 2010; Deleault *et al.*, 2003; Gonzalez-Montalban *et al.*, 2011], and furthermore demonstrated that the different hamster prion strains used in this study are generally dependent on the presence of nucleic acids for PrPres amplification by PMCA.

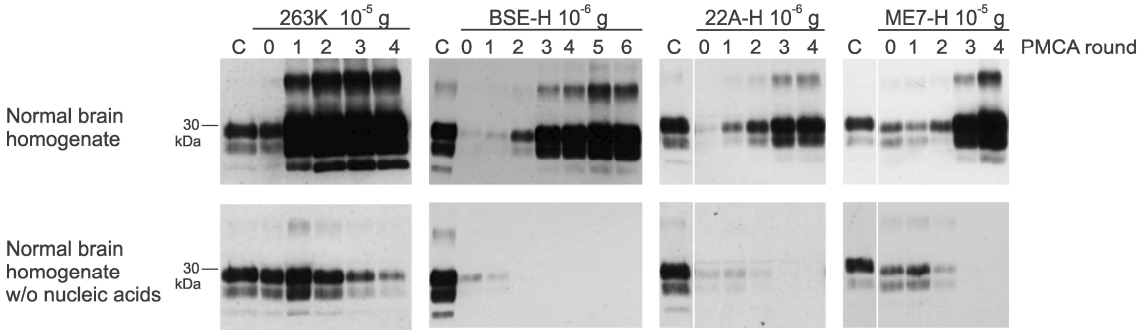

Figure 3-5: Propagation of prion strains is inhibited without nucleic acids in the PMCA substrate. NBH was incubated with the enzyme benzonase to remove all kinds of nucleic acids (normal brain homogenate w/o nucleic acids). Samples were seeded with 10^{-5} or 10^{-6} g infected hamster brain tissue as indicated and samples were subjected to PMCA procedures with incubation times of 2 h for263K, BSE-H and ME7-H and 4 h for 22A-H. In reference samples NBH that still contained endogenous nucleic acids was used. All samples contained 20 mM EDTA. C – Internal Western blot control.

When 263K was used as seeding material in nucleic acid depleted NBH an initial seeding activity was consistently observed in the first PMCA round (see Figure 3-5, detection of PrPres in round 1 vs. round 0). This raised speculations if this seeding activity was mediated by residual nucleic acids in the original 263K seeding material that may have not been destroyed during the incubation with benzonase. In order to address this question the 263K seed was separately incubated with benzonase for 20 h, instead of 2 h, which was as long as the treatment of NBH. Subsequently, the 263K seeding material prepared in this way was mixed with NBH, or with nucleic acid free NBH, and subjected to PMCA. In a parallel PMCA reaction the nucleic acid free NBH was seeded with native 263K scrapie material that was not treated with benzonase. Results are depicted in Figure 3-6. The seeding efficiency of benzonase treated or untreated 263K seeds in nucleic acid depleted NBH were equal. The seeding activity of benzonase treated 263K brain tissue was not impaired as determined by PMCA in NBH and exhibited a similar conversion activity as untreated seeds (see Figure 3-4). Thus, it can be concluded that the initial 263K seeding was either not mediated by residual nucleic acids in the seeding material, or that nucleic acids possibly present in this material were not accessible to benzonase.

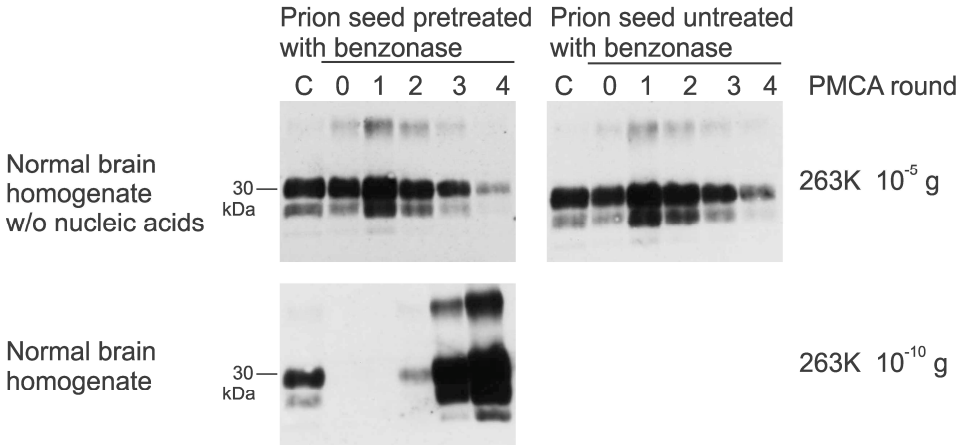

Figure 3-6: Prior to PMCA 263K seeds were incubated 20 h or were not incubated with benzonase. PMCA was performed in NBH that was pretreated with benzonase and was thus free of nucleic acids. For comparison PMCA was also performed in NBH containing endogenous native nucleic acids. C – Internal Western blot control.

A surprising observation was made when larger amounts of BSE-H prion infected tissue were used to seed PMCA in NBH devoid of nucleic acids. Whereas PrPres propagation faded after seeding with 10^{-6} g BSE-H brain tissue (Figure 3-5), an increase in newly formed PrPres was detected after 7 to 8 PMCA rounds when 10^{-4} g BSE-H brain tissue were used for seeding under equal PMCA conditions (Figure 3-7A). The new PrPres species formed in later PMCA rounds propagated despite the depletion of nucleic acids in the PMCA substrate. Further experiments revealed that this PrPres amplificate was able to mediate further PrP conversion when used as seeding material in new PMCA reactions with nucleic acid depleted NBH. They were found to propagate stable in such PMCAs for at least 10 rounds (data not shown). The same PMCA setup was then performed with 2 x 10^{-4} g 263K scrapie brain tissue as seed in nucleic acid free NBH. Interestingly, 263K did not appear to propagate in a similar way independently of nucleic acids. PMCA was performed for 14 rounds. Again, 263K exhibited a residual seeding activity that resulted in a lower decrease of PrPres as observed with BSE-H seeded samples until rounds 8-9. For comparison, the original BSE-H and 263K seeds (PMCA round 0 in Figure 3-7A) were not subjected to PMCA cycles but serially diluted according to the dilutions of serial PMCA rounds (Figure 3-7B). Incubation and sonication steps were not applied to the samples in Figure 3-7B.

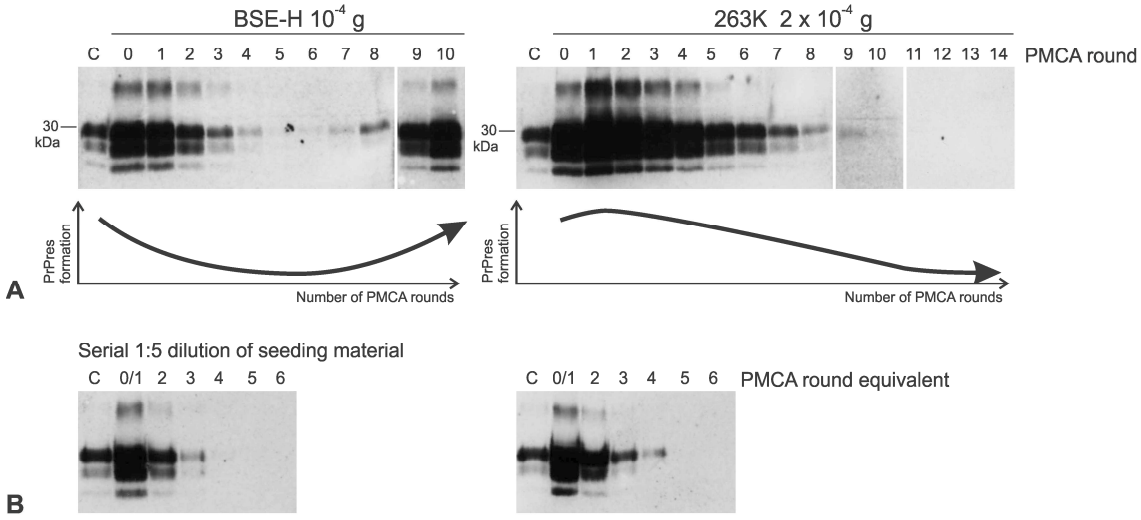

Figure 3-7: (A) PMCA samples were seeded with the indicated amounts of BSE-H or 263K hamster brain tissue and subjected to PMCA in the absence of nucleic acids in the PMCA substrate. For better comprehension the Western blot results were additionally visualized graphically. (B) Samples were seeded with identical amounts of prion tissue as in (A) but were not submitted to PMCA procedure. Instead only 1:5 dilutions were prepared that correspond to the indicated number of PMCA round. C – Internal Western blot control.

3.2.3 PMCA after depletion of nucleic acids in the conversion substrate and subsequent addition of non-metal cofactors

In this chapter the abilities of different putative cofactors to restore PrPres amplification by PMCA after depletion of nucleic acids in the PrP^{C} substrate were assessed individually for all four hamster adapted prion agents. Previous studies gave first evidence that chondroitin sulfate was involved in the biogenesis of PrP^{Sc} in chronically prion infected mouse neuroblastoma cells (ScN2a) [Ben-Zaken *et al.*, 2003], and poly-L-glutamate was reported to stimulate PrPres amplification in an *in vitro* amplification assay with partially purified substrates [Deleault *et al.*, 2005]. Chondroitin sulfate and poly-L-glutamate were therefore assessed as putative cofactors. The following studies were performed on the basis of a suppressed propagation of PrPres in nucleic acid free NBH (Figure 3-5). Polyanionic compounds such as poly(A) RNA, chondroitin sulfate A, poly-L-glutamate and the sphingolipid sphingomyelin were added in different concentrations to prion seeded PMCA reaction mixtures prior to every PMCA round. Compounds were assessed for their reconstitution ability by comparison to Western blot results from reference samples that contained only prion seeds in nucleic acid depleted NBH.

Poly(A) RNA has been described previously to facilitate the propagation of hamster prion agents Sc237 and 139H in PMCA [Deleault *et al.*, 2007]. In accordance with this

report, Figure 3-8 and Figure 3-9 show that poly(A) RNA also restored the amplification of 236K, ME7-H and BSE-H, and furthermore, was found to do so in a dose dependent manner. In Figure 3-8 synthetic RNA was added in final concentrations of 1, 10 or 100 µg/ml to reactions that were seeded with scrapie brain tissue from 263K or ME7-H infected hamsters. 1 µg/ml of the synthetic RNA in 263K samples and 10 µg/ml in ME7-H samples were not sufficient to reconstitute PrPres propagation. However in the presence of 10 µg/ml or 100 µg/ml synthetic RNA, respectively, new PrPres was produced. These results show that reconstitution of PrP conversion by synthetic RNA is dose dependent for the examined prion strains. For reconstitution of PrP conversion ME7-H seeded samples required higher amounts of the polyanionic compound than 236K seeded samples.

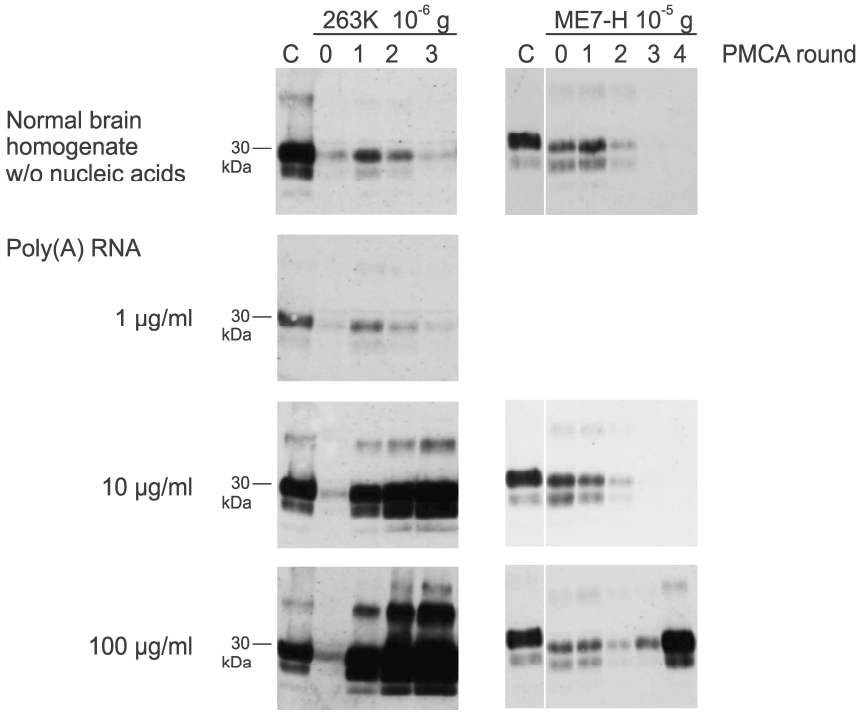

Figure 3-8: Reconstitution of the seeding activities of 263K and ME7-H prions with poly(A) RNA are concentration dependent. Prion seeds were incubated in nucleic acid depleted NBH. Prior to every PMCA round the indicated amount of synthetic RNA was added to the reaction tubes. PMCA incubation time was 1 h for 263K seeded samples and 2 h for ME7-H seeded samples. C – Internal Western blot control.

Furthermore it was found that poly(A) RNA alone was not sufficient to mediate a stable amplification of 22A-H seeded in nucleic acid depleted NBH. Addition of RNA facilitated a prolonged but yet decreasing propagation of 22A-H associated PrPres (Figure 3-9). This amplification was not stable and eventually became undetectable by

PMCA round five. Together with the previous finding that poly(A) RNA inhibited amplification of 22A-H in PMCA with crude brain homogenate (Figure 3-4), this showed that poly(A) RNA did not promote propagation of all the examined hamster prion agents in PMCA. Its effect is therefore strain specific. However amplification of 22A-H was successfully restored by chondroitin sulfate A. 263K and BSE-H seeded samples could be reconstituted with both RNA and chondroitin sulfate A, whereas no reconstitution was observed for ME7-H seeded samples in the presence of chondroitin sulfate in four PMCA rounds (Table 3-2). This again indicates that the ability of synthetic poly(A) RNA or chondroitin sulfate A to compensate for depleted nucleic acids in PMCA depends on the prion strain.

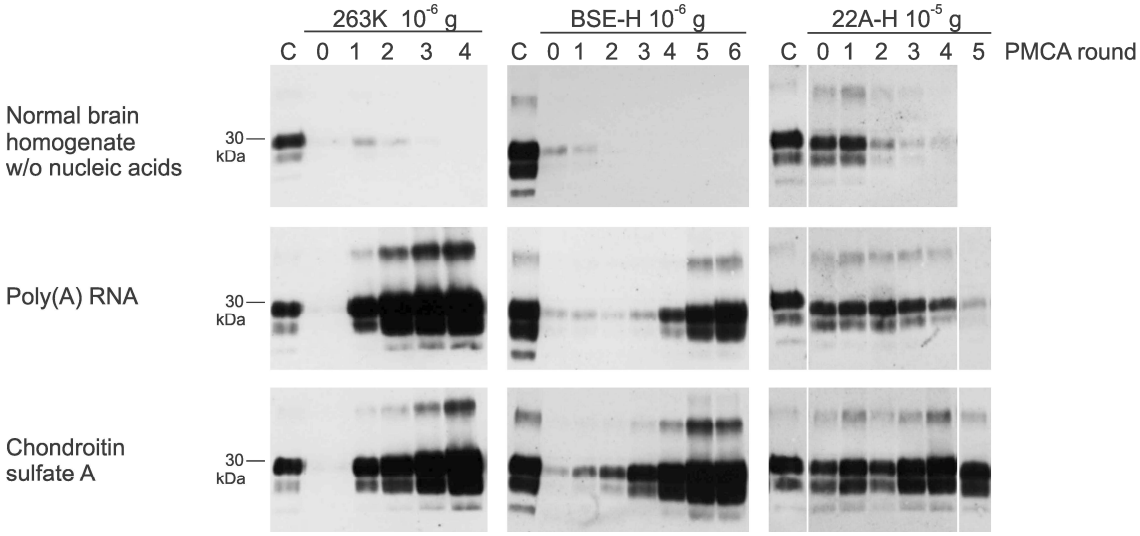

Figure 3-9: Reconstitution of PrPres amplification for 236K, BSE-H and 22A-H prions in NBH depleted of nucleic acids by addition of poly(A) RNA or chondroitin sulfate A. Hamster prion brain tissues were mixed with NBH that was depleted of nucleic acids and samples were subjected to PMCA for the indicated number of rounds. To the sets of samples 100 µg/ml poly(A) RNA or different amounts of chondroitin sulfate A were added before every PMCA round. 263K seeded samples were treated with 100 µg/ml chondroitin sulfate A, and a PMCA incubation time of 1 h. BSE-H and 22A-H seeded samples were treated with 400 µg/ml chondroitin sulfate A and the PMCA incubation time was 2 h and 4 h, respectively. C – Internal Western blot control.

Table 3-2 summarizes the results for all examined putative cofactor molecules. Reconstitution of PrP misfolding was assessed after 4 to 5 PMCA rounds. Poly(A) RNA was found to confer a stable reconstitution of PrP conversion for 263K, ME7-H and BSE-H seeds. Though the seeding activity of 22A-H associated PrP was sustained to some extent, the formation of new PrPres became unsustained in later PMCA rounds. Chondroitin sulfate A was found to reconstitute the seeding activity of 263K, 22A-H

and BSE-H prions but not ME7-H prions. Poly-L-glutamate was added solely to reactions with 263K and BSE-H. The effect by poly-L-glutamate on the seeding activity of 263K was low, and a reconstitution of BSE-H amplification was not determined with this cofactor. Furthermore sphingomyelin was not able to mediate a reconstitution of 263K associated PrP conversion after depletion of nucleic acids in the PMCA substrate.

Table 3-2: Reconstitution effects of putative cofactor molecules on PrP conversion in PMCA. Different hamster prion strains were used as seeds in NBH devoid of nucleic acids. Cofactors were added to reaction mixtures in defined concentrations and prior to every PMCA round.

Cofactor	*263K*		*ME7-H*		*22A-H*		*BSE-H*	
Synthetic poly(A)-RNA	1-300 µg/ml*	++	10-100 µg/ml	++	100 µg/ml	Ø	100-300 µg/ml	+
Chondroitin sulfate A	100-400 µg/ml**	+	400 µg/ml	–	400 µg/ml	+	400 µg/ml	+
Poly-L-glutamate	200 µg/ml	Ø	N/D		N/D		200 µg/ml	–
Sphingomyelin	25-400 µg/ml***	–	N/D		N/D		N/D	

\+ PrPres propagation was successfully reconstituted
++ Reconstitution of PrPres propagation showed concentration dependency on the cofactor
Ø PrPres propagation was prolonged but still decreasing
– PrPres propagation was not reconstituted
* Poly(A) RNA was added at final concentrations of 1, 10, 100 and 300 µg/ml
** Chondroitin sulfate A was added at final concentrations of 100, 300 and 400 µg/ml
*** Sphingomyelin was added at final concentrations of 25, 100 and 400 µg/ml
N/D no data

3.2.4 Metal ions as cofactors

The involvement of metal ions in PrP misfolding and aggregation is being widely discussed and the stimulation by divalent metal ions of the conversion of PrP^C into PrPres in PMCA assays was previously reported [Kim *et al.*, 2005]. Against this background the effect of metal ions on 263K associated PrP conversion in PMCA was investigated. Specific requirements had to be considered to avoid artificial or superposed effects. For these reasons the metal ion chelating agent EDTA was omitted in the PrP^C substrate. Brains of normal hamsters were perfused without EDTA in the perfusion solution and the conversion buffer for brain homogenization was prepared with EDTA-free protease inhibitor tablets. Under these conditions the seeding of 263K

was found to exhibit a lower efficiency. However this could be compensated by seeding the PMCA reaction with a higher seeding dose of 10^{-7} g scrapie brain tissue. Furthermore, artifacts due to an impaired processing by proteinase K in the presence of metal ions had to be considered, as it was reported that Cu^{2+} ions can inhibit the activity of proteinase K [Stone *et al.*, 2007]. PK digestion of PrP^{Sc} in the presence of metal ions in equivalent concentration as used in PMCA experiments revealed no altered PrP processing (Figure 3-10A). Only in the presence of higher concentrations as 10 mM $CuCl_2$ the digestion with PK was found to be incomplete. An addition of 20 mM EDTA restored complete PK digestion (Figure 3-10B).

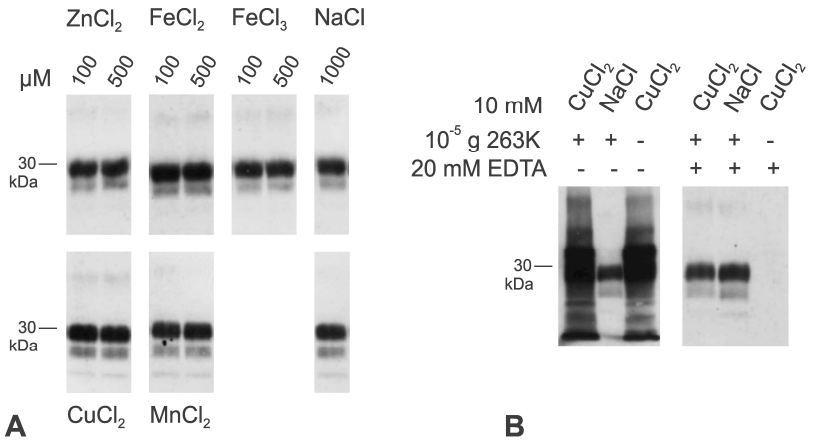

Figure 3-10: (A) PK digestion of PrP^{Sc} was not impaired in 100 or 500 µM metal ion solution. Normal brain homogenate was spiked with identical amounts of 263K brain tissue and PK treatment was carried out in the presence of metal ions as indicated. (B) Normal brain homogenate was spiked with 263K tissue where indicated and limited proteolysis with PK was performed in the presence of 10 mM $CuCl_2$ or NaCl with or without 20 mM EDTA as indicated.

PMCA experiments conducted to assess the effect of metal ions on the seeding activity of 263K prions comprised the following sample types:

- PrP^{C} + PrP^{Sc} (as reference)
- PrP^{C} + PrP^{Sc} + metal ions
- PrP^{C} + metal ions (as control)
- PrP^{C} (as control)

Metal ions were applied to the NBH substrate as aqueous chloride solutions at final concentrations of 100 and 500 µM. These concentrations were chosen to cover a physiologically relevant range. As mentioned in chapter 1.5.2 metal concentrations in

the brain are in a range of 70 µM copper, 350 µM zinc and 340 µM iron [Bush and Tanzi, 2008; Rana *et al.*, 2009]. Generally brain metals are not present as free ions but bound to ligands. As is described in the literature about 300 µM zinc [Assaf and Chung, 1984; Frederickson and Bush, 2001; Howell *et al.*, 1984] are transiently present as free ions in the synaptic cleft in the brain and free copper is present in the extracellular space [Rana *et al.*, 2009; Schlief *et al.*, 2005]. It was reported that copper concentrations of 15 µM are released within the synaptic cleft during synaptic vesicle release and higher concentrations between 100 and 300 µM are achieved during neuronal depolarization [Vassallo and Herms, 2003]. Metal ions were added to NBH prior to every PMCA round and a total of four PMCA rounds were carried out with cyclic incubation times of one hour. None of the tested metal ions in this PMCA setup were present in the conversion buffer used for the preparation of the homogenate substrate. The addition of 500 µM NaCl served as control.

Figure 3-11 shows representative results of the effects of metal ions on the propagation of PrPres from 263K scrapie seeds in the PMCA assay. The Western blot findings showed an inhibitory effect of some ions or none effect on the efficiency of PrP conversion. An increase of PrP conversion was not observed. PrPres formation was never detected in non-seeded control samples, regardless of whether they contained additional metal ions or not. The NaCl control sample showed no deviation from the 263K reference sample that was free of additional metal ions. Zn^{2+}, Fe^{2+}, Fe^{3+} and Cu^{2+} were found to inhibit PrPres formation in a concentration dependent way. Fe^{2+} had the strongest inhibitory effect on 263K associated PrPres propagation followed by Fe^{3+}, Zn^{2+} and Cu^{2+}. Mn^{2+} did not exhibit an effect on the propagation of PrPres.

The previous findings that higher EDTA concentrations promote PrP misfolding in PMCA are in good accordance with the observation that the majority of the applied metal ions generally inhibit PrP conversion.

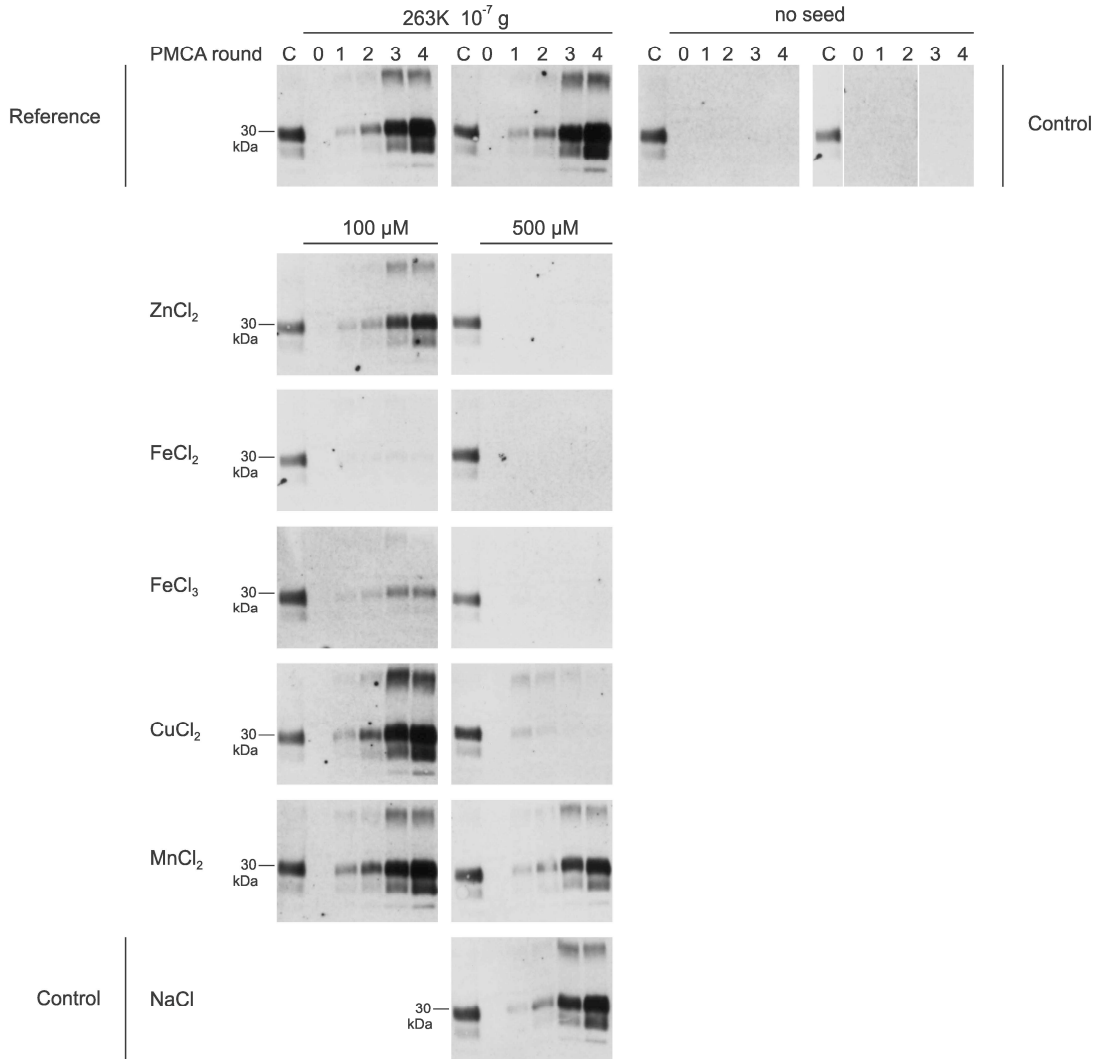

Figure 3-11: Western blot results show the effect of different metal ions on PrPres propagation in PMCA. Metal ions were added to PMCA reactions as aqueous chloride solutions at final concentrations of 100 and 500 µM. Samples were seeded with 10^{-7} g 263K scrapie hamster brain tissue and were subjected to four PMCA rounds. In control samples the 263K seed was omitted. C – Internal Western blot control.

3.3 Infection of glial cell cultures with other hamster adapted prion strains than 263K

A cell culture assay from cerebral glial hamster cells was recently established in our laboratory to qualitatively test and verify 263K associated PrP seeding as a biologically active and replicative principle *in vitro* without experiments in animals [Pritzkow *et al.*, 2011]. In the following this assay was adapted to ME7-H, 22A-H and BSE-H prion agents.

3.3.1 Cell assay with hamster brain homogenate from different prion strains

The cell assay for *in vitro* propagation of PrPres originating from 263K brain homogenate was recently established in our laboratory. Since other hamster prion strains were shown to have different requirements for *in vitro* amplification in PMCA (see chapter 3.1), the propagation of these prion strains in the cell assay was examined in more detail. To this end brain homogenate from hamsters that were infected with 263K, ME7-H, 22A-H, BSE-H, and brain tissue from an uninfected healthy animal, were applied to the cell cultures under equal conditions. Glial cell cultures were prepared 2-3 days prior to inoculation with hamster brain homogenates. 10^{-3} g or 10^{-5} g of homogenized brain tissue, as indicated in Figure 3-12, were added to the culture medium and cells were subsequently incubated for 3 days. At 3 days post exposure (DPE) to the brain tissue, cells were washed to remove inocula. Afterwards, cells were incubated further. At the indicated DPE, cells were harvested and analyzed for PrPres by detection with anti-PrP antibody 3F4 after digestion with PK. The amount of PrPres at 3 DPE thereby was equivalent to the original inoculum that had been attached to the cells. In the Western blot image of 263K infected cells, an increase of PrPres from 3 DPE to 40 DPE and a further increase to 80 DPE is shown, indicating that cells had been successfully infected and further propagated 263K associated PrPres. A propagation of PrPres by glial cells was also observed for 22A-H and BSE-H inoculated cultures. Cell infection with the ME7-H agent was monitored at several time points over an interval of 154 days. Only a decrease of PrPres that originally persisted on the cells was observed, but a propagation of PrPres and thus a successful cell infection could not be detected. Cell cultures that were incubated with normal brain tissue from healthy animals (no seed) are labeled as NBH in Figure 3-12 and served as a negative control. In none of these samples PrPres could ever be found.

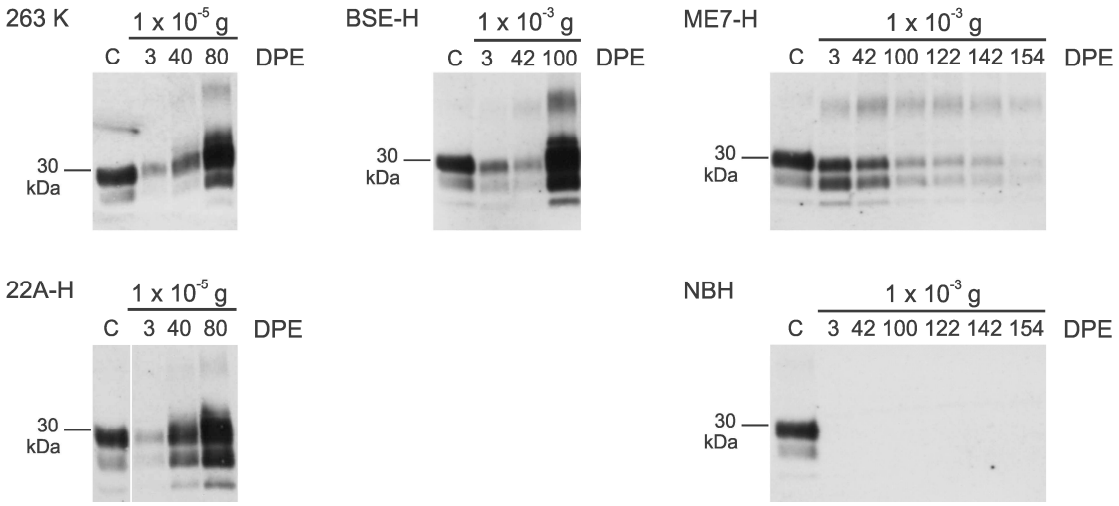

Figure 3-12: PrPres amplification in glial cell cultures after incubation with the indicated amounts of brain tissue from hamsters that had been infected with different prion strains. Cells were incubated with the inocula for three days. Thereafter the inoculum was removed and cells were incubated further. At the indicated days post exposure (DPE) PrPres was detected with an anti-PrP antibody after PK digestion of the cell suspension. NBH – normal brain homogenate of uninfected animals served as control inoculum. C – Internal Western blot control.

In summary, glial cell cultures could be infected with the 263K, 22A-H and BSE-H agent, but not with the ME7-H scrapie agent. It was found that 263K and 22A-H propagated PrPres formation in cell cultures with a similar efficiency, whereas BSE-H exhibited a lower efficiency since a larger amount of tissue was required as inoculum to trigger PrPres formation with a comparable efficiency. These findings correlated only partially with the observed *in vivo* incubation times: In bioassay studies 263K scrapie, 22A-H scrapie and BSE-H prions caused clinically fully developed prion diseases in Syrian hamsters at 83±5, 206±8 and 287±28 days, respectively, whereas the ME7-H scrapie agent has an incubation time of 331±16 days [Thomzig *et al.*, 2004].

3.3.2 Cell assay with PMCA products

After establishment of a cell assay for the detection of biological seeding activity associated with 22A-H and BSE-H prion agents from brain homogenates of Golden Syrian hamsters, it was of interest to determine whether the PMCA derived PrPres species from all four hamster prion strains are able to induce PrPres propagation in the cell cultures. PMCA products contained about 1 % (v/v) Triton X-100 which disrupts cell integrity and was thus toxic to our cell cultures. Therefore all PMCA derived samples had to be ultracentrifuged in order to remove the Triton X-100. Pellets after centrifugation were resuspended in PBS and applied to cell culture media. Cells were

incubated with the inocula for 3 days. Subsequently, inocula were removed and cells were cultured for a duration of 80 days. Cells were analyzed for PrPres at 3, 40 and 80 DPE. Figure 3-13 shows the Western blot results from cell cultures that were infected with PMCA products derived from different hamster prion strains. PMCAs were originally seeded with brain homogenate from 263K, ME7-H, 22A-H and BSE-H infected animals, respectively, and samples were subjected to 4-5 rounds of serial PMCA to ensure a cell exposure to newly formed PrPres. After 4-5 PMCA rounds PrP^{Sc} from the original seeding tissue was diluted below the detection limit in the Western blot. The amount of PrPres from PMCA samples that was utilized for cell infection was approximately identical for the prion strains 263K, ME7-H and BSE-H as determined by Western blot analysis. A lower amount of 22A-H associated PrPres was used for incubation of the cells. Inoculation of cell cultures with PMCA products sampled after 4 rounds of unseeded PMCA (NBH only) served as a negative control. Due to some variations between samples of the same kind, duplicates for each sample are displayed. The results show that PMCA generated PrPres derived from all prion agents induced an accumulation of PrPres in glial cell cultures, hence are infectious to the cell cultures of this assay. Interestingly also ME7-H progeny PrPres was biological seeding active, whereas the original ME7-H brain homogenate previously failed to cause PrPres amplification in the cell culture. As expected, the unseeded PMCA product with NBH only did not produce detectable PrPres in the cell assay.

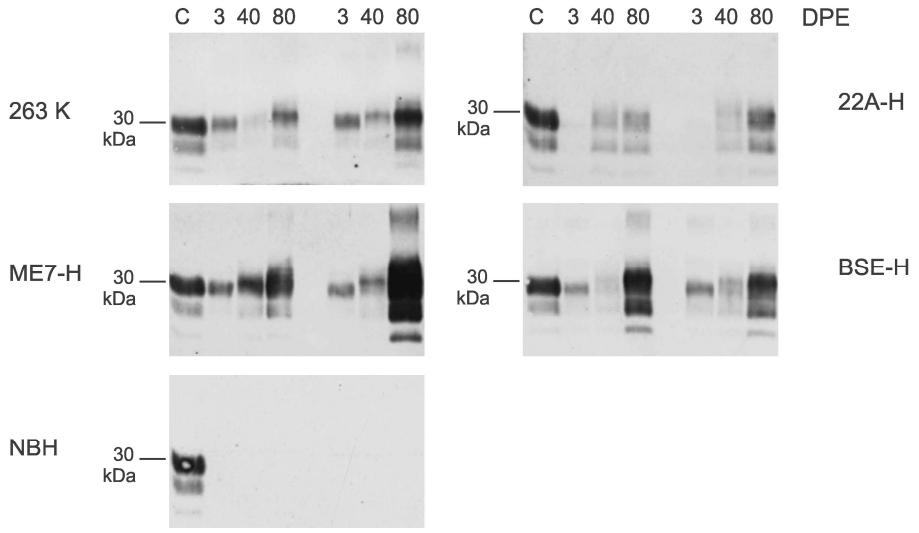

Figure 3-13: Accumulation of PrPres in glial cell cultures after incubation of cells with PMCA products derived from 263K, 22A-H, ME7-H or BSE-H seeds. Cells were incubated with the inocula for three days. Thereafter the inoculum was removed and cells were incubated further up to 80 days. At the indicated days post exposure (DPE) PrPres was detected with an anti-PrP antibody after PK digestion of the cell suspension. NBH – PMCA seed was omitted. C – Internal Western blot control.

Another relevant aspect is the ability of PMCA products that had been derived in the presence or absence of specific cofactor molecules to induce PrPres propagation in glial cell cultures. In the following, cell cultures were exposed to PMCA products which had been obtained under the presence or absence of specific cofactor molecules. PMCA samples were seeded with BSE-H in nucleic acid depleted NBH. As shown in Figure 3-7, BSE-H prions induced an accumulation of PrPres under nucleic acid free conditions. Reaction products after 12 PMCA rounds were used for cell infection. The assay was performed as described above. Figure 3-14 shows an accumulation of PrPres in glial cells after 40 and 80 DPE that was induced by the BSE-H PMCA product generated in nucleic acid depleted NBH. Further PMCA products were generated by seeding nucleic acid depleted NBH with 263K prions in the presence of a polyanion. A reconstitution of PrPres amplification under these conditions could be achieved by the addition of poly(A) RNA or chondroitin sulfate A (Figure 3-9). PMCA samples that were used for cell infection had been produced in presence of 300 µg/ml of each cofactor molecule by four rounds of PMCA. Both of these PMCA samples were found to produce an infection in the cell assay with an accumulation of PrPres until 80 DPE. Thus, all three tested samples were biological seeding active. This demonstrated that poly(A) RNA and chondroitin sulfate A did not only restore the capability to amplify PrPres but also the propagation of infectivity in PMCA. Furthermore, BSE-H associated PrPres preserved a

biological seeding activity, i.e. cell culture infectivity, independently of the depletion of nucleic acids in the PMCA substrate.

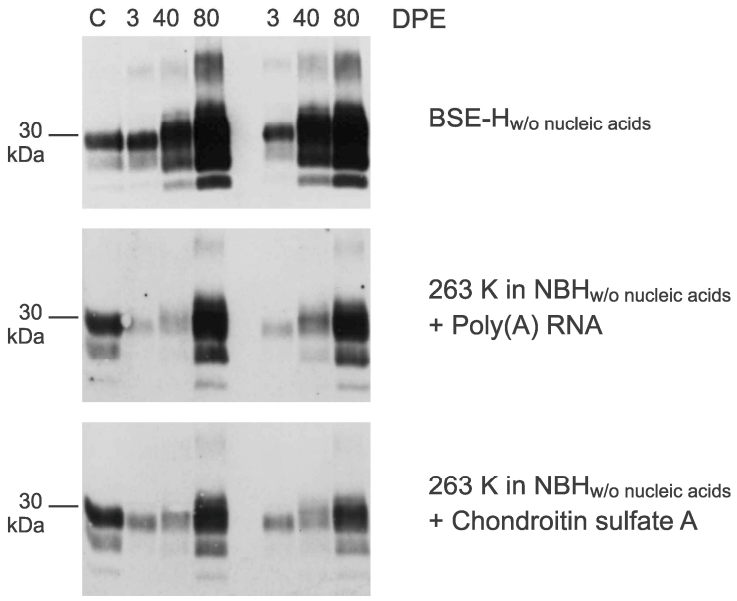

Figure 3-14: PrPres accumulation in glial cell cultures after exposure to PMCA products which had been obtained in the presence or absence of specific cofactor molecules. Cells were incubated with the inocula for three days. Subsequently the inoculum was removed and cells were incubated further. PrPres was detected at the indicated DPE. Cells were infected with BSE-H or 263K seeded PMCA samples in nucleic acid free NBH substrate. 263K seeded PMCA samples were reconstituted with poly(A) RNA or chondroitin sulfate A. C – Internal Western blot control.

3.3.3 Metal cofactors in the cell assay

As shown above, certain metal ions exert an inhibitory effect on PrPres propagation in PMCA (chapter 3.2.4). To address the question whether the same effect of Cu^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+} and Mn^{2+} can be also observed for the biological propagation of 263K prions in the cell culture assay the following study was performed. See Figure 3-15 for a schematic illustration of the experimental design. Glial cells were cultured 2-3 days prior to infection. The cells were then incubated in the constant presence of metal ions starting with the day of inoculation with 10^{-5} g 263K brain tissue, and were cultured up to 74 DPE (Figure 3-15A). Cells were incubated with the 263K inoculum for three days, thereafter the inoculum was removed as described above. Metal ions were applied to the culture medium as aqueous chloride solutions at final concentrations of 20 and 50 µM. According to the manufacturer the cell culture medium DMEM did not contain any of the metal ions that were applied in the assay, except for 6.4 g/l (~110 mM) NaCl and

0.1 mg/l (~0.25 µM) iron(III) nitrate nonahydrate ($Fe(NO_3)_3 \cdot 9H_2O$). The concentration of approx. 0.25 µM $Fe(NO_3)_3 \cdot 9H_2O$ was well below the lowest applied concentration of 20 µM $FeCl_3$ and is therefore negligible. Similar concentrations of metal ions were used in studies that involved primary neuronal cells [Cuajungco *et al.*, 2000; Lovell *et al.*, 1999]. Higher concentrations resulted in cell damage, as was observed by microscopic monitoring. With the renewal of culture medium every week the metal ion solutions were again newly applied.

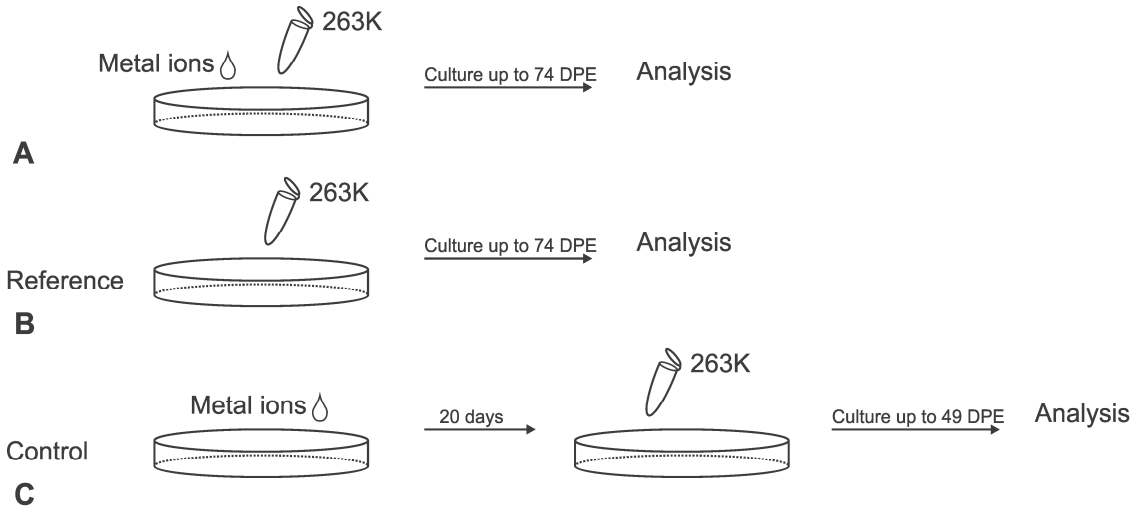

Figure 3-15: Experimental design for the determination of metal ion mediated effects on prion propagation in the cell assay. (A) Cells were cultured in the presence of metal ions for the whole duration of the cell infection experiment beginning with the day of exposure to 263K hamster brain homogenate. (B) Cells were incubated with 263K brain tissue and further cultured without any additional metal ion solutions. (C) Metal ions were added to the culture medium for 20 days before infection. Thereafter cells were infected with 263K and further cultured without metal ions.

Cell cultures that were incubated with 263K brain tissue without any additional metal ion solutions served as a reference to evaluate metal ion mediated effects (Figure 3-15B). To assess whether metal ions might have implications on cell metabolism which in turn could possibly result in an impaired ability to produce PrPres, an additional control was included. Cultures were incubated for about 20 days in the presence of metal ions. Thereafter cells were infected with 263K and were cultured further 49 days in normal medium without additional metal ions (Figure 3-15C). PrPres formation in these cultures was compared to reference cultures that were not exhibited to metal ions but inoculated with 263K after 20 days incubation in normal culture medium. These controls are marked with * in Figure 3-16. A thorough examination of cells by at least weekly microscopy was of high importance to assess cell membrane integrity, since

there are no data available that describe the long-term effect of metal ions on cell vitality of isolated hamster glial cells (appearance of cell death by apoptosis or necrosis). Indeed manganese was found to be toxic to cells in this assay even at a concentration as low as 20 µM. A constant loss of cells exposed to manganese was observed upon microscopic inspection. Therefore results for manganese were excluded from further analyses.

Figure 3-16 shows the Western blot results. Reference samples showed a constant increase in PrPres levels up to 74 DPE. Cells that were infected 20 days later had higher PrPres contents at 49* DPE than cells after 47 DPE. Apparently, PrPres was more efficiently propagated by these cells. Furthermore a solution of NaCl was applied as a mock control. As the cell culture medium DMEM already contained 6.4 g/l (~110 mM) NaCl, this was used as general control to exclude that any effects are caused by the pure addition of exogenous chloride solutions, e.g. on cell vitality. The detected PrPres levels for these controls were found to be similar to the reference samples with only minor variations. The susceptibility to prion infection after 20 days culture with $ZnCl_2$ was not impaired, as assessed by the amount of PrPres at 49* DPE. When cell infection was performed in the presence of 20 µM $ZnCl_2$ no change in PrPres formation was observed, whereas PrPres production was decreased when 50 µM $ZnCl_2$ were present. An inhibitory concentration dependent effect of $ZnCl_2$ on the seeding activity of 263K prions was already observed in the PMCA assay. For the ions Fe^{2+}, Fe^{3+} and Cu^{2+} also lower levels of PrPres were detected at 47 and 74 DPE as compared to the reference and control samples. The inhibitory effect of 20 µM $FeCl_2$ could not be enhanced in the samples containing 50 µM $FeCl_2$. The control at 49* DPE for 50 µM $CuCl_2$ did not show a proper PrPres production compared to the reference which might be due to impaired cell metabolism. A damage of cells was not observed by microscopy.

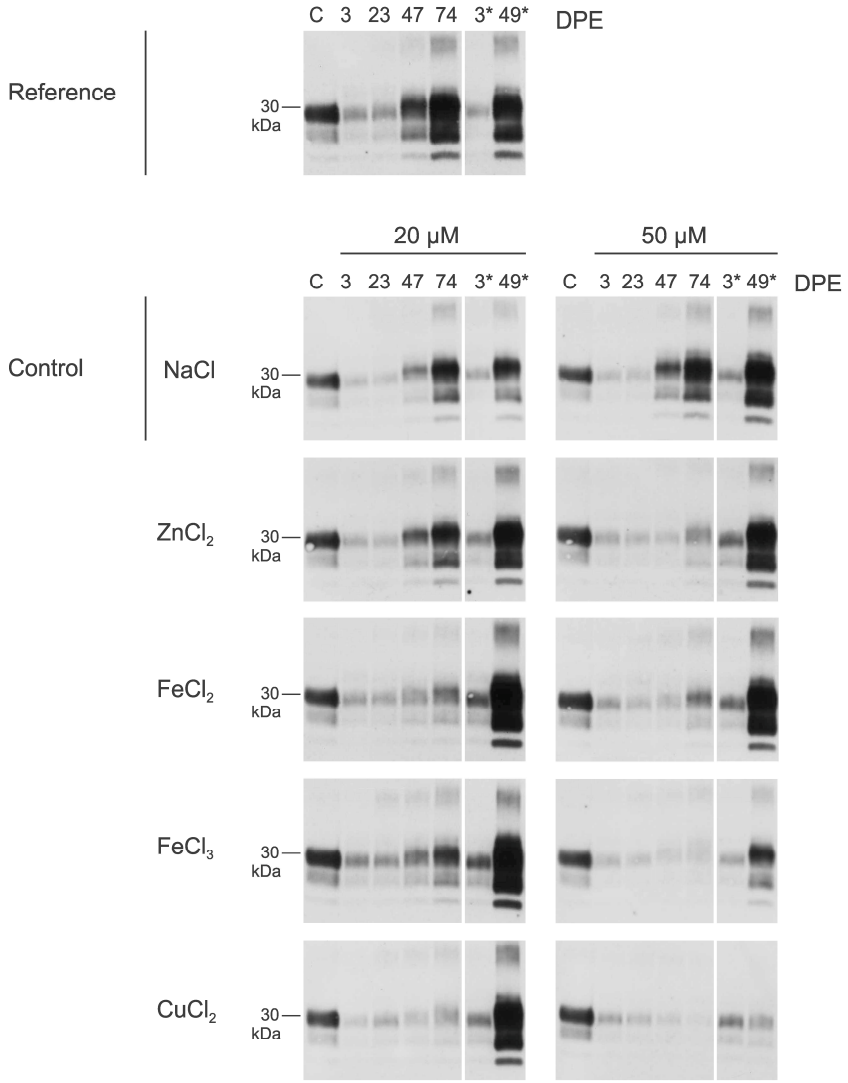

*Figure 3-16: Glial cell assay with 263K scrapie prions to assess the impact of different metal ions on PrPres propagation. Cell infection was performed in the presence of 20 or 50 µM metal ions, except for references or controls. Metal ion solutions were added directly to the culture medium. PrPres was detected at the indicated DPE. The signal at 3 DPE resulted from 263K inocula that remained attached to the cells. Samples marked with * were cultured for 20 days in the presence of metal ions prior to infection with 263K. Thereafter, cell infection and subsequent cultivation of control cell cultures was performed in the absence of additional metal ions. C – Internal Western blot control.*

In accordance with the findings from PMCA assays all examined metal ions were found to inhibit the propagation of 263K associated PrPres in glial cell cultures. This effect appeared to be dose dependent between 20 and 50 µM for Zn^{2+} and Fe^{3+}. In contrast to PMCA, Cu^{2+} (20 µM) had the strongest inhibitory effect on 263K amplification followed by Fe^{2+}, Fe^{3+} and Zn^{2+}.

3.4 Comparative biochemical and biophysical characterization of native prion seeds and their progeny PrPres from PMCA- or cell assays

Brain derived PrP^{Sc} associated with 263K, ME7-H, 22A-H and BSE-H were shown to exhibit distinct combinations of biochemical and physical properties [Thomzig *et al.*, 2004]. In the following chapter biochemical and biophysical features of PrPres generated by PMCA or in glial cell cultures were determined and compared to the parental prion agents.

3.4.1 Amplification efficiency of parent and progeny seeds in PMCA

The following procedure allowed a parallel monitoring of the seeding efficiencies of PrP^{Sc} from 263K and ME7-H associated brain homogenate and their respective PMCA derived PrPres. A PMCA was carried out with identical treatment of all samples. Samples were seeded with identical amounts of PrPres from brain homogenate or PMCA products. Identical amounts of PrPres seeds were adjusted based on comparison of Western blot signal intensities. PMCA derived PrPres was generated beforehand by subjecting 263K and ME7-H brain homogenates to three rounds of PMCA (see Figure 3-17A). Comparative PMCA with brain- and PMCA derived seeds was subsequently performed for four rounds and in identical NBH substrate for all samples. The Western blot results are presented in Figure 3-17B. Staining intensities of PrPres from samples that were seeded with 263K brain homogenate or its derived PMCA product are identical for same PMCA rounds. Thus both seed types exhibit similar seeding efficiency in PMCA. In contrast, the formation of PrPres in samples seeded with ME7-H brain homogenate increases very slowly, starting with the second round of PMCA. However, PMCA derived ME7-H seeds have a higher efficiency in the production of PrPres which already started in the first PMCA round. The seeding efficiency of this material is similar to that of 263K associated prion seeds. Hence, brain ME7-H and its PMCA product differ from each other with respect to their seeding efficiency.

Furthermore a change in the glycosylation profile of the PMCA derived ME7-H PrPres was observed. The ME7-H prion strain displays similar staining intensities of the diglycosylated and the monoglycosylated protein band [Thomzig *et al.*, 2004], whereas its PMCA product shows a stronger appearance of the diglycosylated protein band, as can be clearly seen in the start sample (round 0).

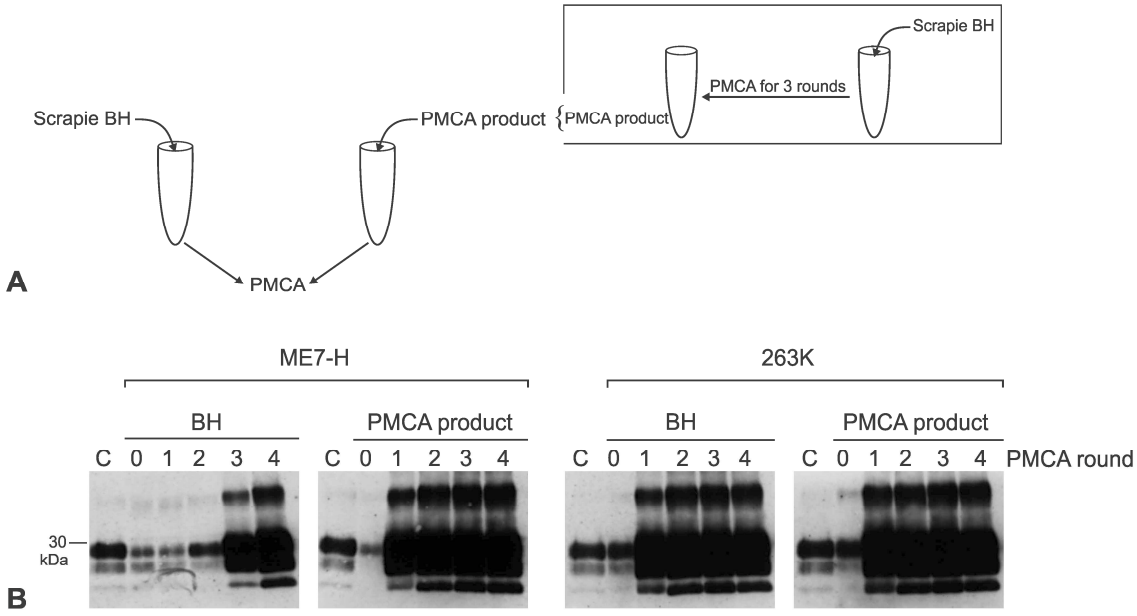

Figure 3-17: Amplification efficiencies of native 263K and ME7-H seeds and their progeny PrPres from PMCA assays. (A) Schematic representation of experiment. (B) PMCA reactions were seeded with 1 x 10^{-5} g brain tissue (referred to as BH) from ME7-H or 263K infected Syrian hamsters or with an identical amount of PrPres from a 3-round PMCA (referred to as PMCA product) that was seeded with native ME7-H or 263K, respectively. C – Internal Western blot control.

3.4.2 Impact of cofactors on the seeding activity of parent and progeny seeds

The same experimental design was applied to assess the efficiency of the polyanions poly(A) RNA and chondroitin sulfate A to reconstitute PrP conversion by PMCA in nucleic acid depleted NBH. Identical amounts, in terms of PrPres, of brain homogenate and PMCA products of 263K and ME7-H associated PrP were seeded in nucleic acid free NBH and samples were subjected to four rounds of PMCA (Figure 3-18, first line). As mentioned above (chapter 3.2.2) a propagation of misfolded PrP could not be achieved under nucleic acid depletion in NBH for both prion strains. Figure 3-18 shows that this also applied to samples seeded with PMCA derived PrPres of both prion strains. Nevertheless a change in one detail was observed. The samples that had been seeded with native 263K prions exhibited a residual seeding activity that caused a transient amplification of PrPres in the first PMCA round (see chapter 3.2.2). A similar effect was also observed in samples that had been seeded with PMCA derived ME7-H, but was absent with the ME7-H brain homogenate. Propagation of 263K parent (from brain) and progeny (from PMCA) seeds was efficiently restored by the addition of synthetic poly(A) RNA molecules (Figure 3-18, middle line). A similarly efficient reconstitution was achieved for the amplification of PMCA derived ME7-H seeds, whereas reconstitution was only weak for the ME7-H brain seed in this experiment,

with a recovery of PrPres formation in the fourth PMCA round (cp. Figure 3-8 where the same effect appears in a more pronounced way). A further difference was observed when PrPres propagation was effectively restored with chondroitin sulfate A in samples that had been seeded with native and progeny 263K material as well as the sample that had been seeded with PMCA derived ME7-H associated PrPres, but not in the sample that had been seeded with ME7-H brain tissue (Figure 3-18, last line).

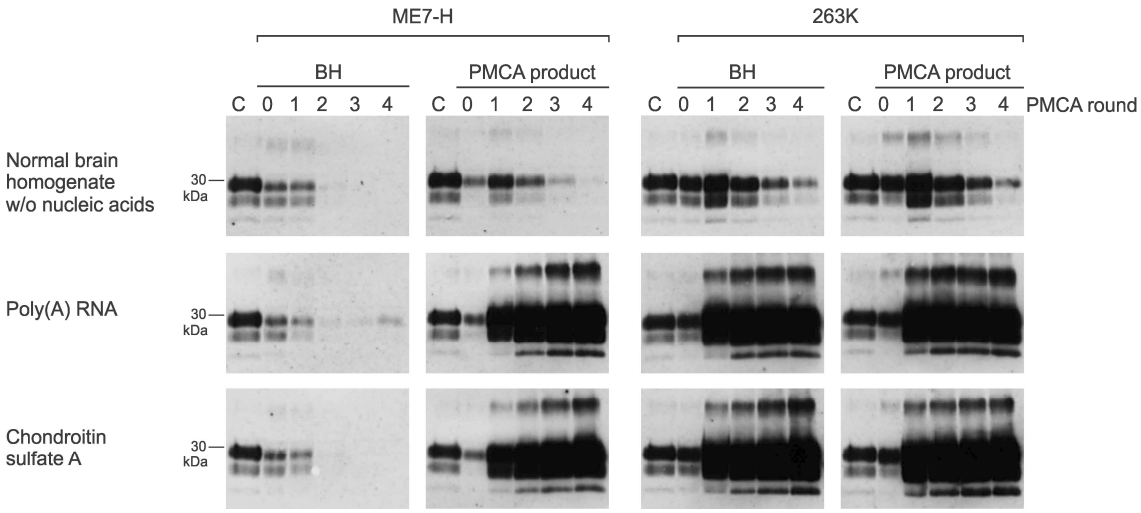

Figure 3-18: Impact of cofactors on the seeding activity of native 263K and ME7-H seeds and their PMCA derived progeny seeds in PMCA. PMCA was performed in NBH that had been depleted of nucleic acids and in similar samples to which 100 µg/ml poly(A) RNA or 400 µg/ml chondroitin sulfate A were added during PMCA. C – Internal Western blot control.

3.4.3 Electrophoretic mobility of deglycosylated parent and progeny seeds in SDS-PAGE

One biochemical method to distinguish 263K and BSE-H is an enzymatic deglycosylation of both PrPres species with PNGase F. After incubation with this enzyme the prion protein shows a single band upon detection with an anti-PrP antibody in the Western blot. After digestion with PK the unglycosylated band of 263K and BSE-H exhibit a different electrophoretic mobility due to different molecular masses of approx. 20 kDa and 19 kDa, respectively [Thomzig *et al.*, 2004]. In the following PrPres produced *in vitro* by PMCA or in cell cultures was analyzed, and compared to the native PrPres from 263K- or BSE-H brain homogenates by this approach. Analyses were performed with reaction mixtures after at least four serial PMCA rounds or with

PrPres from harvested cells. In both cases the amount of starting seeding material or inoculum was below the detection limit of the Western blot.

In Figure 3-19A the electrophoretic mobilities of unglycosylated PrPres from 263K, BSE-H, ME7-H and 22A-H originating from brain homogenate or derived by PMCA after four PMCA rounds are shown. Discrimination between brain homogenates from BSE-H and all other prion strains on the basis of the characteristic band shift was possible. Furthermore a direct comparison of BSE-H derived PMCA product with brain BSE-H shows that the characteristic band shift for BSE-H was still preserved in its PMCA derived progeny PrPres. The PMCA derived BSE-H PrPres is clearly different from all PMCA products that had been seeded with 263K, ME7-H or 22A-H. Next, BSE-H associated PrPres that had been generated in previous PMCA experiments under various cofactor conditions (chapter 3.2) was analyzed for its electrophoretic mobility. In Figure 3-19B BSE-H seeded PMCA samples are directly compared to 263K seeded PMCA samples. The first PMCA sample was seeded with BSE-H brain tissue in normal hamster brain homogenate and additional 200 µg/ml poly(A) RNA (Figure 3-4). The other BSE-H seeded samples were generated in nucleic acid depleted NBH and by addition of 100 µg/ml poly(A) RNA or 400 µg/ml chondroitin sulfate A, respectively. The polyanions were required for amplification in nucleic acid depleted NBH as presented above (Figure 3-9). The characteristic shift of the unglycosylated protein band was also preserved in PMCA samples after six amplification rounds that were generated in the presence of synthetic RNA or after PMCA reconstitution with synthetic RNA or chondroitin sulfate A, respectively. Since the different molecular masses of 263K and BSE-H of approx. 20 kDa and 19 kDa after digestion with PK probably reflect differences in protein conformation, the findings indicated that characteristic conformational features of native brain BSE-H were transferred to the PMCA generated PrPres in the presence of poly(A) RNA or chondroitin sulfate A. In contrast, the PMCA generated BSE-H species that was found to propagate in nucleic acid depleted NBH (Figure 3-7) did not differ with respect to the electrophoretic mobility of its unglycosylated band from 263K as shown in Figure 3-20B. This indicated that a lack of nucleic acids in the substrate of BSE-H PMCA resulted in the amplification of conformationally changed PrPres.

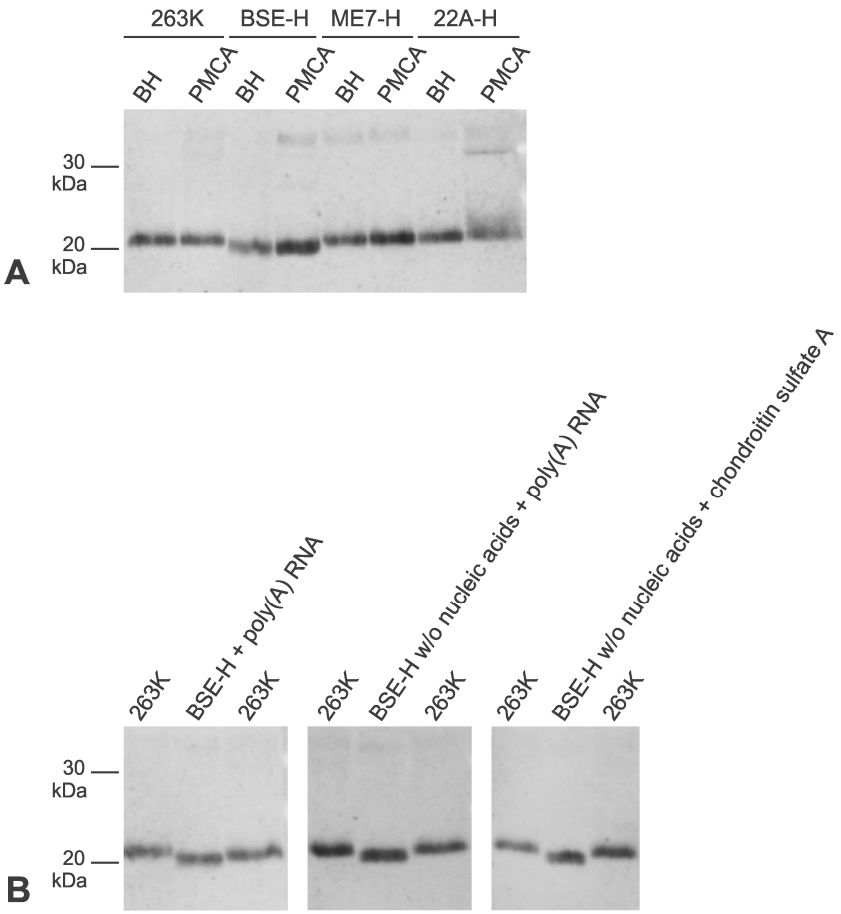

Figure 3-19: Electrophoretic mobility of PrPres species before and after PMCA. PrPres was deglycosylated with PNGase F. (A) Brain homogenate (BH) of 263K, BSE-H, ME7-H and 22A-H infected hamsters was used to seed PMCA reactions and was compared to its corresponding PMCA derived progeny PrPres (PMCA). (B) Progeny PrPres derived from native 263K or BSE-H seeds by PMCA under the indicated cofactor conditions.

Electrophoretic mobilities of PMCA samples that were used as inocula for the infection of glial cell cultures and of the corresponding cell culture derived PrPres are depicted in Figure 3-20. PrPres produced by glial cells after incubation with PMCA derived BSE-H inoculum also exhibited an electrophoretic migration pattern different from that of 263K PMCA- and cell products (Figure 3-20A). In contrast, the PMCA generated BSE-H that had been amplified in the absence of endogenous nucleic acids in NBH substrate showed no different molecular mass from 263K. The same hold true for PrPres that had been produced by cell culture infection with this particular BSE-H derived PrPres. However, for this material the protein band was not as dense as expected and a thinner ghost band appeared repeatedly with a slightly lower molecular mass (Figure 3-20B). This result was therefore ambiguous and remained to be further investigated.

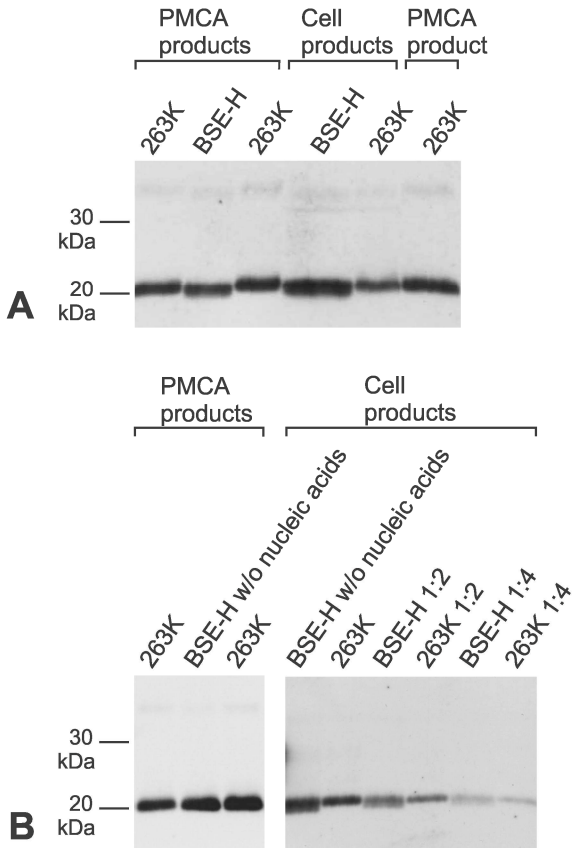

Figure 3-20: Comparison of the electrophoretic mobility of PrPres species used for infection of glial cell cultures and the glial cell-amplified PrPres harvested at 80 DPE. After digestion with PK samples were enzymatically deglycosylated using PNGase F. (A) PMCA derived BSE-H and 263K PrPres prior to and after amplification in the cell culture. (B) Cell infection was performed with PMCA derived BSE-H PrPres produced by PMCA with nucleic acid depleted NBH. Some samples were serially diluted for better resolution.

3.4.4 Secondary structure analysis of parent and progeny seeds by FT-IR spectroscopy

The hamster adapted prion strains 263K, ME7-H, 22A-H and BSE-H were recently described to be distinguishable by the biophysical method of Fourier transform-infrared (FT-IR) spectroscopy [Thomzig *et al.*, 2004]. This method enabled a swift differentiation of TSE agents, even of ME7-H and 22A-H which were not distinguishable by biochemical, clinical or neuropathological features but only by incubation time. Differentiation with FT-IR spectroscopy was based on conformational diversity, attributed mainly to differences in β-sheet structure and to variations in other secondary structure elements of PrPres from different hamster prion strains. The biochemical characterization of BSE-H progeny seeds produced by PMCA in nucleic acid depleted NBH described above (chapter 3.4.3) indicated that the lack of nucleic acids in the reaction substrate resulted in amplification of conformationally changed

PrPres. To investigate if the presence or absence of specific cofactors during PMCA caused detectable alterations in protein conformations, FT-IR spectroscopy was used in order to structurally characterize PrPres amplificates. For this purpose PMCA products were purified according to a protocol by Daus *et al*. and dried protein samples were subjected to Fourier transform-infrared micro-spectroscopy established by this author [Daus *et al.*, 2013]. By this novel approach, it was possible to analyze very small amounts of PrPres as 25-75 ng per dried sample spot. This is relevant due to the small protein amounts produced by PMCA.

PrPres was purified from PMCA products to obtain extracts suitable for FT-IR measurements as described in materials and methods (chapter 2.2.11). This procedure included a very mild digestion with PK in order to degrade other proteins in the suspension with a high yield of PrPres (which maintains a PK-resistant core for longer periods of time). Quality of PrPres extracts was analyzed by Western blotting and silver staining of gels. As determined by Western blot detection with the anti-PrP antibody 3F4, some extracts still contained full-length PrP, presumably not degraded PrP^C. In order to improve digestion with PK in these samples, all remaining extracted pellets of the same PMCA product were pooled and were again subjected to the purification protocol starting with the PK digestion step. This is a modification of the protocol by Daus *et al*. (2013). However, only extracts in which PrP^C was completely degraded were eventually subjected to FT-IR spectroscopic analysis. Samples that were repeatedly digested with PK are indicated in Table 3-3 and Table 3-4. Due to the difficulties with PK digestion and due to remaining impurities revealed in the silver stained gels, all data shown in this chapter have to be considered preliminary and were obtained under not yet optimal conditions.

PMCA products derived from 263K seeds using nucleic acid depleted NBH but with addition of 100 µg/ml poly(A) RNA or 300 µg/ml chondroitin sulfate A, respectively, were generated in three independent PMCA experiments and with different ultrasonic devices. Each PrPres sample was amplified to a final volume of 1 ml in six PMCA rounds. Purification of PrPres was also performed in independent runs, and quality of purified protein samples was analyzed by Western blot and silver staining prior to measurement by FT-IR spectroscopy. Although silver gels still showed the presence of impurities in the PrPres extracts spectra were tentatively obtained from dried protein samples in a set of preliminary pilot experiments as exemplarily shown in Figure 3-21.

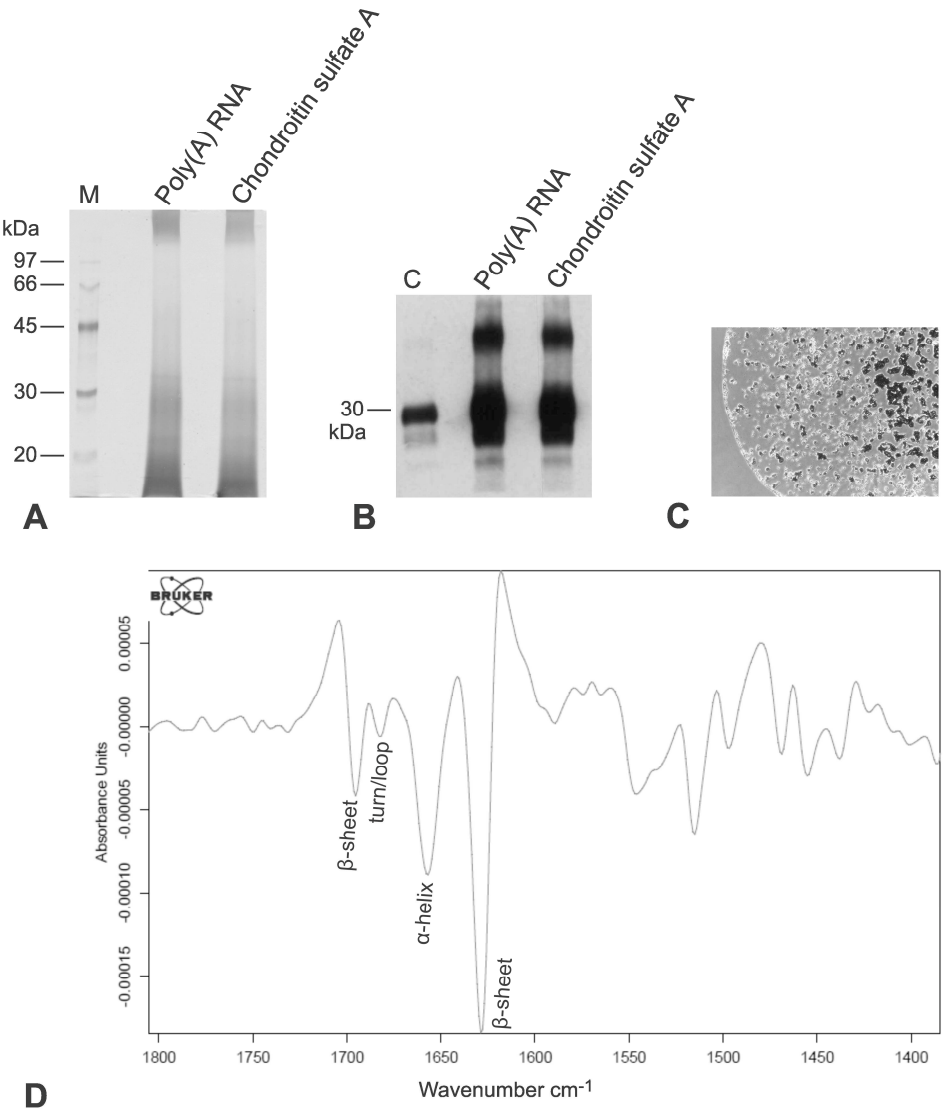

Figure 3-21: (A) Silver staining of PrPres extracts from PMCA samples. (B) The presence of PrPres after purification was confirmed by Western blot. (C) Purified PrPres was transferred onto CaF_2 slides and dried samples were subjected to FT-IR spectroscopy. (D) FT-IR absorption spectra were obtained of dried protein samples. Amide I region encompasses the range from 1610-1700 wavenumbers (cm^{-1}).

The peak positions that were identified in the spectra for each sample are listed in Table 3-3. Peak positions of the 263K seeded samples reconstituted with polyanions are compared to available data [Daus *et al.*, 2013] from i) 263K parent seeds and ii) 263K progeny seeds that had been produced by standard PMCA. Samples that were extracted and purified in three independent runs showed nearly identical peak positions in their spectra. From the PMCA experiment in which the NBH had been first depleted for endogenous nucleic acid and was subsequently reconstituted with exogenous RNA only two sample preparations were suitable for FT-IR measurement. Mean values of

absorption maxima were calculated and are listed in bold and marked with ∅. To assess putative spectral interference of cofactors that had been added to reconstituted PMCA substrate, solutions of poly(A) RNA and chondroitin sulfate A were separately dried and subjected to FT-IR measurement. No absorption peaks were observed for chondroitin sulfate in the amide I region. One peak was obtained for pure poly(A) RNA at 1656 cm^{-1} which however differed from the protein peak position and was only obtained with high concentrations of RNA that were equivalent to the reaction conditions during PMCA. The extraction of PrPres from PMCA products involved nucleic acid degrading procedures, so that theoretically no residual nucleic acids should be present in the final extract.

Table 3-3: FT-IR absorption maxima of purified 263K parent and progeny PrPres in the amide I region. Samples marked with * had been repeatedly digested with PK.

	Structure component peak positions (cm^{-1})			
	ß-sheet (low frequency)	*α-helix*	*turns/loops*	*ß-sheet (high frequency)*
Cofactor molecules				
Poly(A) RNA	-	1656	-	-
Chondroitin sulfate A	-	-	-	-
BH				
263K [Daus *et al.*, 2013]	**1626/1635**	**1659**		**1694**
PMCA products				
263K [Daus *et al.*, 2013]	**1627**	**1658**	**1681**	**1694**
263K + poly(A) RNA*	1628	1657	1682	1695
	1628	1658	1682	1695
∅	**1628**	**1657.5**	**1682**	**1695**
263K + chontroitin sulfate A*	1629	1657	1682	1695
	1629	1657	1681	1695
	1628	1658	1681	1695
∅	**1628.7**	**1657.3**	**1681.3**	**1695**

The data do not allow a clear discrimination between samples from PMCA that had been reconstituted with RNA or chondroitin sulfate. Determination whether these spectral data are significantly different from 263K progeny seeds that had been generated under conventional PMCA conditions is, due to little sample number, not feasible yet and remains to be done in future studies. However, all progeny PrPres forms produced by PMCA were clearly different from authentic brain associated 263K PrPres. The latter showed a spectral double peak at the position that is assigned to

β-sheet (low frequency), whereas all progeny PrPres forms have a single peak in this part of the amide I region of the spectrum.

Additionally, first preliminary FT-IR spectral data were obtained for PMCA produced PrPres derived from ME7-H, 22A-H and BSE-H under various cofactor conditions. Table 3-4 shows these preliminary data that were obtained from one or two sample preparations. At this point, a clear and robust differentiation of these PMCA products from each other is not possible.

However, FT-IR spectra of PrPres from parent brain homogenates of different prion strains are clearly distinguishable from those of the respective PrPres amplificates. Differences were observed with respect to peak positions indicating β-sheets (low frequency). For example ME7-H parent PrPres showed a double peak at 1626/1633 cm^{-1}, whereas a single peak at 1629 cm^{-1} was found for the ME7-H derived PMCA product. For further structural analyses the spectral database has to be further improved and expanded.

Table 3-4: FT-IR absorption maxima of ME7-H, 22A-H and BSE-H associated parent and progeny PrPres in the amide I region. Samples marked with * had been repeatedly digested with PK.

	Structure component peak positions (cm^{-1})			
	ß-sheet (low frequency)	*α-helix*	*turns/loops*	*ß-sheet (high frequency)*
BH (M. Daus unpublished)				
ME7-H	1626/1633	1659	1680	1695
22A-H	1631	1659	1682	1695
BSE-H	1627	1659	1680	1695
PMCA products				
ME7-H*	1627	1658	1682	1695
	1629	1659	1681	1695
22A-H*	1628	1655	1682	1696
BSE-H	1627	1659	1683	1695
PMCA w/o nucleic acids				
BSE-H	1627	1656	1683	1696
	1627	1655/59	1683	1696
Reconstituted PMCA products				
22A-H + chondroitin sulfate*	1626	1658	1681	1695
BSE-H + poly(A) RNA*	1628	1658	1682	1695
BSE-H + chondroitin sulfate*	1628	1654	1684	1696

In summary, the ME7-H derived progeny PrPres differed from its ME7-H parent seeds with respect to the glycosylation pattern, the seeding activity, the ability to induce glial cell infection and the dependency on specific cofactors for PrP conversion. For BSE-H associated PrPres a typically lower molecular mass of the unglycosylated band was exhibited by parent and progeny seeds, when the latter were generated in the presence of polyanionic factors. In contrast, BSE-H progeny seeds produced in the absence of such cofactors did not show such lower molecular mass of the unglycosylated PrPres band. Biophysical analysis of purified PrPres using FT-IR spectroscopy revealed spectral differences between parent and progeny PrPres for prion strains 263K, ME7-H, 22A-H and BSE-H. The preliminary findings from this FT-IR analytical pilot study need to be validated and expanded in the future.

3.5 PMCA and cell culture assays as alternative *in vitro* methods for prion infectivity titrations

The findings presented below evolved during this dissertation project and perspectively facilitate the reduction or even replacement of animal bioassays in prion research. This part of the doctoral thesis was published recently in the journal Laboratory Animals [Boerner *et al.*, 2013] and is presented in brief in the following paragraphs.

It has been recently demonstrated that the PMCA assay is not only a simple method for the qualitative detection of 263K seeding activity but that this assay also allows a quantitative determination of 263K seeding activity [Chen *et al.*, 2010; Pritzkow *et al.*, 2011; Wilham *et al.*, 2010]. This previously established PMCA procedure could be further adapted for an efficient amplification and detection of BSE-H and 22A-H prions as described above. These adapted PMCA assays are also suitable for a quantitative titration of the seeding activity of both prion strains. Figure 3-22 shows that propagation of PrPres from BSE-H and 22A-H in PMCA is consistently dose-dependent. A quantitative PMCA assay for 263K is shown comparatively. By endpoint titrations of prion seeding activity using these PMCA assays median PMCA seeding doses (SD_{50}) can be determined. One SD_{50} is the dose of seeding activity that converts PrP^C into PrPres in 50% of PMCA samples. SD_{50} values of unknown samples can be determined by parallel endpoint titrations of reference and unknown samples in PMCA as described by Boerner *et al*. (2013).

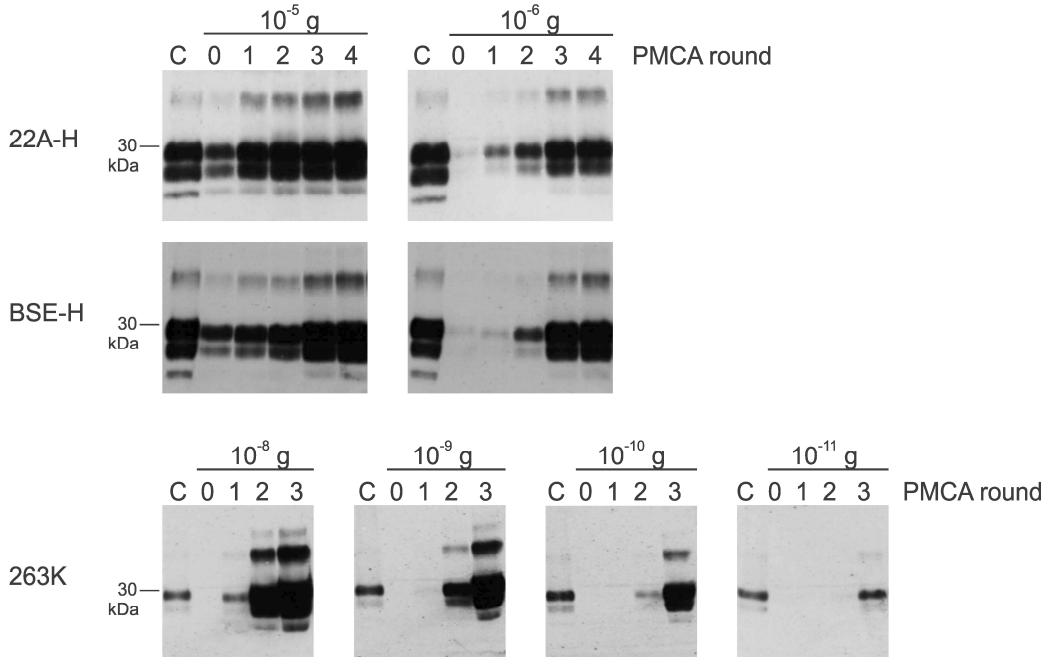

Figure 3-22: Dose-dependent propagation of BSE-H- and 22A-H-derived PrPres by PMCA. PMCA assays were seeded with 10^{-5} and 10^{-6} g 22A-H or BSE-H brain tissue. For comparative reasons, a quantitative PMCA assay for 263K prions is shown as well. The amounts of seeding tissue are indicated. PMCA protocols were specifically adapted for efficient propagation of PrPres from 22A-H and BSE-H (see chapter 3.1). C – Internal Western blot control.

Similarly to the titration of seeding activity by PMCA, 263K scrapie infectivity can be quantitatively titrated by the glial cell assay. As shown above, glial cell cultures can also be infected with other hamster prion strains. BSE-H and 22A-H, but not ME7-H are able to infect such cultures. Furthermore, though the cell assays for these prion strains were not yet fully developed regarding a quantitative titration of prion associated infectivity, they hold the potential for this purpose as shown in Figure 3-23. Cells showed a clear correlation between the amount of formed PrPres at defined incubation times and the prion inoculation dose. In analogy to the determination of SD_{50} values by PMCA, the cell assay can be used for a determination of the median cell culture infective dose ($CCID_{50}$). One $CCID_{50}$ is the dose of prions that causes infection (in terms of PrPres propagation) in 50% of inoculated cell cultures. $CCID_{50}$ values of unknown samples can be determined by parallel endpoint titrations of reference and unknown samples in the cell assay as described by Boerner *et al*. (2013). In order to increase the sensitivity of BSE-H infections in glial cell cultures further improvements could be made that have not been published yet. Due to an optimization in the procedure for proteinase K digestion (see chapter 2.2.10.5) an amount of 10^{-5} g brain tissue inoculum was sufficient to produce a detectable PrPres amplification after

80 DPE. This additionally reduced the overall processing time of the assay for future BSE-H titrations.

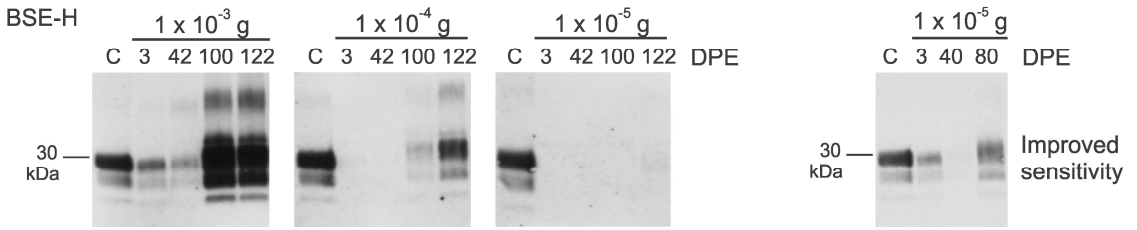

Figure 3-23: PrPres amplification in glial cell cultures after incubation with the indicated amounts of BSE-H brain tissue is dose dependent. An optimized PK processing protocol devised based on the published work by Boerner et al. (2013) improved the sensitivity and reduced the incubation time. C – Internal Western blot control.

"Quantitative prion bioassays in animals usually determine infectivity titers in terms of the median infective doses (ID_{50}) that had been present in the inoculated sample material. One prion ID_{50} is the dose of prions that causes infection in 50% of inoculated animals. Based on empirically established quantitative correlations among SD_{50}, $CCID_{50}$ and ID_{50} in reference standards such as homogenized 263K scrapie hamster brain tissue, SD_{50} and $CCID_{50}$ values detected in test samples can be tentatively translated into ID_{50} values. PMCA and cell assays represent profoundly different cell-free and cell-based test principles for the biochemical and biological titration, respectively, of prion activity *in vitro*. Therefore, if these assays independently deliver consistent ID_{50} assessments, this substantially backs up the overall test reliability as compared with titrations based on either PMCA or cell assays alone. An integrated approach for the reduction and replacement of prion bioassay titrations in animals that includes both the measurement of seeding activity and the determination of cell culture infectivity by quantitative, sensitive and robust PMCA and cell assays, respectively, is suggested" [Boerner *et al.*, 2013].

4. Discussion

4.1 Propagation of ME7-H, 22A-H and BSE-H prion strains in PMCA and cell assay

4.1.1 PMCA

The hamster prion strain 263K scrapie is often used as a model prion agent for research purposes in laboratories. Very efficient propagation of 263K in serial PMCA with an optimized protocol was recently described by our group [Pritzkow *et al.*, 2011]. However, other hamster adapted prion strains, such as ME7-H, 22A-H and BSE-H could not be amplified, or amplified only with poor efficiencies, using this PMCA protocol. As mentioned above (chapter 3.1) these prion strains were distinguishable by their respective *in vivo* incubation times and by biophysical FT-IR spectroscopic measurements. As previously reported, these prion strains differed in part with respect to clinical, neuropathological and biochemical features [Thomzig *et al.*, 2004].

Amplification of ME7-H, 22A-H and BSE-H by the PMCA technique could only be achieved by addition of the metal chelator EDTA and a prolongation of the cyclic incubation time. Each prion strain had slightly different minimal requirements with respect to amplification conditions. Whereas 22A-H required an incubation time of 4 hours, amplification of BSE-H and ME7-H required only 2 hours intervals of incubation time. This indicates that BSE-H and ME7-H exhibit faster seeding activities than 22A-H under the applied PMCA conditions.

According to the “protein-only” hypothesis the infectious prion agent consists mainly if not entirely of misfolded protein without coding nucleic acids [Prusiner, 1982]. Since different prion strains exist in a single species which share the same amino acid sequence, they must originate from different protein conformations or be due to different cofactor attachments or effects [Collinge *et al.*, 1996; Geoghegan *et al.*, 2007; Safar *et al.*, 1998]. Extension of the incubation times during PMCA cycles was beneficial for the amplification of strains with long *in vivo* incubation times (ME7-H, 22A-H and BSE-H).

Nucleation-dependent, or seeded, PrP polymerization was proposed by Jarrett and Lansbury [Jarrett and Lansbury, 1993] as the molecular mechanism underlying PrP fibril elongation. It was assumed that fibrils are elongated by template-mediated selection of the amyloidogenic conformation from a pre-existing equilibrium of different PrP conformers [Bhak *et al.*, 2009]. If there are multiple conformers present in

an equilibrium in the PrP substrate, then the need for appropriate selection of molecules with the complementary conformation to the template could plausibly influence the efficiency of fibril elongation. Alternatively, PrP^C monomers may experience a structural transition to the amyloidogenic conformation complementary to the template upon binding. This transition may occur slower for ME7-H, 22A-H and BSE-H than for 263K, and thus result in a delayed elongation of seeds. Furthermore, it was speculated that PrP^{Sc} fibrils require further maturation after breakdown into aggregates by the sonic treatment before efficient conversion of PrP^C can take place [Eiden *et al.*, 2006]. If the sonication is applied too early, this would interfere with the elongation or maturation of PrP^{Sc} seeds, respectively, and would impair the amplification process. In any case, differences in prion strains are reflected by differing individual requirements for efficient PMCA.

In contrast, amplification of prions from various strains and various species with an apparently unchanged PMCA protocol was described by Soto and others [Barria *et al.*, 2011; Castilla *et al.*, 2008b; Deleault *et al.*, 2010; Piro *et al.*, 2009]. In accordance with the data presented in this thesis, different seeding efficiencies for different hamster prion strains in serial PMCA have been described recently by others [Gonzalez-Montalban and Baskakov, 2012] and were experimentally addressed by using adapted dilution ratios for serial transmission steps between PMCA rounds [Gonzalez-Montalban *et al.*, 2011].

The addition of EDTA in a concentration up to 20 mM instead of 5 mM to reaction mixtures yielded higher amplification rates of all four prion strains in this study. The standard PMCA procedure does not use 20 mM EDTA, it was however recommended to include 5 mM EDTA in the conversion buffer, which is used to prepare the NBH [Barria *et al.*, 2012; Castilla *et al.*, 2006]. By using elevated EDTA concentrations and optimizing the incubation times both parameters additively yielded higher amplification efficiencies (Figure 3-3). A putative mechanism for the action of EDTA is described in chapter 4.2.4.3.

A serial PMCA procedure for a successful and efficient amplification of PrPres from BSE-H and 22A-H prions was not reported previously. However, Ayers *et al*. showed an amplification of 22A-H associated PrPres after one round of PMCA and therefore demonstrated that this prion agent in principle can propagate in a one round PMCA assay [Ayers *et al.*, 2011]. Our group recently demonstrated that the PMCA assay allows a quantitative determination of 263K seeding activity [Boerner *et al.*, 2013; Pritzkow *et al.*, 2011]. Moreover the results in this thesis and in [Boerner *et al.*, 2013] demonstrate that propagation of ME7-H, 22A-H and BSE-H is dose dependent (though the sensitivity for 263K prions is much higher), and thus a quantitative assay similar to 263K titrations in PMCA may also be established.

4.1.2 Cell assay

Autocatalytic propagation of prions by PMCA is a very fast method for the biochemical amplification of prions *in vitro* under cell-free non-physiological conditions. In contrast, glial cell cultures represent a biological *in vitro* amplification assay for prions under physiological conditions. They provide information about the biological seeding activity of prions in terms of biological infectivity. The susceptibility of primary hamster glial cell cultures to 263K prions was described previously [Pritzkow *et al.*, 2011]. In this thesis, ME7-H, 22A-H and BSE-H prions were applied to cell cultures as well. Cells were susceptible to infection with 22A-H and BSE-H prions [Boerner *et al.*, 2013] but not to ME7-H prions. Infection of primary cells or cell lines with hamster adapted prion agents ME7-H, 22A-H or BSE-H has not been reported previously. Similar efficiencies of cell infection by 263K and 22A-H prions were observed. For the BSE-H agent a lower efficiency of infection was determined. The cell assay findings show a trend that is consistent with bioassay data from studies in hamsters: In bioassay studies 263K scrapie, 22A-H scrapie and BSE-H prions caused the full-blown clinical picture of prion diseases in Syrian hamsters at 83±5, 206±8 and 287±28 days, respectively [Thomzig *et al.*, 2004], whereas the ME7-H scrapie agent had the longest incubation time of 331±16 days. The inability of ME7-H to propagate in glial cell cultures may be explained by these bioassay findings. In contrast, ME7-H amplification by PMCA with whole brain homogenate was efficient. One explanation of this apparent discrepancy may be that biological replication of ME7-H depends on a specific cell tropism not met in the glial cell preparations, while a molecular fit between PrP^{Sc} and PrP^{C} in the PMCA reaction was possible. Interestingly, Ayers *et al*. recently investigated the deposition of PrP^{Sc} from different hamster prion agents in the brain tissues by antibody staining and observed a low immunoreactivity of PrP^{Sc} within neurons, astrocytes and microglia in ME7-H infected animals compared to the other hamster prion agents [Ayers *et al.*, 2011] while the deposition in the neuropil was rather the same as for the other prion strains. On the other hand, ME7-H replication may rely on other conversion factors that are available in PMCA but not in the cell assay, e.g. due to cell compartmentalization. Similar observations were reported for the mouse RML prion strain [Herva and Weissman, 2012]. While RML prions could replicate in the PK1 cell line, $R33_{2H11}$ cells were resistant to infection. The efficiency of RML propagation in PMCA with cell lysates as substrate was however equal for both cell lines. This showed that susceptibility of cells to infection with a prion strain was not reflected in PMCA and implicated that other factors additionally to PrP^{C} are involved in the PrP seeding activity. Cell dependent factors, which are critical for susceptibility to prion infection include specific clearance of PrP^{Sc} [Ayers *et al.*, 2011]. Alternatively, co-internalization of PrP with other proteins has been suggested, and several potential receptors involved in the binding and uptake of PrP^{Sc} by cells have been proposed, including

glycosaminoglycans (GAGs) [Grassmann *et al.*, 2013]. Thus, if prion strains arise from association with different cofactor molecules such as GAGs, it would be conceivable that cofactors influence receptor mediated PrP internalization.

We recently demonstrated that PMCA and glial cell assays allow a quantitative determination of 263K seeding activity and infectivity [Boerner *et al.*, 2013; Pritzkow *et al.*, 2011]. Since the efficiency of BSE-H cell infection depends on the administered inoculation dose (Figure 3-23) a prospective quantitative assay for BSE-H titrations in cell cultures may be established as well. The findings show, that glial cell cultures can not only be used for the titration of 263K infectivity, but in principle also for the titration of infectivity associated with BSE-H prions (and 22A-H). A further increase of the cell assay sensitivity might be achieved by successive infection passages [Arellano-Anaya *et al.*, 2011]. As shown in this thesis an increase in sensitivity of BSE-H in the glial cell assay, could already be achieved by an optimization of the PK digestion step (Figure 3-23).

4.2 Role of cofactor molecules for prion propagation in PMCA

In this study the suitability of synthetic poly(A) RNA to enhance the *in vitro* conversion and amplification process of prions from four hamster strains was assessed by serial PMCA. The *in vitro* amplification of 263K and ME7-H prions by standard PMCA could be enhanced by addition of synthetic RNA to whole crude brain homogenates, while the effect on BSE-H propagation was negligible. In contrast, the addition of synthetic RNA to PMCA reaction mixtures was found to reduce the seeding efficiency of 22A-H. Thus, RNA did modulate the seeding efficiencies of four hamster prion strains in a different way.

However, PrP^{Sc} from all four prion strains was unable to propagate efficiently in nucleic acid depleted hamster brain homogenate, which demonstrates that all examined prion agents rely on endogenous nucleic acids as a cofactor for propagation. A subsequent addition of exogenous synthetic RNA, after previous depletion of nucleic acids in the reaction substrate, did reconstitute a stable amplification of 263K, ME7-H and BSE-H prions, pointing to RNA as a putatively essential cofactor molecule.

In this setup the amplification of 22A-H prions was prolonged but eventually became unsustainable. Synthetic RNA showed a substitution effect in the first PMCA rounds but was not sufficient for a longer stable propagation. This indicates that 22A-H either requires other certain kinds of nucleic acids than the precise synthetic poly(A) RNA, or that this prion strain requires another conversion cofactor than nucleic acids for stable propagation in PMCA that was destroyed alongside with nucleic acids during benzonase treatment.

Another polyanionic compound, chondroitin sulfate A, facilitated a stable amplification of 22A-H. In contrast, for ME7-H prions a successful reconstitution was observed with RNA but not with chondroitin sulfate A. These results indeed support the notion that prion strains can use and rely on cofactor molecules quite selectively for their replication at least in PMCA reactions.

In addition, it was found that BSE-H could propagate in a stable manner and completely independent of nucleic acids in nucleic acid depleted hamster brain homogenate when large amounts of seeding tissue and a high number of PMCA rounds were used. Hence the BSE-H agent was not entirely dependent on nucleic acids or other polyanions, in PMCA, but was able to adapt to nucleic acid free replication conditions.

Other candidate cofactors such as poly-L-glutamate and the sphingolipid sphingomyelin were not found to be able to successfully reconstitute the amplification reactions with 263K scrapie.

263K seeded samples that were reconstituted with synthetic RNA or chondroitin sulfate A were also biologically seeding active as was demonstrated in cell culture infection assay. Furthermore, also BSE-H associated PrPres that replicated independently of nucleic acids in PMCA could be subsequently propagated by glial cells. This provides a strong indication that these *in vitro* generated PrPres species may be also infectious *in vivo* in Syrian hamsters.

4.2.1 Cofactors are required for *in vitro* prion propagation

The findings from this work, that a depletion of nucleic acids from the PMCA substrate inhibits the cell-free prion propagation from all hamster prion strains, are in accordance with results published elsewhere. It was reported that treatment of crude brain homogenates with RNase or micrococcal nuclease, which digests DNA and RNA, abolished Sc237 hamster scrapie amplification in serial PMCA experiments using purified PrP^C [Deleault *et al.*, 2007; Deleault *et al.*, 2010; Deleault *et al.*, 2003]. In a recent study from Baskakov and colleagues, amplification of six hamster prion strains (including 263K and ME7-H) was shown to be suppressed in PMCA after RNase treatment of brain homogenate [Gonzalez-Montalban *et al.*, 2011]. This dependence on nucleic acids seems to be a general requirement for all hamster prion strains. In contrast, the absence of nucleic acids was not found to abolish amplification of mouse prions in serial PMCA experiments (own results, not shown and [Deleault *et al.*, 2010]), leading to the assumption that the requirement for nucleic acids may be species dependent. However more recent experimental data from Saa *et al*. show that RNA depletion by RNase A impairs *in vitro* amplification of mouse prions in a prion strain dependent

fashion [Saa *et al.*, 2012]. RNA was shown to enhance *in vitro* amplification of nine murine prion strains. Therefore, the effect of RNA as a cofactor for PMCA is not restricted to hamster prions, but seems to be a prion specific phenomenon.

4.2.2 Nucleic acids are not essential for BSE-H prion propagation in PMCA and infectivity in the cell assay

In this study it was found that BSE-H could propagate, under specific experimental conditions, independently of nucleic acids in the PMCA substrate. Such phenomenon has not yet been reported in the scientific literature for a hamster prion strain. Together with the initial observation that addition of synthetic RNA could not enhance BSE-H amplification in normal PMCA with whole brain homogenate (unlike 263K or ME7-H), this indicates that the BSE-H agent is not entirely dependent on RNA (or other nucleic acids) for replication. However, a long lag phase without apparent amplification was observed for BSE-H propagation in nucleic acid depleted PMCA substrate. Only at later PMCA rounds amplification became very efficient.

For propagation of prions in PMCA it is commonly assumed that prion seed fragmentation by sonication results in the formation of novel seeds. In this manner an exponential growth can be assumed under optimal PMCA conditions [Saborio *et al.*, 2001]. A lag phase was not observed for BSE-H propagation under normal PMCA with nucleic acids. There are several different explanations for the occurrence of the lag phase in BSE-H amplification in nucleic acid depleted PMCA substrate.

Firstly, the findings suggest that some kind of BSE-H prion adaptation occurred. Such adaptations could encompass changes of the protein conformation an in cofactor selection. It is thought that different prion strains in a mammalian species are enciphered essentially in different PrP conformations. Accordingly, the original "BSE-H protein conformation" may have changed during the first PMCA rounds under nucleic acid-free conditions and may have adapted to a different conformation that is able to propagate in such environment. Indeed the observed differences in electrophoretic mobility after limited proteolysis (Figure 3-20B) indicate another conformation of the BSE-H PMCA product that had been generated in the absence of nucleic acids. This is reflected in the altered accessibility of the PK cleavage site in the N-terminal region of the respective PrPres. A pilot analysis of the PrPres structures by FT-IR spectroscopy did not clearly reveal structural differences between PMCA amplificates of BSE-H that had been either produced in the presence or absence of nucleic acids. However, slight spectral differences were seen with respect to an absorption peak at 1655 cm^{-1} that was present in the BSE-H PMCA product produced in the absence of nucleic acid but absent in the PMCA product generated in the presence of nucleic acids.

Secondly, non-nucleic acid cofactors such as GAGs or lipids present in the PMCA substrate may compensate for nucleic acid deficiency in a way that may or may not influence the protein conformation. In this sense nucleic acids may have been substituted by other molecules. This explanation is also conceivable, since prion strain diversity may also be based on different cofactors that act during prion replication or are attached to the prions. In PMCA prion strains do not only rely on a single cofactor molecule but exhibit some strain dependent preferences for different molecules. As was shown in this study, amplification of BSE-H could be reconstituted with RNA and chondroitin sulfate. ME7-H was reconstituted with RNA but not with chondroitin sulfate whereas RNA was not effective to mediate a lasting amplification of 22A-H that, however, could be reconstituted with chondroitin sulfate.

Thirdly, BSE-H amplification under nucleic acid deficiency was only observed when large amounts of seeding tissue were used. It has been suggested that prion strains consist not only of a single protein conformation but comprise a pool of different conformations, so called quasi species [Collinge, 2010; Li *et al.*, 2010]. Based on this concept it is assumed that a quasi species has a dominant component with recognizable biochemical and biological properties and that subspecies are present in a lower abundance. Under conditions of natural selection as they may be present in hosts that are unable to propagate the dominant species, a subspecies may be preferentially replicated accompanied by changes in biochemical and biological properties of the prion strain. In PMCA artificial selection may be created by nucleic acid deficiency, hence only formerly invisible subspecies of the pool may be able to propagate efficiently. Thus, the lag phase of BSE-H was possibly required to select the proper subspecies which achieves high amplification efficiency in later PMCA rounds. The subspecies eventually amplified under the selection pressure of nucleic acid deficiency then differed in its conformation and/or certain cofactor utilization as well as further phenotypic properties.

In this study a cell assay which was performed with BSE-H progeny seeds produced under nucleic acid deficiency demonstrated that this BSE-H amplificate was infectious to glial cells. This seeding material was propagated over 80 days, and PrPres accumulated in the cells. Infectivity in the cell assay suggests that the tested BSE-H derived amplificate is also infectious in animals. Furthermore, the cell assay data showed that nucleic acids were not necessarily required for infectivity and replication of the BSE-H agent.

Another recent study used a different approach and showed that nucleic acids are not essential for maintaining infectivity of Sc237 seeded PMCA prions generated from purified PrP^C [Piro *et al.*, 2011]. In the study by Piro *et al*. synthetic photocleavable nucleic acids were required for amplification of prions by PMCA and were incorporated

into PMCA generated prions. Prior to inoculation of PMCA generated prions into reporter animals the incorporated photocleavable nucleic acids were degraded into 5 base fragments by UV light. Nucleic acids with a length of 5 bases are ineffective as cofactors for PrP conversion in PMCA. However, in the animal bioassay all recipient hamsters developed prion disease. In ongoing experiments of the same group it was recently demonstrated that PrPres could be formed in a seeded PMCA reaction with recombinant PrP as substrate in the absence of any cofactor molecules [Deleault *et al.*, 2012b]. In the mentioned study a recombinant prion strain was propagated by PMCA in the presence of α-helical recombinant prion protein and phospholipids for a number of rounds. This strain was subsequently subjected to PMCA in the presence of rPrP alone. An adaptation to the cofactor-depleted conditions was described and propagation was maintained. This adapted prion strain did appear to have a lower molecular weight than the parental prion strain after PK treatment, also indicating altered protein conformations. However, a bioassay in mice showed that this adapted recombinant prion strain was not infectious, irrespective of its seeding activity detected in PMCA. Only PMCA products that were produced in the presence of phospholipids were infectious in animals.

In this study, the cell assay with BSE-H progeny seeds that had been generated in the absence of nucleic acids demonstrated that the adapted BSE-H was infectious to glial cells. However, PMCA was performed in brain homogenate, i.e. not with purified substrates. Considering the findings from the study by Deleault *et al*. this indicates that other cofactor molecules such as possibly phospholipids were involved in BSE-H amplification and infectivity under experimental conditions of nucleic acid depletion.

As mentioned above the replication of some mouse prion strains in PMCA without nucleic acids is not impaired. Saa *et al*. showed that amplification of RML prions in serial PMCA remained unaffected for up to 9 rounds in RNase treated substrates. Subsequent inoculation of wildtype mice with the resulting PMCA products showed that RML prion strain identity was maintained after *in vitro* amplification under conditions of RNA depletion. However, an unusual clinical symptom was observed with a statistically higher incidence in the group of mice inoculated with RML prions amplified under RNA-depleted conditions. The authors concluded that this observation cannot be considered as a strain change but was attributed to the possibility that a shift, below the threshold of a true strain change, had occurred in the PrP quasi species under the particular replication conditions, that resulted in a transient change of the clinical phenotype [Saa *et al.*, 2012].

When assessing the electrophoretic mobility of the BSE-H PMCA product, that had been generated in the absence of nucleic acids after its propagation by glial cell cultures, the precise molecular size of the unglycosylated band could not be clearly determined

(Figure 3-20B). Though this band of the "nucleic acid independent" BSE-H PMCA product used for cell culture infection did not exhibit a lower molecular mass compared to 263K, the cell-derived protein band appeared together with an additional "ghost" band of lower molecular mass. The molecular mass of this "ghost" band appeared identical to that of the unglycosylated band of authentic BSE-H. This suggests that this original biochemical feature may re-emerge from the PMCA products upon biological propagation in a cell culture. Accordingly, the altered electrophoretic mobility observed after 12 PMCA rounds may be only transiently present as long as *in vitro* propagation is performed under nucleic acid depletion. However, further clarification of this question would require additional studies.

In such future studies, the cell derived BSE-H isolate should be subjected to further passages in glial cell cultures. Subsequently, the electrophoretic mobilities of the cell-derived isolates could be assessed and compared as described above. After 12 PMCA rounds the original BSE-H tissue is practically diluted out due to serial PMCA, and only newly formed PrPres should be present in reaction samples. Accordingly, cells were inoculated only with newly generated PrPres. This excludes the possibility that remnants of original BSE-H could have caused a (partial) reversion of the electrophoretic mobility to the original pattern. Therefore one might speculate that this original feature is still preserved, and may thus potentially re-emerge from the PMCA derived PrPres. The conformational change in PMCA derived PrPres that was underlying the altered electrophoretic mobility obviously resulted from the selection pressure of nucleic acid deficiency during PMCA. In the cell assay such selection pressure is absent due to the presence of nucleic acids in this biological system. Thus, if the original conformational information coding for a smaller digestion product after limited proteolysis is still present, then it can be propagated by the cells. If this feature is generally favored under biological conditions of nucleic acid presence, then it will be re-established in a few cell culture passages. PMCA under nucleic acid deficiency may thus be regarded as a transient artificial disturbance that forces the BSE-H agent into a different conformation or a transient quasi species. If the original electrophoretic mobility of BSE-H were eventually re-established in the "nucleic acid independent" BSE-H isolate upon cell culture passages this would suggest that nucleic acids are not essentially required to maintain strain specific information in the BSE-H seeds.

In other PMCA studies of this thesis with 22A-H prions it was demonstrates that the addition of synthetic RNA to the standard PMCA in whole brain homogenate suppressed PrPres amplification. Furthermore, a sustained amplification of 22A-H in nucleic acid depleted homogenate was not mediated by synthetic RNA. These observations indicate that 22A-H may also be able to propagate under nucleic acid deficiency. However, this has not been examined and remains to be further explored in future studies.

As can be seen from the Western blots in Figure 3-7 263K showed an initial apparently authentic amplification under nucleic acid deficiency. Nevertheless, this amplification could not be sustained. This shows once more, that prion strains have different requirements in terms of conversion cofactors. The initial seeding ability of 263K is discussed below (chapter 4.2.3).

4.2.3 Different molecules can substitute for cofactor function in PMCA

It was demonstrated above that under standard PMCA conditions all examined hamster prion strains rely on nucleic acids for *in vitro* propagation. PMCA reconstitution, after nucleic acid depletion, with different polyanionic compounds was successful in this work which is in agreement with previous studies by others [Deleault *et al.*, 2005; Deleault *et al.*, 2007; Deleault *et al.*, 2010]. Propagation of 263K, ME7-H and BSE-H was successfully reconstituted with synthetic RNA, and propagation of 263K, 22A-H and BSE-H could be reconstituted with chondroitin sulfate A (Table 4-1). Glycosaminoglycans such as chondroitin sulfate are linear polyanions with a structure that resembles single-stranded nucleic acids. This demonstrates that, in principle, different polyanions can act as stimulators of PrPres amplification in PMCA. The observed differences for ME7-H and 22A-H prions may reflect strain specific preferences for certain conversion cofactors, and indicate higher cofactor selectivity for both strains as compared to 263K and BSE-H which could be efficiently amplified by addition of either synthetic RNA or chondroitin sulfate. In accordance with these findings different cofactor selectivities were recently observed for different mouse prion strains in RNA depleted PMCA [Saa *et al.*, 2012]. While amplification of mouse RML was restored by the addition of poly(A), poly(G) or DNA, amplification of mouse ME7 was only reconstituted by poly(A) but not by poly(G) or DNA.

In any case such data indicate that several different molecules can substitute for natural conversion cofactors and mediate amplification in PMCA. This indicates that prions may be potentially able to use a variety of different cofactor molecules for their propagation *in vivo*. Furthermore, it can be speculated that strains with a broader cofactor specificity might replicate more effectively and faster than strains with higher selectivity. As demonstrated in this study, 263K amplification was enhanced by synthetic RNA, and its propagation was restored by RNA as well as chondroitin sulfate. This was unique for 263K among the prion strains examined in this thesis. All other strains showed a higher degree of cofactor selectivity and have substantially longer *in vivo* incubation times than 263K. Table 4-1 summarizes the effects of cofactor molecules on PMCA with the hamster strains examined in this study. BSE-H amplification after nucleic acid depletion was restored by RNA and chondroitin sulfate, but its propagation was not enhanced by addition of RNA to PMCA substrate that had

not been depleted of nucleic acids. ME7-H amplification was enhanced as well as restored by RNA, but reconstitution of the PMCA reaction with chondroitin sulfate was not possible after nucleic acid depletion. In contrast, amplification of 22A-H was even inhibited by RNA. After nucleic acid depletion, PMCA could be reconstituted by chondroitin sulfate but not by RNA.

Table 4-1: Summary of cofactor effects on the propagation of PrPres from different hamster prion strains by PMCA.

	In vivo incubation times in days*	*Effect of synthetic RNA on propagation in PMCA with NBH*	*Reconstitution with synthetic RNA after nucleic acid depletion*	*Reconstitution with chondroitin sulfate A after nucleic acid depletion*
263K	83 ± 5	+	+	+
22A-H	206 ± 8	–	–	+
BSE-H	287 ± 28	±	+	+
ME7-H	331 ± 16	+	+	–

* *In vivo* incubation times according to [Thomzig *et al.*, 2004]

\+ Promotion

– Inhibition or no reconstitution

± None effect

Abid *et al*. showed that various types of molecules can act as conversion factors *in vitro*. The authors utilized a one-round PMCA assay with purified PrP^{C} and PrP^{Sc}, in which PrP conversion was basically only facilitated by addition of whole brain homogenates from mammalian species, e.g. rabbit or PrP knockout mice but not by the sole presence of normal conversion buffer. They reported that treatment of brain homogenates with various enzymes that specifically destroy certain classes of molecules, including nucleic acids (benzonase), proteins, lipids (lipase A) and proteoglycans (heparinase II and III and chondroitinase ABC) did not impair the reconstitution of PrP conversion [Abid *et al.*, 2010]. These results suggested that each putative cofactor may partially replace the conversion factor activity *in vitro*.

However, the results by Abid *et al*. seem to be contradictory to the findings reported in this thesis for benzonase treated samples, and to findings from other groups who performed RNase treatments of PMCA substrates [Deleault *et al.*, 2010; Gonzalez-Montalban *et al.*, 2011]. One explanation for the apparent discrepancies might be that a single PMCA round only was performed in the study by Abid *et al*., which may have lead to artifactual or misleading results. One consistent observation in this thesis is that 263K exhibited a limited intrinsic seeding activity when propagated in benzonase

treated brain homogenate. This resulted in an initial increase of PrPres in the first PMCA round but not in subsequent PMCA rounds (Figure 3-5, Figure 3-7). This effect was never observed for the other hamster prion strains and thus seemed to be 263K strain specific. Theoretically, the residual seeding activity of 263K prions in nucleic acid depleted PMCA substrates may have originated from remaining nucleic acids in the infected seeding tissue. But a longer treatment of the seeding tissue with benzonase for 20 hours did not reduce the effect (Figure 3-6), which excludes the possibility that freely accessible nucleic acids mediated the initial 263K seeding. A similar effect is also visible in experimental data from Deleault and colleagues [Deleault *et al.*, 2007; Deleault *et al.*, 2010]. However, this observation has not been discussed by these authors. Deleault *et al*. used purified Sc237 and PrP^{C} substrates and conversion was only facilitated by addition of RNA or whole PrP knockout brain homogenate, similar to the PMCA system of Abid and colleagues. Recently, Piro *et al*. [Piro *et al.*, 2011] showed that a 100-base oligonucleotide was incorporated into complexes of PrP^{Sc} molecules during serial PMCA reactions and became resistant to benzonase digestion by this incorporation. Several lines of evidence indicate that polyanionic molecules such as RNA may be incorporated into stable complexes of PrP molecules during the formation of native hamster prions [Geoghegan *et al.*, 2007; Simoneau *et al.*, 2009]. In such nucleoprotein complexes nucleic acids are possibly not accessible for benzonase digestion due to protection by PrP and can thus co-purify with brain PrP^{Sc} used as seeding material for PMCA experiments.

RNAs were shown to promote the conversion of PrP^{C} into PrP^{Sc} *in vitro* [Deleault *et al.*, 2005; Deleault *et al.*, 2007; Deleault *et al.*, 2010; Wang *et al.*, 2010]. Furthermore, in this thesis it was demonstrated that the amplification efficiency of 263K and ME7-H in PMCA can be enhanced by addition of synthetic RNA. In a study from Baskakov and colleagues, six hamster prion strains (including 263K and ME7-H) were shown to be dependent on RNA in PMCA [Gonzalez-Montalban *et al.*, 2011]. However, studies that have determined reconstitution capacities of other different polyanionic compounds for the set of different hamster prion agents examined in this thesis have not yet been reported.

Moreover, a reduction of the amplification efficiency due to the addition of synthetic RNA has not been reported before to the best of the author's knowledge. In this thesis it could be clearly demonstrated that addition of RNA suppressed 22A-H propagation by PMCA in whole brain homogenate. In addition, synthetic RNA was not sufficient to mediate a sustained amplification in nucleic acid depleted brain homogenate. This strikingly shows that poly(A) RNA is not a generic accelerator of hamster prion propagation, but rather facilitates or impedes amplification in a strain dependent manner. In this regard, an application of polyanions or putative cofactor molecules in prion detection assays with the aim of increasing the speed and/or sensitivity of prion

detection as suggested previously [Deleault *et al.*, 2005; Deleault *et al.*, 2003; Yokoyama *et al.*, 2011] should be considered very carefully. Since chondroitin sulfate mediated a stable restoration of PrP amplification in nucleic acid depleted brain homogenate, it may exhibit an accelerating effect on 22A-H propagation under standard PMCA conditions. However, this remains to be determined in future studies. Furthermore it was demonstrated in this thesis that elevated EDTA concentrations yielded higher conversion efficiencies of all tested hamster prion strains and thus facilitated a quicker detection of amplified PrPres than previously used standard PMCA procedures. If this effect applied to other prion strains as well, this may generally improve the sensitivity of PMCA detection assays.

It was recently reported that phospholipids are required for replication of infectious mouse related PrP^{Sc} [Deleault *et al.*, 2012a; Deleault *et al.*, 2012b; Wang *et al.*, 2010]. Sphingolipids such as galactosylceramide and sphingomyelin were previously detected in scrapie hamster prions [Klein *et al.*, 1998] and were therefore experimentally added to hamster brain PMCA substrate in this study. A sphingomyelin-mediated reconstitution of 263K propagation in PMCA after nucleic acid depletion was not possible, nor were sphingomyelin and galactosylceramide able to mediate an accelerated prion amplification in standard PMCA carried out without nucleic acid depletion.

Taken together the results of this thesis show that prion strains have different requirements with respect to the amplification conditions in PMCA. Under nucleic acid deficiency the examined prion strains exhibited different abilities to use certain polyanions for the reconstitution of PrP conversion. Thus, these prion agents clearly showed differences in PMCA cofactor selectivity. It has to be considered, however, that PMCA cofactor molecules could merely function as artificial helpers for PrPres amplification under unphysiological cell-free conditions. But if the cofactor findings from the PMCA studies are indicative of the mechanisms underlying prion replication *in vivo*, prion strains may also require different cofactors, or respond differently to them, under “real-life” conditions. 263K scrapie prions seem to have a broader ability to use a variety of cofactors for their replication than the other hamster prion strains. This may also provide one explanation for their extremely fast replication *in vivo*.

4.2.4 Impact of metal ions on *in vitro* prion propagation

The presented experiments also assessed the role of the metal ions Zn^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+} and Mn^{2+} in 263K seeded PrPres amplification in two independent and profoundly different cell-free and cell-based assays, serial PMCA and glial cell cultures. The addition of 100 or 500 µM metal ion solution to PMCA reaction mixtures affected the propagation of 263K differently. While Zn^{2+}, Fe^{2+}, Fe^{3+} and Cu^{2+} were found to suppress PrPres formation in a concentration dependent way, Mn^{2+} did not exhibit an effect on the propagation of 263K (Figure 3-11). Fe^{2+} had the strongest inhibitory effect on PMCA-mediated 263K amplification followed by Fe^{3+}, Zn^{2+} and Cu^{2+}. An acceleration of PrP misfolding was not observed with any of the ions. Moreover, PrPres formation was never detected in non-seeded control samples that contained additional metal ions. In accordance with the findings from PMCA assays metal ion solutions were found to suppress propagation of 263K in glial cell cultures. This effect appeared to be dose dependent for Zn^{2+} and Fe^{3+} (Figure 3-16). In contrast to PMCA, Cu^{2+} had the strongest inhibitory effect on 263K propagation in glial cell cultures followed by Fe^{2+}, Fe^{3+} and Zn^{2+}. Due to cellular toxicity the effect of Mn^{2+} on glial 263K propagation could not be examined.

4.2.4.1 Discussion of PMCA findings

The addition of metal ions to PMCA reaction mixtures created an excess of specific metal ions in the NBH substrate. Under these conditions an overall tendency of metal ions to suppress 263K propagation was observed. This is in good accordance with the previous findings that higher EDTA concentrations up to 20 mM promoted PrP conversion in PMCA of four different hamster adapted prion agents. This clearly demonstrated that free metal ions can suppress prion propagation *in vitro*. Consistent with this, a formation of PrPres was not induced by any metal ion in PMCA samples that contained only PrP^C substrate without a prion seed. This, however, is in contrast with published data by Kim *et al*. [Kim *et al.*, 2005] who showed that conversion of PrP^C from normal hamster brain homogenate into a protease resistant form could be induced by Mn^{2+}, Cu^{2+} and Fe^{2+} during PMCA in the absence of a prion seed. It is important to note that these authors added very high concentrations of metal ions to the brain homogenate, up to 350 mM $MnCl_2$ and up to 87.5 mM $CuCl_2$ and $FeCl_2$. Even when 10 mM $CuCl_2$ was present during seeded PMCA in the experimental setup of this thesis, an inhibition of 263K propagation was observed (data not shown, PK digestion was performed in the presence of 20 mM EDTA). Furthermore, Kim *et al*. used a different experimental setup in regard to the buffer of brain homogenate, incubation times, number of PMCA cycles and conditions for PK digestion compared to the

experiments described in this thesis. Under treatment with PK authentic PrP^{Sc} is usually processed to an N-terminally truncated protease resistant core fragment of 27-30 kDa, while non-PK treated full-length PrP possesses a molecular weight of 33-35 kDa. However, in the report by Kim *et al*. the molecular weight of PrPres generated in the presence of metal ions seemed unaltered before and after PK treatment, hence a PK-mediated truncation of PrP at the N-terminus, which is typically observed with conventional PrP^{Sc}, was not apparent. Additionally, Bocharova *et al*. reported that Cu^{2+} ions increased the resistance against proteolysis of full-length recombinant non-amyloidogenic PrP without changing its α-helical conformation, as was assessed by CD spectroscopy [Bocharova *et al.*, 2005]. This PK resistant PrP was also not digested to a PrP27-30 like fragment. As demonstrated in Figure 3-10B the presence of 10 mM $CuCl_2$ during limited proteolysis by PK resulted in an inhibited digestion of PrP. The samples in this figure had not been subjected to PMCA. Addition of EDTA restored complete PK digestion. Since it was reported that Cu^{2+} ions can inhibit the activity of PK [Stone *et al.*, 2007] this is another plausible explanation for the observed differences between this and the study by Kim *et al*.. Unfortunately, it has not been examined previously if other metal ions can also inhibit PK activity. Therefore, in this thesis a PK digestion of PrP^{Sc} in the presence of metal ions in equivalent concentration as used in PMCA experiments was performed (Figure 3-10A). This revealed no altered PrP processing in these samples. To answer, however, the question whether higher concentrations of metal ions specifically mediate an authentic resistance of PrP^{C} against PK, or whether merely the enzyme activity is impaired by the ions, a simple experiment can be performed:

The samples in which PrP^{C} was detected, though they had been digested with PK (in the presence of millimolar amounts of metal ions and without EDTA), could be analyzed, in future studies, for the presence of other brain proteins that are usually degraded by PK (e.g. glial fibrillary acidic protein, actin or glyceraldehyde 3-phosphate dehydrogenase) with specific antibodies. If PK activity were inhibited by the metal ions, other proteins from NBH would not be degraded and could be detected. Alternatively, the total protein content in these samples could be determined and compared to the total protein content in samples that had not been PK digested. However, in the experiments of this thesis only micromolar amounts of metal ion solution were applied, and PK digestion was not affected by these concentrations.

The finding that specifically Zn^{2+} and Cu^{2+}, but not Mn^{2+} suppressed PrPres formation is in agreement with previous reports by Bocharova *et al*. and Orem *et al*. [Bocharova *et al.*, 2005; Orem *et al.*, 2006]. Using an amplification system with purified PrPres different from PMCA, Orem *et al*. showed that the seeded amplification of PrPres with a purified PrP^{C} substrate was potently inhibited by $CuCl_2$ in a dose dependent way. $ZnCl_2$ had also inhibitory effects, but with a 20-fold lower potency. An inhibition by

$MnCl_2$ was not observed. Moreover the authors determined that even an excess of Mn^{2+} could not prevent the Cu^{2+} mediated inhibition of PrPres amplification, whereas metal chelators did. The authors suggested that the inhibitory effect of Cu^{2+} was likely mediated by a metal binding site on PrP^C. Furthermore, Bocharova *et al*. found that Cu^{2+} and Zn^{2+} (to a lesser degree) but not Mn^{2+}, inhibited a conversion of bacterially expressed full-length PrP into amyloid fibrils [Bocharova *et al.*, 2005]. They suggested that Cu^{2+} inhibited the structural conversion of PrP by stabilizing a non-amyloidogenic PK-resistant form of α-helical recombinant PrP (as mentioned above). In this context, it would be very interesting to know whether different metal ions can specifically mediate resistance of PrP^C against PK as mentioned above and thereby suppress PrP conversion.

In a PrPres amplification system with purified substrates Cu^{2+} was the most potent inhibitor of PrP conversion, followed by Zn^{2+}. In this system Mn^{2+} did not show any effect. These results correlated with the binding affinities of the ions to the octapeptide repeat region of PrP. Binding of metal ions to the octarepeats of PrP was found to occur with different affinities. Cu^{2+} has a high affinity while Zn^{2+} binds with an affinity that is two to three orders of magnitude lower [Pan *et al.*, 1992]. Mn^{2+} binds with a yet lower affinity [Bocharova *et al.*, 2005; Brazier *et al.*, 2008].

However, the PMCA experiments performed in this thesis with whole brain homogenate led to different results, since Fe^{2+} was the strongest inhibitor of 263K amplification followed by Fe^{3+}, Zn^{2+} and Cu^{2+}. This may be due to binding of Cu^{2+} and Zn^{2+} to other proteins in the homogenate, resulting in a different availability of these ions for PrP^C. Binding sites and affinities of PrP for iron have not been established yet. It was suggested that the octarepeat region is not essential in this context, and that iron binding to PrP may depend on its conformation or may be very transient [Singh *et al.*, 2010]. Furthermore it was suggested that iron is associated with PrP^{Sc} in form of insoluble PrP^{Sc}-ferritin complexes [Singh *et al.*, 2009]. However, the addition of iron ions to PMCA experiments in this thesis resulted in a strong inhibition of PrPres amplification.

Further investigations might use chelators that are specific for a certain metal ion to reveal the contribution of individual ions to the *in vitro* conversion of PrP in PMCA experiments using normal brain homogenate as PrP^C substrate.

4.2.4.2 Discussion of cell culture findings

In this study metal ions were applied for the first time to primary hamster glial cell cultures to investigate metal effects on prion propagation for a long-term duration of approx. 80 days. Because free metal ions may be toxic to cells depending on the specific kind of ion and its concentration they were applied to cell cultures with caution. A regular, at least weekly, inspection of cell cultures by microscopy was of high importance to assess cell vitality, since there are no data available that describe the long-term effects of metal ions on the viability of glial cells. The applied µmolar concentrations of metal ions on cell cultures were adjusted to amounts published in the literature. Similar concentrations of metal ions (20 µM $ZnCl_2$ or $CuCl_2$) had been used in studies that involved primary neuronal cells [Cuajungco *et al.*, 2000; Lovell *et al.*, 1999]. In these studies cell viability was determined after shorter incubation time intervals of about 24-72 h. Cell viability is reflected in a variety of parameters, but is commonly measured either directly at the cellular level by staining of living or dead cells (e.g. MTT [Mosmann, 1983] or trypan blue, respectively), or by analysis of the culture supernatant. Damaged cells release components from the cytoplasm into the culture medium, e.g. nucleic acids or lactate dehydrogenase, which can be determined. However these compounds underlie a naturally occurring degradation. For this reason, assessment of cell viability by analysis of culture medium compounds is rather suitable for assays with shorter incubation times. In contrast, the glial cell assay was performed for durations of up to 80 days, which makes an analysis of compounds in the culture medium not straightforward. Therefore, in addition to regular microscopic inspection for detecting the occurrence of cell damage, it is suggested to perform the cell culture experiments with multiple replicates of the same sample (at least 4 replicates). At the appropriate DPE of PrPres detection, two replicates of the sample are used for Western blot detection and the other replicates are used for cell viability measurements preferentially with an appropriate staining method. Alternatively, metabolic activities of cells could be determined, e.g. by measurement of ATP levels or reduction of tetrazolium salts [Roche-Diagnostics, 2008].

In accordance with the findings from PMCA assays metal ions were found to suppress amplification of 263K in glial cell cultures. Cu^{2+} had the strongest inhibitory effect on 263K amplification in the cell assay. Copper in form of $CuSO_4$ had been previously applied to a permanently scrapie infected neuroblastoma cell line (ScN2a) [Hijazi *et al.*, 2003], which resulted in a reduced accumulation of PrP^{Sc}. Cultivation of normal N2a cells in the presence of copper resulted in increased accumulation of total PrP. Furthermore, Hijazi *et al.* showed that cultivation of N2a cells in the presence of 500 µM $CuSO_4$ 24 h prior to cell infection with scrapie brain homogenate inhibited the binding and internalization of PrP^{Sc}. Knowing that copper can enhance PrP^{C} internalization into cells [Pauly and Harris, 1998; Perera and Hooper, 2001], it was

hypothesized that PrP^C internalization by copper may hinder interaction with PrP^{Sc} and thereby prion propagation. Thus the observation presented in this thesis that PrPres formation was impaired in glial cell cultures that had been incubated in the presence of 50 µM $CuCl_2$ 20 days prior to cell infection (control sample, Figure 3-16) can be possibly explained by a Cu^{2+} induced PrP^C deficiency on the cell surface. As a damage of cells was not visible by microscopy the inhibition of cellular PrP conversion by Cu^{2+} does not seem to be due to copper cell toxicity.

In addition to copper, Zn^{2+} was shown to stimulate PrP^C endocytosis, though to a lesser extent than Cu^{2+} [Perera and Hooper, 2001]. The study presented in this thesis found no impairment of prion propagation when cultures were incubated 20 days in the presence of Zn^{2+} ions prior to cell infection. However, Zn^{2+} mediated inhibition of prion propagation was effective only in the constant presence of 50 µM $ZnCl_2$ during cell incubation.

In a study by Basu *et al*. human neuroblastoma cells were exposed to $FeCl_2$ which bound to PrP^C and transformed it into a PrPres-like form [Basu *et al.*, 2007]. Another recent publication reported that an internalization, conversion into PrPres and accumulation and of exogenously administered recombinant PrP was induced in cells by Fe^{3+} but not Fe^{2+} [Choi *et al.*, 2013]. In contrast to these findings, the administration of $FeCl_2$ and $FeCl_3$ to PMCA samples or cell cultures in this study did suppress the formation of PrPres in both *in vitro* assays. In this regard, $FeCl_2$ as well as $FeCl_3$ were similarly effective. The results of this thesis provide no indication that iron ions promoted prion propagation. This discrepancy to published data could be due to different experimental designs and assays used in this and the published studies. In the studies by Basu *et al*. and Choi *et al*. relatively high concentrations of 100 µM-300 µm $FeCl_2$ and 600 µM ferric ammonium citrate (Fe^{3+}), respectively, were examined for effects on the conversion of PrP. In this thesis, however, concentrations of 20 and 50 µM were used (since 100 µM were found to cause cell damage in long-term experiments) together with authentic PrP^C as conversion substrate. In this context it has to be noted that the data on the influence of metal ions on PrP conversion presented in this thesis are still preliminary and need to be further substantiated in future studies.

4.2.4.3 Conclusion and outlook

There are many reports that address the involvement of copper in the etiology and pathogenesis of prion diseases. In the literature it has been discussed that elevated extracellular copper levels cause PrP^C to attain a PrP^{Sc} like structure that accumulates on the cell surface and eventually forms amyloid like aggregates (for review see [Rana *et al.*, 2009]). This assumption was supported, inter alia, by the findings that the application of Cu^{2+} chelators delayed the onset of prion disease in infected mice

[Sigurdsson *et al.*, 2003] and that Cu^{2+} *in vitro* increases the resistance of PrP against PK [Kim *et al.*, 2005; Sigurdsson *et al.*, 2003]. However, the specific role of copper in prion diseases may be complex. In this study copper ions consistently decreased the propagation of PrP^{Sc} seeds by PMCA and in glial cell cultures.

Though PMCA and cell assay represent profoundly different cell-free and cell-based test principles for the biochemical and biological propagation of prion activity *in vitro* they delivered basically similar findings when metal ions were applied. Due to the absence of cellular structures, PMCA experiments provide insight into the effects of metal ions on prion replication on the molecular level. In the cell culture assay, however, additionally to molecular effects cell mediated effects of metal ions, such as ion induced PrP internalization, influence prion propagation. Therefore, findings from PMCA and cell assay do not need to be identical. Indeed, the suppressing effect of copper was relatively stronger in the cell assay and less pronounced in PMCA. This indicated that the cell mediated inhibition of prion propagation by copper is more effective than its mere action on PrP misfolding at the molecular level. While $FeCl_2$ was the most effective compound in suppressing PrP misfolding in PMCA it did not cause a similarly strong inhibition of PrPres formation in the cell assay, showing that an inhibitory molecular effect on PrP misfolding in PMCA is not necessarily sufficient to prevent biological prion propagation. Together, these data suggest that a combined application of $FeCl_2$ and $CuCl_2$ could best prevent prion propagation in cell cultures, by a molecular as well as cell mediated non-cytotoxic mode of action.

The experimental results in this thesis showed examples for the *in vitro* inhibition of seeded prion propagation by elevated metal ion concentrations. Primary PrP nucleation, i.e. the *de novo* formation of a prion seed, has not been addressed in this experimental setup. In the context of the nucleation-polymerization model of prion replication, the observed inhibitory effects of metal ions are rather explained by a metal ion-mediated interference with seed elongation than metal ion-mediated impairment of seed fragmentation. The experiments of this thesis could, however, not clearly identify by which precise mechanisms PrP conversion had been inhibited by metal ions. Metal-induced structural stabilization of PrP^{C} has been hypothesized to mediate inhibition of PrPres amplification [Levin *et al.*, 2005]. This would affect the interaction with PrP^{Sc} seeds in the course of seed elongation, and is a conceivable mode of action in the PMCA experiments of this thesis. Figure 4-1 depicts a hypothetical diagram of the possible mechanisms that explain the metal ion-mediated inhibition of PrP conversion in seeded PMCA experiments observed in this thesis. PrP^{C} is known to bind copper ions *in vivo*, whereas PrP^{Sc} becomes depleted of copper and enriched in manganese [Brown *et al.*, 1997; Thackray *et al.*, 2002; Wong *et al.*, 2001a]. Inhibitory effects of Cu^{2+} on PrPres formation observed by others were suggested to be mediated by binding of Cu^{2+} to PrP^{C} and a structural stabilization of the PrP^{C} [Levin *et al.*, 2005; Orem *et al.*, 2006].

It has been hypothesized that copper binding to PrP^C would affect PrP^C structure and the ability to aggregate [Levin *et al.*, 2005]. Additional evidence comes from a study by Thakur *et al.*, who showed that the inhibition of formation of PrP amyloid fibrils by copper at physiological temperature is accompanied by two novel intra-protein interactions that render the PrP structure more compact and thus stabilize it [Thakur *et al.*, 2011]. If binding of metal ions, in particular copper, with high affinity to PrP^C stabilizes the structure of this protein and make it therefore more resistant to adaptation to the PrP^{Sc} conformation, conversion into and amplification of PrPres would be plausibly inhibited. The addition of EDTA captures metal ions. In the presence of EDTA the structure of PrP^C would not longer be stabilized by metal binding, and an adaptation to the PrP^{Sc} conformation would be facilitated. However, manganese may bind to PrP in either conformation, but does not stabilize PrP^C. Hence, conversion would not be affected. A pro-aggregatory effect of manganese that was previously described in the literature [Kim *et al.*, 2005; Levin *et al.*, 2005] which may result in accelerated PrPres formation was however not observed in the PMCA assay in this thesis.

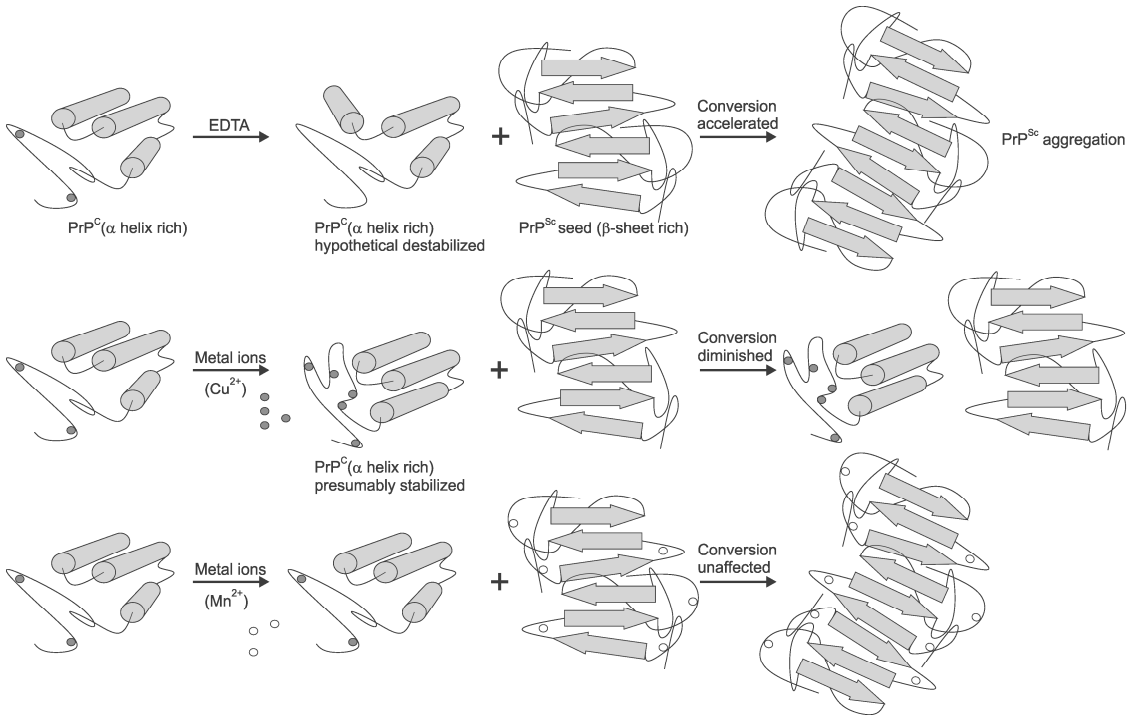

Figure 4-1: Hypothetical model of a molecular mechanism of metal ion mediated inhibition of PrP amplification in seeded PMCA. Metal ions may affect the elongation step of PrP^{Sc} seeds by stabilizing PrP^C substrate conformation and thus impeding PrP misfolding and attachment to the PrP^{Sc} seed. Addition of EDTA captures metal ions and facilitates accelerated PrP^{Sc} propagation. Manganese may bind to PrP^C and PrP^{Sc} but without implications for conversion of PrP and propagation of PrP^{Sc}.

It has been established by a variety of studies that different modes of action exist with respect to the influence of metal ions on PrP conversion. Manganese has been discussed to propagate prion replication by inducing structural changes in PrP, binding to monomeric PrP^{Sc} thereby stabilizing it and propagating its aggregation, or by inhibiting proteasomal activity and increasing the PrP^{Sc} levels [Choi *et al.*, 2006]. The findings in this work did however not indicate a stimulation of PrP misfolding and prion replication by Mn^{2+}.

Furthermore, metal ions may play a role in the prion strain phenomenon [Wadsworth *et al.*, 1999], or may affect the incubation times of prion strains. Within the scope of the present study, PMCA and glial cell assays for other hamster adapted prion strains 22A-H, BSE-H and ME7-H, respectively, have been established. Using the same experimental setup of PMCA and cell assay as applied for 263K, the effect of metal ions on PrP misfolding associated with these other prion strains can be examined easily in future studies.

4.3 Characterization of the amplified PrPres species

Comparison of biochemical properties of brain PrP^{Sc} and PMCA generated PrPres from each prion strain revealed some alterations from the original properties. During *in vitro* propagation of ME7-H a change from the original glycosylation profile was observed. The diglycosylated protein band was most pronounced in PMCA derived PrPres, while mono- and diglycosylated protein bands showed an approximately equal intensity in brain PrP^{Sc}. Similar observations were reported previously by others [Gonzalez-Montalban and Baskakov, 2012] using PMCA with other hamster prion agents. PMCA derived PrPres was reported to be depleted of monoglycosylated PrP and the percentages of diglycosylated PrP glycoforms was increased compared to brain PrP^{Sc}. However, diglycosylated PrP^{Sc} was already predominant in the original PrP^{Sc} seeds. The authors suggested that PMCA propagates the same molecular features in an undifferentiated manner, i.e. at least partially independent of the strain characteristics of PrP^{Sc} parent seeds, and that the properties of PrPres derived from different prion strains basically converge during *in vitro* amplification [Gonzalez-Montalban and Baskakov, 2012]. As discussed above, PMCA conditions cause an artificial environment and selection pressure for PrPres replication. Under such amplification conditions diglycosylated PrP may be the favored protein species for prion replication. Furthermore, it was demonstrated that propagation of hamster prions requires diglycosylated PrP^{C} whereas propagation of mouse prions requires unglycosylated PrP^{C} in PMCA [Nishina *et al.*, 2006]. On the other hand, however, other authors had reported

that PrP glycosylation is not required to maintain strain-specific properties such as patterns of cerebral PrP^{Sc} deposition and neuronal vacuolation [Piro *et al.*, 2009]. Accordingly, it can be hypothesized that the change in the glycoform pattern of PMCA derived ME7-H observed in this thesis has no implications for its strain specific properties *in vivo*. Yet, the PMCA derived ME7-H differed also with respect to other properties from the original inoculum. Compared to brain PrP^{Sc} PMCA derived ME7-H exhibited a higher efficiency to induce PrP conversion in PMCA (Figure 3-17B). Moreover, amplification of PMCA derived ME7-H under nucleic acid deficiency was equally well restored by synthetic RNA and chondroitin sulfate, whereas propagation of brain ME7-H PrP^{Sc} was facilitated by synthetic RNA only and with a lower efficiency than found for PMCA derived ME7-H. Amplification under nucleic acid deficiency revealed a residual seeding ability of PMCA derived ME7-H that was otherwise only observed for the fast replicating 263K scrapie strain. The most striking difference was observed in the cell assay. While authentic ME7-H from hamster brains failed to replicate in glial cell cultures, the PMCA derived ME7-H had gained the ability to do so. Several studies found that PMCA generated PrPres exhibited extended incubation times when inoculated into animals [Klingeborn *et al.*, 2011; Piro *et al.*, 2009]. The findings shown in this thesis, that PMCA derived ME7-H acquired a faster seeding efficiency in PMCA, and more importantly, that it gained the ability to infect glial cell cultures suggests that the ME7-H PMCA product may have an increased or "faster" infectivity in animals (i.e. PMCA derived ME7-H may well cause disease after shorter incubation times than authentic ME7-H which does only after about 330 days). Structural analyses of FT-IR spectra from brain ME7-H and PMCA derived ME7-H revealed different spectral features of PMCA derived PrPres that, however, resembled those of all other examined PMCA samples. This further shows that PMCA derived ME7-H structurally differs from its brain ME7-H parent seed. From the data available so far it cannot be concluded whether the observed altered properties of PMCA derived ME7-H are permanent or transient (the latter seems to be likely for the BSE-H PMCA product produced under nucleic acid deficiency, see chapter 4.2.2 above). Again, the findings for ME7-H may reflect a dynamic selection process in terms of preferentially propagated PrPres species under certain amplification conditions, as discussed above for BSE-H. Further clarification of this issue will require additional studies.

Apart from these findings, it was demonstrated in this study that other strain specific properties were preserved during PMCA. The electrophoretic mobility of BSE-H from brain or PMCA was similar, and was distinguishable from brain PrP^{Sc} and PMCA derived PrPres associated with 263K, ME7-H or 22A-H. The findings for BSE-H were only different, when PMCA conditions had been specifically modified for permanent nucleic acid deficiency (discussed above). When, however, BSE-H was generated in

PMCA under an excess of synthetic RNA, or when its amplification under nucleic acid deficiency had been restored with synthetic RNA or chondroitin sulfate, the electrophoretic mobility was still unchanged. Thus, if suitable cofactor molecules were continuously present during amplification, the electrophoretic mobility typical of original BSE-H seeds was preserved in PMCA. Since in the present study only a proportion of the biochemical, biological and structural properties of PMCA generated PrPres species were characterized and compared to the corresponding brain PrP^{Sc}, it cannot be ruled out that further differences with respect to other properties exist.

There is an ongoing debate whether PMCA propagates all prion strain characteristics faithfully. First publications reported that major characteristics are preserved [Castilla *et al.*, 2008b; Castilla *et al.*, 2005a]. Such properties were assessed by different methods on different PMCA propagated prion strains. This included analyses of the electrophoretic mobility, glycosylation profile, resistance to PK and resistance to denaturation with guanidine hydrochloride, as well as inoculations of animals and subsequent analyses of the incubation times until development of disease and the clinical symptoms. However, recently evidence has accumulated that some properties of parent PrP^{Sc} seeds are modified in PMCA products [Daus *et al.*, 2013; Gonzalez-Montalban and Baskakov, 2012; Klingeborn *et al.*, 2011]. The analysis of structural components of brain PrP^{Sc} and PMCA derived PrPres by FT-IR spectroscopy aimed to determine whether alterations in protein structure can be detected and may be attributed to strain specificities and certain experimental cofactor conditions in PMCA. FT-IR spectroscopy has been used to distinguish 263K, ME7-H, 22A-H and BSE-H [Thomzig *et al.*, 2004]. In this thesis, FT-IR spectroscopy was applied to investigate if the presence or absence of specific cofactor molecules during PMCA caused detectable alterations in protein conformations. Analysis of 263K generated by PMCA under different cofactor amplification conditions (263K in standard PMCA or PMCA reconstituted with synthetic RNA and chondroitin sulfate, respectively) indicated no major differences in structural components between these samples based on preliminary FT-IR data. However, due to limited sample number this has to be considered only as a tentative indication, that needs further substantiation. If future measurements provide similar results, this would indicate that protein conformations are similar and that PMCA products derived by PMCA from the same parent strain may share similar conformations after generation under different cofactor conditions. The ability of the different 263K PMCA products (standard PMCA or PMCA reconstituted with synthetic RNA and chondroitin sulfate, respectively) to induce PrP conversion in glial cell cultures was not impaired, which demonstrates that the biological seeding activity was unaffected by the substitution of endogenous cofactor molecules using synthetic RNA or chondroitin sulfate. However, it has to be noted that all 263K associated PMCA

generated PrPres samples are clearly different from brain 263K. In FT-IR spectra brain PrP^{Sc} differs from PMCA derived PrPres of 263K scrapie in certain peak positions [Daus *et al.*, 2013]. Preliminary data from PMCA derived PrPres associated with ME7-H, 22A-H and BSE-H (in standard PMCA, PMCA under nucleic acid deficiency or PMCA reconstituted with synthetic RNA and chondroitin sulfate, respectively) showed an overall spectral similarity for all samples. Spectra of brain PrP^{Sc} of these prion strains were clearly distinguishable and different from the corresponding PMCA generated PrPres samples. However, a clear and robust differentiation of these PMCA products from each other was not possible. On the one hand this may be due to the limited number of samples. On the other hand it may also indicate a previously unrecognized challenge for the differentiation of PMCA products by FT-IR spectroscopy. PMCA seems to select PrP subspecies that have a high replication efficiency under the specific amplification conditions. As discussed above, it has been suggested by Gonzalez-Montalban and Baskakov (2012) that basically converging molecular features are being propagated in PMCA. If similar subspecies present in the quasi species ensemble of PrP conformers of different prion strains are selected under equal PMCA conditions, this could result in the propagation of identical or similar PrP subspecies from different prion strains and thus complicate the differentiation of PMCA products by FT-IR spectroscopy. Such effect could account for the similar spectra of PMCA derived PrPres from different prion strains, and for the spectral differences to the corresponding parental brain PrP^{Sc}. However, additional experiments are required to definitely resolve this issue.

4.4 Contribution of PMCA and cell culture assays for the reduction of animal bioassays

On the basis of joined applications of PMCA and cell culture *in vitro* assays, titrations of prion infectivity of unknown samples can be performed *in vitro* without the need of titrations in laboratory animals [Boerner *et al.*, 2013]. This was demonstrated with established PMCA and cell culture assays for 263K hamster prions and was perspectively shown for other prion strains. By parallel endpoint titrations of samples of known and unknown prion seeding activity in PMCA, SD_{50} values can be determined. In analogy to the determination of SD_{50} values by PMCA, the cell assay is used for determinations of cell culture infectivity in terms of $CCID_{50}$ by parallel endpoint titrations of reference and test samples in the cell assay. Due to correlations among SD_{50}, $CCID_{50}$ and ID_{50} in reference standards such as homogenized 263K scrapie hamster brain tissue, SD_{50} and $CCID_{50}$ values detected in test samples can be tentatively translated into ID_{50} values. PMCA and cell assays are established for each prion strain and the correlation factors between SD_{50}, $CCID_{50}$ and ID_{50} have to be determined individually. By this approach seeding- and cell culture infectivity titers can be determined *in vitro* and tentatively translated into animal infectivity titers without the need for ethically critical, expensive and time consuming titrations in animals.

"This rationale and methodology for a progressive reduction and eventual replacement of prion bioassay titrations in laboratory rodents will also facilitate studies on the anti-prion effectiveness of (re-)processing procedures for medical devices and biological products, and help to further harness prions as test agents for the development of broad-range disinfectants and as bio-indicators for sterilization processes [Wagenfuhr and Beekes, 2012]. However, in order to definitely prove that combinations of PMCA and cell assays provide an effective or even superior alternative to bioassays in laboratory rodents, prion titre estimates from such jointly performed *in vitro* assays remain to be further validated by actual ID_{50} data from – preferably completed – *in vivo* studies." [Boerner *et al.*, 2013]

This rationale and methodology for the reduction and replacement of animal bioassays in prion research was submitted as a proposal to the contest for the Scientific Award for the Protection of Laboratory Animals of Berlin (Berliner Forschungspreis für Alternativen zu Tierversuchen) in October 2012, and was appreciated as the second best contribution.

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Berlin, Susann Boerner

Erklärung

Ich erkläre, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe.

Berlin, Susann Boerner