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„ Biochemical analysis of an *in vivo* immunoprecipitation complex of the prion protein “

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Abstract

The prion protein is a cell surface GPI-anchored protein, best known for causing transmissible spongiform encephalopathies (TSE) when conformationally converted into its protease resistant and β sheet rich scrapie form (PrP^{Sc}). Its physiological function remains largely unknown, however its expression and membrane anchoring is indispensable for the development of TSEs. Numerous biochemical efforts have been made recently to address PrP's involvement in signaling pathways, by testing direct protein-protein interactions of PrP. To this end, an *in vivo* immunoprecipitation (IP) complex was isolated using mouse monoclonal antibodies (POM2) and eluted with synthetic peptides specific for the POM2 binding region. Native gels (and size-exclusion chromatography) revealed a high-molecular weight complex of approximately 800kD. Subsequent mass spectrometry analyses suggested the presence of multiple proteins in this complex.

Here, I tried to verify the interaction of the candidate proteins with PrP in the complex via Western blotting. So far, no specific interaction could be verified. In addition, silver staining under denaturing conditions revealed a single band on a SDS-PAGE corresponding to PrP, suggesting an absence of other proteins/peptides in the complex. Control experiments with monomeric detergent-free purified and phospholipase-cleaved bovine PrP further confirmed that the HMW band is not a mere electrophoretic migration artifact. Furthermore, chemical cross linking with cell impermeable agents in cell culture yielded multiple band shifts, potentially indicating a multimeric organization of PrP at the cell surface.

Taken together, these results suggest that there is a homotypic interaction of cellular PrP, which might be necessary for exerting its physiological function in signal transduction. Conversely, a disruption of this type of interaction might subvert PrP-mediated signal transduction thus causing severe neurotoxicity reminiscent of the phenotype resulting from genetic ablation of the central domain. In summary, the multimeric organization of PrP might provide new insights in understanding PrP physiology and more importantly help to better understand the initiation of toxic events in prion-mediated pathologies.

Zusammenfassung

Das Prion-Protein (PrP^{C}) ist ein Glykosyl-Phosphatidylinositol-verankertes Membranprotein, welches durch Konformationsänderung zur β–Faltblatt-reichen und proteaseresistenten Skrapie-Isoform (PrP^{Sc}) zum Auslöser der transmissiblen spongiformen Enzephalopathien (TSE) werden kann. Die physiologische Funktion von PrP bleibt hingegen weitgehend unbekannt. PrP-Expression und Verankerung an der Zellmembran sind für die Entwicklung der Pathologie unerlässlich. In zahlreichen Arbeiten wurde die Rolle von PrP in Signaltransduktionswegen anhand von direkten Protein-Protein Interaktionen untersucht. Durch Immunpräzipitation mithilfe der monoklonalen Antikörper POM2 wurde für diesen Zweck ein *in vivo* Immunpräzipitationskomplex (IP-Komplex) isoliert, welcher anschliessend mittels synthetischer Peptide spezifisch für die POM2-Bindungsstelle eluiert wurde. Durch native Gele und Gel-Permeations-Chromatographie (GPC) wurde die Grösse des IP-Komplexes (800kD) bestätigt. Anschliessende massenspektroskopische Analysen deuteten eine Beteiligung mehrerer Proteine im Komplex an.

In dieser Studie habe ich versucht die Interaktion der Kandidatenproteine mit PrP im IP-Komplex mithilfe des Western Blotting nachzuprüfen. Eine spezifische Interaktion konnte bis jetzt nicht bewiesen werden. Ausserdem konnte nach der Silberfärbung unter denaturierenden Bedingungen im SDS-PAGE Gel nur eine einzige Bande nachgewiesen werden, die der Grösse des PrP selbst entspricht und praktisch die Präsenz anderer Proteine ausschliesst. Kontrollexperimente mit monomerem detergenzfrei aufgereinigtem bPrP zeigten, dass die 800kD Bande nicht infolge der beeinträchtigten elektrophoretischen Migration entstanden ist. Zusätzlich haben *in vitro* Experimente unter Einsatz von chemischen Crosslinkern nahegelegt, dass PrP in multimeren Clustern an der Zelloberfläche vorkommen könnte.

Zusammenfassend weisen diese Ergebnisse auf eine homotypische Interaktion des zellulären PrP hin, die möglicherweise für die Ausübung der physiologischen Funktion von Bedeutung ist. Anderseits könnte eine Unterbrechung der physiologischen Signaltransduktion zu Neurotoxizität führen, worauf genetische Ablationen bereits hindeuten. Die Erkenntnis der multimeren Organisation von PrP könnte helfen die Physiologie und viel wichtiger die Aktivierung der pathologischen Vorgänge besser zu verstehen.

1. Introduction

1.1 Prions and prion diseases

Prion diseases or transmissible spongiform encephalopathies (TSEs) are fatal and hitherto incurable neurodegenerative illnesses that have gained enormous public attention primarily due to the bovine spongiform encephalopathy (BSE) crisis in the past two decades. They are caused by a conformational conversion of the cellular prion protein (PrP^{C}) into its scrapie form PrP^{Sc} (Prusiner, 1998). Unlike other known infectious diseases, prion infections are caused only by a single protein which is able to replicate and transmit infections without the help of encoding nucleic acids (Aguzzi et al., 2008), hence the name prion, an anagram for "proteinaceous infectious only".

Different TSEs have been identified in humans, with the most prominent being Creutzfeldt-Jakob Disease (CJD), as well as Gerstmann-Sträussler-Scheinker syndrome (GSS), Kuru and Fatal Familial Insomnia (FFI) and scrapie disease in sheep, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in deer and elk and a variety of transmitted forms of BSE in captive animals. The variant form of CJD, the disease believed to be trasmitted from BSE to humans caused the death of hundreds of people in Europe and provoked immense fear in the 1990s, threatening to become an epidemic with unprecedented and disastrous implications. The other forms of CJD are sporadic (sCJD) accounting for 90% of cases, and genetic CJD. Much excitement surrounded the discovery that polymorphisms occuring at codon 129 of the *PRNP* gene (located on human chromosome 20), coding for methionine or valine (M129V) determine the susceptibility of developing CJDs. While vCJD exclusively requires methionine homozygosity, codon 129 heterozygositiy is protective for sCJD (Palmer et al., 1991).

Like transmissibilty, another common feature of the prion disease spectrum is the severe and widespread neurodegeneration, which translates into progressive motoric and cognitive disorders, mostly concomitant with the formation of amyloid plaques of PrP^{Sc}. Histopathologically, they are associated with conspicuous intraneuronal vacuoles containing membrane fragments and occassionally degenerating organelles (Aguzzi and Steele, 2009).

The molecular determinants that give rise to vacuolation remain vastly unknown. It also remains to be clarified whether they precede and therefore propel neuronal cell death.

These pathologic features are also common in the other non-transmissible forms of prion diseases that are genetically inherited (usually genetically dominant) with the gene encoding the prion protein and are ususally described as prionopathies (Aguzzi et al., 2008). Many of the described mutations associated with PrP have been studied *in vitro* (Harris, 2003) and surprisingly not all of them exhibited PrP^{Sc} biochemical features such as amyloid formation, protease K and detergent resistance or resistance to its GPI-anchor cleavage by phosphatidyl inositolphosphate specific phospholipases (Prusiner 1982). Strikingly, some of the disease-causing mutants act biochemically identically to PrP^{C} pointing to distinct properties of PrP in terms of infectivity and pathogenicity, as illustrated by the example of the insertional mutations in the octapeptide repeats (Chiesa et al., 2003).

Despite intense research in the past three decades, the molecular mechanisms initiating the devastating neurodegeneration entailed by prion encephalopathies remain largely unidentified and hence cannot yet be therapeutically targeted, leaving TSEs incurable. Notwithstanding that, a major breakthrough has been achieved with regard to post-exposure prophylaxis. This is mainly thanks to the seminal work carried out by the Aguzzi lab in discovering the mechanisms by which the prions infect the organisms and invade the central nervous system (CNS). That research led to the understanding of the cross-talk between the immune system and CNS, thus identifying the lymphotoxin pathway as a key mediator of prion replication prior to “neuroinvasion” by prions. This pathway can effectively be targeted by lymphotoxin beta receptor inhibitors (reviewed in Aguzzi et al., 2007).

1.2.1 Prion Structure and Chemistry

While less success has been reported in elucidating the toxic mechanisms and the cellular responses to the formation of PrP^{Sc} plaques (see the subsequent section), the findings concerning the structure and chemistry of the scrapie agent have been more promising

culminating with reports about the synthesis of infectious prions from prokaryotic PrP (Legname et al., 2005).

Deposition of protein aggregates is commonly associated with the more prominent neurodegenerative diseases such as Alzheimer's and Parkinson's diseases, but prions, are thus far the only agent that is transmissible both within and across species (Aguzzi and Rajendran, 2009). A three-dimensional structure of the infectious prion doesn't exist yet, mainly due to aggressive oligomerization of PrP^{Sc} and formation of very heterogenous aggregates which make both X-ray crystallography and NMR studies difficult (Govaerts et al., 2004). Electron microscopy studies of the two-dimensional and partially proteinase K (PK) digested PrP^{Sc} fragment (PrP 27-30) have suggested a stacked, left-handed β helical structure of the infectious form (Nguyen et al., 1995). Optical spectroscopy analyses revealed an enriched content of β-sheet structure (up to 45%) compared to the only 3% of the physiological PrP(C) (Caughey et al., 1991).

The traditional view has been that the accumulation of the infectious scrapie agent is the toxic species per se and has correlated its extent of accumulation with the extent of the pathology. This view has however been challenged by insights gained from two other elegant *in vivo* studies. Namely, Silveira et al. (2005) demonstrate biochemically employing flow field-flow fractionation (FlFFF) that the large prion fibrils are by far not the most toxic and it is rather the smaller non-fibril PrP particles (14-28 PrP molecules) instead that require shorter incubation times for the outbreak of scrapie pathology.

The other study was done in transgenic mice carrying the aforementioned insertional mutations in the ocapeptide repeat (see subsequent section) which also lead to formation of protease resistant prion protein aggregates and hence develop spontaneous pathologies reminiscent of scrapie, but fail to transmit the disease (Chiesa et al., 2003). These mice are susceptible to Rocky Mountain Laboratories (RML) strain of prions endowing them with a highly infectious variant which conformationally resembles PrP with extra octapeptide repeats (14 instead of 5), but has a different quaternary structure. Namely, the infectious variant constitutes a large and tightly packed multimer (up to 100-200 PrP molecules) with a sedimentation coefficient between 50 and 120S whereas the non-transmissible toxic variant consists largely of 20-30 monomeres and even up to 10-15% monomers, stressing again the

different properties of PrP required for infectivity and toxicity and pointing to smaller β–pleated sheet rich oligomers of being more toxic species.

1.2.2 Prion protein's structure and chemistry

Structure analysis of recombinant PrP unraveled an unstructured, ill-defined and floppy N-terminus comprising the amino acid residues 23-125 and a well-defined, globular carboxy-terminus extending from residues 126-231 (Riek et al., 1996; Hornemann et al., 1997) attached to the plasma membrane by a glycosylphosphytidylinositol (GPI) anchor (Stahl et al., 1990). The N-terminal polypeptide chain consists of two positively charged clusters (denoted as CC1 and CC2), flanking a stretch of proline- and glycine-rich octapeptide repeats (OR), uniquely found in the prion protein, and finally a region containing hydrophobic residues termed hydrophobic core (HC).

The carboxy-terminus consists of three α–helices (H1-H3) and two short β–sheets between H2 and H3 and undergoes a two-fold post-translational N-linked glycosylation at the 181 and 197 asparagine residues (Bolton et al., 1985). Some cell culture studies had suggested that glycosylation is important for preventing PrP monomers to aggregate (Korth et al., 2000; Priola and Lawson 2001), however the knock in transgenic mice with a constitutive or double mutations at the glycosylated asparagine residues (Cancelloti et al., 2005) did not display any pathology although this did impair the translocation of PrP to the outer leaflet of the membrane.

The glycosylphosphytidylinositol (GPI) anchor of the PrP plays a putative role in exerting its physiological function, given that the transgenic mice expressing anchorless PrP (ΔGPI) displayed a peripheral demyelination similar to the phenotype PrP KO mice described in the study of Bremer and colleagues (2010). These mice however express only one 1/4 of WT PrP. So it remains unclear however whether in the case of ΔGPI mice the demyelination arises from the low expression level of PrP or is a constitutive phenotype of the GPI-anchor deficiency (see "Lessons from PrP KO mice").

This phenotype notwithstanding, the GPI anchor certainly does play a pivotal role in initiating the toxic events following prion inoculation-, given that ΔGPI mice do not develop clinical scrapie (see the subsequent section). It's been reported that GPI-anchored proteins are harbored in specialized detergent resistant membrane compartments called lipid rafts, where also many other signaling molecules reside, including small lipid messenger molecules such as phoshpatidyl-inositol-bisphosphate (PIP2), small GTPases and some nonreceptor-type kinases (reviewed in Anderson, 1993). Indeed, several lines of evidence have shown that PrP^{Sc} translocates preferably to these detergent resistant domains (Naslavsky et al., 1997; Meier et al., 2003) and conversely pharmacological depletion of lipid rafts largely inhibits the formation of the scrapie form (Taraboulos et al., 1996).

1.3.1 Prion neurotoxicity and the role of PrP

As predicted by the protein only hypothesis, mice lacking the *Prnp* gene (*Prnp*$^{0/0}$ mice, Büeler et al., 1992) were resistant to scrapie when inoculated with mouse scrapie prions (Büeler et al., 1993), and neurotoxicity could only occur when PrP^{C} was expressed by host neurons (Brandner et al., 1996). Moreover, increased scrapie susceptibility and shortened incubation time (60 days instead of 150) in transgenic mice overexpressing PrP^{C} (Tga20 mice) coincided with almost halved presence of PrP^{Sc} (Fischer et al., 1996), further disqualifying PrP^{Sc} plaque accumulation as the sole cause for the neurodegeneration and emphasizing again the impact of the cellular PrP in inducing neurotoxic pathways.

Furthermore, depleting endogenous neuronal PrP^{C} after intracerebral prion inoculation of the mice reversed neurodegeneration and spongiosis, despite massive accumulation of extra-neuronal PrP^{Sc}, indicating that endogenous neuronal PrP-mediated signalling is required so that neurotoxic pathway can unfold (Mallucci et al., 2003). In addition, transgenic mice that express a secreted form of prion protein (ΔGPI), do not exhibit neurotoxicity despite sustained prion replication and reasonable accumulation of PrP^{Sc} in their brains, demonstrating that membrane anchoring of endogenous PrP is a pre-requisite for initiating neurotoxic pathways (Chesebro et al., 2005).

Conversely, intra-cerebral injection of anti-PrP^C antibodies induced cerebellar and hippocampal neuronal death (Solforosi et al., 2004) emphasizing the importance of PrP membrane anchoring in initiating toxic events, hence suggesting an involvement of cellular PrP in a signal transduction pathway. A modification of the cell surface anchored PrP might subvert this signalling pathway finally rendering it toxic. The authors ascribe this to a possible PrP molecule crosslinking by their antibodies and suggest a similar scenario in prion diseases with PrP^{Sc} evoking also a crosslinking of PrP^C molecules. However, they do not provide a direct evidence that PrP molecules were indeed crosslinked and data from our lab also indicate that single chain peptides and Fab fragments of carboxy-terminus antibodies also induce neurotoxicity (Sonati et al., submitted), suggesting that PrP crosslinking is not required for initiating the death signalling.

1.3.2 Lessons from PrP mutants

1.3.2.1 Point mutations

To learn more about prion toxicity many research laboratories have expressed some of the naturally occurring point mutations in mice or cell lines in an attempt to mimic human prion diseases. In many cases, a toxic phenotype wasn't reproducible and in none of them has a successful transmissibility been reported, except for the mice expressing the P101 variant, a naturally occurring mutation causing GSS (reviewed in Harris, 2003). Most of the point mutations leading to genetic CJD occur in the carboxy-terminus, primarily affecting the region flanked by the first two the α helices (see panel below)

Also, most of the knock-in animal models carrying point mutations in PrP, normally causing prionopathies don't display any visible toxic phenotype (Aguzzi et al., 2008). Some of the mutants biochemically display PrP^{Sc}-like properties, some show altered localization compared to wildtype PrP^C and as mentioned above, some behave virtually identically to PrP^C in biochemical terms and yet induce a severe toxic phenotype, reiterating the involvement of PrP in a signal transduction pathway which could be modified through modest alterations and thus exert toxic signaling.

1.3.2.2 PrP topology and neurotoxicity

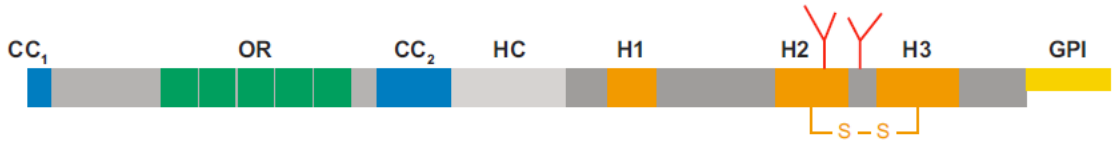
PrP is usually anchored to the outer leaflet of the cell membrane via the GPI anchor. Other membrane topological variants have also been reported to be associated with prion pathologies, namely the ^{Ctm}PrP conformation, where the carboxy-terminal polypeptide chain remains located in the lumen of the endoplasmic reticulum (ER) and its inverted conformation termed ^{Ntm}PrP, with both topological variants being able to span the cell membrane (Hegde et al., 1998).

Several forms of GSS are associated with mutations within the central domain (CD) encompassing the charge cluster 2 (CC2) and the hydrophobic core (HC) of human PrP thus enabling the protein to span the cell membrane within amino acids 102-127 (Hegde et al., 1998; Collinge, 2001). Transgenic mice expressing these transmembrane domains of PrP develop cerebellar degenerative alterations similar to those seen in most of the prion diseases. Other transgenic mice (L9R/3AV) with additional substitutions in the signaling peptide express only the Ctm conformation (Stewart et al., 2001) also develop ataxia, cerebellar and neuronal loss, particularly aggravating the phenotype with the expression of endogenous PrP, in contrast to the toxic phenotypes caused by deletion mutations (discussed in the subsequent section).

Interestingly, the expression of endogenous PrP also aggravates the granular cell degeneration in the transgenic mice expressing the GPI-anchored N-terminus of PrP, another mouse model generated in the Aguzzi lab to confine the PrP-induced neurotoxicity. The preliminary data strongly indicate that this PrP variant undergoes as well a transmembrane conformation as well (Dametto et al., drafted).

It was originally speculated by Hegde and colleagues that infectious prion pathologies generate an enhanced ^{Ctm}PrP conformation, thus explaining the neurotoxicity of transmissible variants. This tantalizing scenario certainly presents a plausible model of how aberrantly membrane-anchored PrP might kill neurons. This wasn't however supported in the following *in vivo* studies (Stewart and Harris, 2003). In addition, there hasn't been any

evidence that mutations occurring outside the hydrophobic core that also lead to prion pathologies, adopt any ^{Ctm}PrP topology.



Panel 1. Human prion protein with its domains (adopted from Aguzzi et al., 2008)

1.3.2.3 Deletion mutations cause spontaneous neurodegeneration

The Aguzzi lab has primarily pursued the transgenesis path to gain insights about prion-mediated neurotoxicity and PrP's physiology. The initial idea was to create numerous transgenic mice with truncated forms of prion protein (on a PrP KO background) and thus map out the PrP sequences necessary for restoring susceptibility to prions. Unexpectedly, this approach provided more encouraging clues than the PrP-deficient mice regarding the physiological function of PrP, since some of the deletion mutants spontaneously developed severe neurological disorders, reminiscent of the prion infected mice, which indicated an involvement of distorted PrP in cell death pathways.

The transgenes with larger deletions in the N-terminus (Δ32-121 and Δ32-134) developed ataxia and cerebellar granular cell degeneration (Shmerling et al., 1998). Co-expression of the endogenous PrP^{C} largely rescued the toxic phenotype. The pathology was also ameliorated in the shorter mutants encompassing the central domain by the expression of the endogenous PrP. Baumann and colleagues (2007) deleted the central domain (Δ95-134; denoted as ΔCD) of the prion protein, a region that encompasses the second charged cluster (CC2) and hydrophobic core (HC). The mice developed a marked white matter vacuolar degeneration that resulted in death within the first three weeks of life. Strikingly, transgenes expressing a deleted central domain but lacking the GPI anchor are completely innocuous, stressing the importance of membrane anchorage of PrP in exerting a toxic signaling

function (Baumann et al., 2009). Of note, unlike ΔF (Shmerling et al., 1998), ΔCD didn't display granular cell degeneration.

In conjunction with these observations, the deletion mutant from another study (Li et al., 2007) which contains a shorter deletion in the central domain i.e. partial deletion of the charged cluster 2 and the hydrophobic core (Δ105-125), displayed a virtually identical phenotype, and mice died within the first week of life. Interestingly, sole deletion of the hydrophobic core (ΔHC) also displays a lethal phenotype (Bremer et al. unpublished). Both these studies indicate that these two sequences in the central domain are required for normal functioning of PrP, potentially suggesting that this could be a ligand binding domain that subverts the trophic signaling of endogenous PrP (since its expression rescues the phenotype) into toxic signaling. Given that the anchorless deletion mutants do not display a toxic phenotype, a putative subversion acquisition must therefore be cell membrane-associated.

However, not only the N-terminal deletions comprising the central domain are inherently toxic. Genetic ablations in the second (H2) or third helix (H3) at the carboxy terminus or their simultaneous double deletions also display spontaneous ataxia and several other neurological disorders, resembling those of the N terminal deletion mutants and TSEs (Muramoto et al. 1997; Supattapone et al. 2001)

Taken together, the studies listed above strongly suggest that prion toxicity requires the engagement of a membrane associated cellular prion protein. Furthermore, they strongly point to its involvement in a signal transduction pathway (thoroughly reviewed in Aguzzi, 2005). However, questions remain as to whether toxic signaling arises from a loss; gain or subversion of PrP's physiological signaling. The mild phenotypes of the knockout mice (see subsequent section) would barely justify the tremendous phenotype of the prion-mediated pathologies. Traditional gain of function in prion disease is unlikely, as this implies that PrP^{Sc} should be toxic independent of endogenous PrP, as it is the case in Alzheimer's and Parkinson's diseases, for example. However, this doesn't rule out the possibility that a gain-of-function causes the non-transmissible, PrP-mediated pathologies. A likely scenario is that PrP^{Sc}, some antibodies directed against the carboxy-terminus and the PrP deletions mutants

alter conventional signaling normally mediated by the prion protein, leading to a toxic cellular response (Harris and True, 2006). Be that as it may, the physiology of PrP remains just as mysterious as the prion toxicity. In the subsequent section I will summarize the findings that have been already reported regarding the function of cellular PrP.

1.4 Physiology of prion protein

1.4.1 Lessons learned from the PrP knockout mice.

Twenty-five years after the *Prnp* gene was cloned (Oesch et al., 1985; Basler et al., 1986) the physiological role of prion protein still remains the Riemannian equation of neurobiology. A great deal of effort has been committed to defining its role, both genetically and biochemically. The first PrP KO mice made in Charles Weissmann's lab termed $Prnp^{0/0}$ [Zürich I] (Büeler et al., 1992) did not show any phenotype. Lines generated later did show neurological disorders, but it was soon discovered that this phenotype derives from a genetic artefact, i.e. the overexpression of doppel, a PrP paralogue, after being put under the control of the PrP promoter (reviewed in Aguzzi et al., 2008).

1.4.2 PrP and signal transduction involved in myelin maintenance

The normal development and longevity of PrP-deficient mice (Büeler et al., 1992) landed a big blow to the hopes of discerning the physiological role of this GPI-anchored cell surface protein. So far, the only robust phenotype described in the PrP KO mice is a chronic demyelinating neuropathy (CDP) at 60 weeks of age that all the mice develop regardless of their genetic background (Bremer et al., 2010).

Genetic reintroduction of PrP^{C} *via* crosses to *tga20* mice, or introduction of one *Prnp* allele fully prevented this polyneuropathy. Electron microscopic analysis of 60 week-old KO mice showed characteristic ultra-structural signs of demyelination. In electrophysiological investigations, the nerve conduction velocities were significantly reduced in KO mice. The group further showed that KO mice expressing the *tg*NSE-PrP transgene which drives PrP^{C}

expression from the neuron specific enolase promoter had normal sciatic nerves, indicating that neuronal expression of PrP^C is required for myelin sheath maintenance.

1.4.3 Cleavage and endocytosis of PrP

Full-length PrP^C is processed by cleavage at amino acids 111/112 (α-site cleavage) to generate the GPI-anchored C-terminal fragment C1 and the soluble N-terminal fragment, N1. Interestingly, transgenic mice lacking the GPI-anchor-domain of PrP^C (ΔGPI) fail to undergo α-site PrP^C cleavage and exhibit CDP similar to KO mice.

It's been reported that PrP displays higher detergent solubility compared to some other GPI-anchored proteins such as Thy-1. This most likely occurs due to a differing lipid environment between PrP and Thy-1 with PrP containing larger extent of unsaturated long chain fatty acids as the Brugger et al. (2004) study shows. The proportion of unsaturated fatty acids on the other hand largely determines the solubility of the GPI-anchored proteins (s. Discussion). Whether GPI-anchored proteins dwell in different raft domains remains largely unknown, as does the very composition and the biological function of membrane domains. Controversial remains also the internalization dynamics of the prion protein. Cryoimmunogold electron microscopy has shown PrP localized to caveolae-like domains (Peters et al., 2003), the membrane invaginations thought to carry out the clathrin-independent endocytosis. Moreover, pharmacological cholesterol depletion has been reported to inhibit caveolae endocytosis (Parpal et al., 2001).

Nevertheless, most neuronal cells do not express caveolin-1 and don't display caveolae or caveolae-like invaginations (reviewed in Taylor and Hooper, 2005), so caveolin-dependent internalization of PrP has been questioned. On the other hand, two lines of evidence suggest that PrP endocytosis is clathrin-mediated, reminiscent of what has been described for another GPI-anchored protein, urokinase-type plasminogen activator receptor (uPAR). First, autoradiography and electron microscopy (EM) studies have identified chicken PrP to be recycled via clathrin-coated pit in murine neuroblastoma cells (N2a) (Shyng et al., 1993; Shyng et al., 1994). In support of clathrin mediated PrP internalization, these studies

revealed that PrP co-localizes with the transferrin receptor and low density lipoprotein receptors, two markers of clathrin-mediated endocytosis as shown by EM (Sunyach et al., 2003). In addition, observations from the GFP-tagged PrP clearly demonstrated a dynamin-dependent internalization (a crucial protein in the cascade of clathrin-mediated endocytosis), unlike many other GPI-anchored proteins (Magalhaes et al.,2002).

There have been attempts to reconcile this opposing data, by attributing this contradictory behavior of PrP to its proposed function i.e. the regulation of copper metabolism. It was reported that PrP is able to bind copper ions via its four octapeptide repeats (Aronoff-Spencer et al., 2000). This copper binding is believed to induce its endocytosis (Taylor et al., 2005) by loosening its association with the raft domains and relocating it to detergent-soluble membrane regions, hence initiating its internalization. However, the endocytosis question still remains unanswered just as the function of PrP still does.

1.5 Proposed functions of prion protein

Copper trafficking is not the only function that has been proposed for the prion protein. Evidence has been provided for its involvement in macrophage phagocytosis, given that PrP KO mice display impairments in this regard (Zhang et al., 2006), but this is most likely a genetic artifact and based on the studies in our lab, this impairment most likely originates from the neighboring locus of Sirpα gene (Nuvolone et al., drafted). Cell-cell adhesion has also been suggested to be facilitated by PrP based on biochemical evidence of direct interaction of PrP with proteins known to be involved in cell adhesion such as NCAM, EGF and also from genetic knockdown studies in zebra fish demonstrating hampered gastrulation due to improper cell contacts (Malaga-Trillo et al., 2009; see Results and Discussion).

1.5.1 PrP and the synapse

PrP's involvement in synaptic function and organization seems to be a plausible scenario, considering its high expression in the CNS. There is solid biochemical evidence for its

presence in the pre-synapse (Herms et al., 1999). One of the early signs of TSEs is the loss of synapses and deposition of PrP^{Sc} plaques in the synaptic terminals, mostly preceding spongiosis, neuronal loss and gliosis, thus inferring that synaptic dysfunction may be a consequence of corrupting PrP's physiological function.

1.5.2 PrP engaged in neuroprotection

It has been reported that cultured neurons derived from PrP KO mice are less viable than wild-type neurons (Kuwahara et al., 1999), and this is consistent with observations made in our lab (O'Connor et al., unpublished data). In addition, there have been several lines of evidence suggesting a cytoprotective and/or anti-apoptotic role of the physiological PrP. First it was shown that PrP antagonizes the effects of overexpression of the apoptotic factor Bax in primary neurons (Bounhar et al., 2003). In addition, a study from the same research group showed *in vitro* that PrP specifically inhibits the activation of Bax, although it doesn't co-localize with Bax during apoptosis or under normal conditions in primary neurons (Roucou et al., 2005). The absent co-localization potentially suggests a membrane-association of PrP in executing or initiating its anti-apoptotic function, at least in cultured neurons.

Consistent with this, several *in vivo* studies have demonstrated that brains from PrP KO mice show larger lesions than the wildtype brains after ischaemic induction (Hoshino et al., 2003; McLennan et al., 2005; Weise et al., 2005). McLennan et al., (2005) also reported an increased deposition of PrP^{C} in the penumbra zone of human autopsies of adult patients (whose recent cerebral insults had directly contributed to their death) and the hypoxic regions of patients with perinatal hypoxic-ischaemic injuries. Conversely, Shyu et al., (2005) report an increased immunostaining of PrP in the penumbra of rat brains. Moreover, the overexpression of PrPC in the rats undergoing ischaemia reduced the infarct size compared to the untreated controls. So far, there are no studies showing a similar rescue phenotype in PrPKO mice after ischemic injury while overexpressing PrP. In concert with existing evidence of prion protein involvement in the neuronal excitatory system, Rangel et al., (2007) show that PrP KO mice display significantly increased susceptibility to kainate-induced seizures

and increased neurodegeneration histopathologically compared to the wildtype C57BL6J strain.

The aforementioned *in vitro* and *in vivo* data strongly indicate that PrP might be regulated by neuronal stress and might be physiologically involved in maintaining neuronal membrane homeostasis. Therefore, PrP may be part of a mechanism that signals aberrant occurrences at the neuronal cell membrane intracellularly, prior to the formation of reactive oxygen species. Brown et al., (2002) examined a variety of agents that generate reactive oxygen species or led to their generation. They could show that hydrogen peroxide and NOC18, a generator of nitric oxide were not more toxic to the cultured cerebellar neurons from PrP KO mice than to the wildtype cells, unlike glutamate, which was slightly more toxic in the knockout neurons. Consistent with that, the incubation of cerebellar neurons in high extracellular potassium (a well-established apoptosis inducer in cultured cerebellar neurons) caused a significantly greater reduction of knockout neuron viability compared to wild-type.

Clearly, these data indicate that "membrane events" precede the activation of toxic pathways through oxidative stress. On the other hand, *in vivo* data from our lab (Hutter et al., 2003) and several other *in vitro* studies have basically ruled out any superoxide dismutase (SOD)-like activity of PrP, which again would support the hypothesis of membrane interactions in initiating toxic events.

1.6 Combined efforts to elucidate prion-mediated neurotoxicity

In summary, a large body of evidence unambiguously suggests that cellular prion protein exerts a neuroprotective function. This most likely occurs via acting as a cell surface signal transducer or mediator, quite common for many GPI-anchored proteins. Prion-driven toxicity, toxicities arising from deletion mutations, transmembrane conformations, as well as antibody toxicity, unequivocally require membrane association of PrP in order to unfold. It becomes clear that understanding the molecular mechanisms of prion diseases and therefore therapeutically targeting them is far from conceivable without knowing the physiological processes that cellular PrP impacts.

The Aguzzi lab has devoted a great deal of effort to illuminating the mechanisms of PrP-mediated pathologies and thus has shifted their focus from neuroinvasion to neurotoxicity. For this reason the lab has established a novel *ex vivo* assay using mouse organotypic cerebellar slice cultures (POSCA; Falsig and Aguzzi, 2008) grossly recapitulating the CNS prion disease hallmarks i.e. prion replication coupled to progressive neurodegeneration (5-8 weeks) alongside inflammation and gliosis (Falsig et al., submitted). To address the prion protein's physiology, the lab has primarily employed transgenesis, generating deletion mutants in search of effector domains of PrP.

Recently and most importantly, the lab has been keen on elucidating the upstream events in prion-mediated neurotoxicity. The lab has pursued a biochemical path to address this question, aiming to isolate a native PrP complex *in vivo* so that one can identify direct interacting factors and thus narrow down the pathways in which PrP might be involved.

Aim of the study

So far, the lab has been able to isolate a native immunoprecipitation (IP) complex of over 700kD size (Figure 7A), with was confirmed by size-exclusion chromatography. Also, mass spectrometry analysis of the isolated complex (from a bovine brain) was performed, a biased approach to identify PrP's interacting factors present in the IP complex. The chief aim of this study was to characterize this complex. More precisely, the assignment consisted of verifying the presence of proteins suggested by the mass spectrometry analysis. Furthermore, the presence of other factors reported in the literature (based mainly on *in vitro* findings) was also tested. Finally, the presences of proteins that have been reported to be involved in cellular processes, which seem to be hampered in PrP knockout mice, were also tested.

2. Results

Immunoprecipitation (IP) was performed as described below in *Materials and Methods*. Subsequently, the IP complex was denaturated and the candidate proteins were tested via immunoblotting.

2.1 PrP and the neuronal excitatory system. Interaction with receptors and channels of the glutamatergic system

The majority of the candidates that mass spectrometry suggested were proteins involved in regulation of neuronal excitability, clearly suggesting that PrP could also engage in the complex mechanisms assigned to regulate neuronal membrane depolarization. These mechanisms are also believed to be implicated in higher cognitive functions. In the *Introduction* already mentioned, several independent studies have provided evidence that PrP too might engage in these complex mechanisms. Electrophysiological measurements have shown a decreased neuronal inhibition and impaired long-term potentiation (Collinge et al., 1994; see Discussion). Later, in support of this finding, Khosravani and colleagues (2008) attribute the increased neuronal excitability to a deficient inhibition of N-Methyl-D-aspartate (NMDA) receptors, a prominent family of excitatory receptors, allowing a Ca^{2+} influx. The authors also show biochemically that PrP interacts with NR2D, NMDA-subtype 2D, and suggest that it directly inhibits it. Finally, concerning prion-mediated neurotoxicity, the data from our lab, employing mouse organotypic cerebellar slice cultures, point to a Ca^{2+} dependent calpain activation as key conductor of pathological events (Sonati et al., submitted).

Taken together, the mass spectrometry data, in concert with literature reports of PrP's involvement in regulating calcium mediated excitatory events as well as calcium's pathologic implications strongly pinpointed to a potential interaction of PrP with some of the channels or receptors carrying out the glutamatergic excitatory processes. To this end, I decided to first test the presence of the following channels in the PrP immunoprecipitate; the voltage-

gated sodium channel, α subunit (VGSC), SK2.2 and TREK-1 channels. However, as Figure 1 illustrates, despite the numerous phenotypical indications PrP is not biochemically associated with any of these membrane channels. At least the interaction might not be as strong as to endure the harshness of an IP, or maybe not present in amounts detectable by immunoblotting.

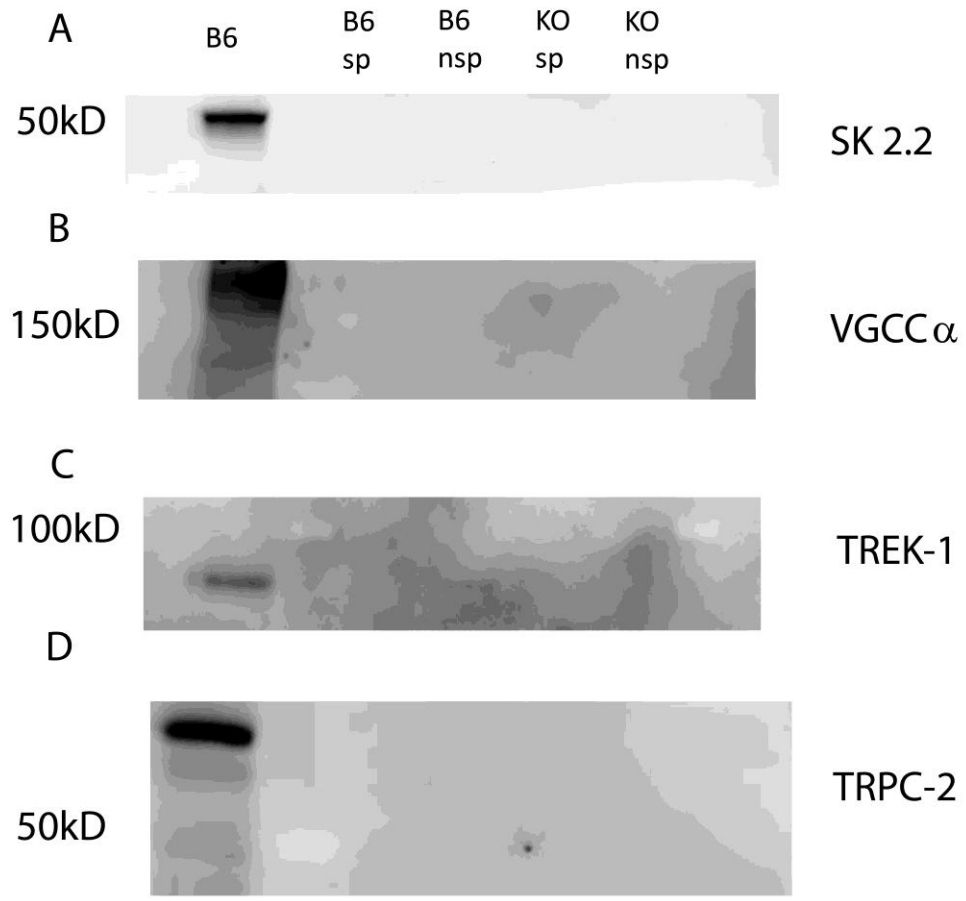


Figure 1a. PrP does not interact with cationic membrane channels. Immunoprecipitation (IP) complex was denatured and run on a SDS-PAGE gel and stained with specific antibodies against candidate proteins. IPs were performed on B6 and $Prnp^{0/0}$ as a negative control. Scrambled elution peptide (NSP) was used to test the specificity of the pulldowns. The **sp** lanes indicate the IPs eluted with the specific peptides spanning the octapeptide region i.e. the POM2 binding site. The brain homogenate lane (on the left **B6**) serves as a positive control showing antibody's target recognition signal. The blots shown here (**A-D**) prove the absence of these membrane channels in the PrP IP complex.

Of the many phenotypes reported about the PrP knockout mice, the one that stands out concerning behavioural alterations was its putative role in olfactory discrimination (Le Pichon et al., 2009). In conjunction with this observation, PrP is known to be highly expressed in bulbus olfactorius, more precisely in the axons of peripheral sensory neurons and mitral cells. An excessive sniffing of PrP overexpressing (Tga20) mice has also been observed in our lab (Petra Schwarz, personal communication).

Consistent with this, mass spectrometry analyses suggested TRPC-2 to be part of the HMW complex. Transient receptor potential (TRP) channels are a superfamily of cationic channels, traditionally consisting of six transmembrane domains. Specifically, TRPC-2 has been reported to be expressed in the vomero-nasal organ (Kim et al., 2010) and therefore important for proper olfactory signal transduction. It becomes increasingly evident that TRP channels play an important role in neuronal ischaemic death, being directly involved in the delayed calcium deregulation following excitotoxic events (reviewed in Tymianski 2010).

Taken together, the *in vivo* indications concerning the olfactory impairments of PrP KO mice and given that Ca^{2+} dependent calpain activation has been identified as the initiator of prion toxicity, which could engage TRP channels, we decided to test for the presence of TRPC-2 in the IP complex. However as **Figure 1C** clearly demonstrates, this channel is likewise not detectable via immunoblotting.

Unlike the membrane channels, staining for 2D subunit of the NMDA receptors did yield a band. However the same signal appeared in the scrambled peptide lane and the KO lanes of the SDS-PAGE gel (Figure 2, upper panel), suggesting that this might not represent a specific interaction of these two proteins. The brain homogenate lane showed that there are two extra bands running at slightly under 150 and 100 kD, respectively. This indicated that this antibody might recognize unspecific targets too. Nevertheless, I tested whether this signal also appears at the molecular weight of the IP complex on a native gel. And indeed, the native gel showed the same band (roughly 800kD) when stained with the NR2D antibody (Figure 2, middle panel, NR2D blot). However, this band appeared also on the non-specific lane of B6, which in this experiment is not so surprising since the anti-PrP staining was also

positive in the NSP lane (middle panel, POM1 blot) but is still an indication of a non-specific signal.

On the other hand, the specific peptide KO lane showed also a band, which runs lower than the B6 bands. This together with the triple bands in the brain homogenate lanes pointed to an unspecific recognition of IgGs by this mouse antibody. To test this, I first ran control blots using reducing conditions in the sample buffer, and this indeed shifted the triple bands, showing that the mouse antibody does recognize mouse IgGs (data not shown). To circumvent the problem of non-specifically recognizing mouse IgGs, I assessed the presene of NR2D in the complex using rabbit NR2D antibodies. This unambiguously showed that the previous bands were not specific NR2D signals and most likely mere IgGs since the rabbit antibody did not yield any band on the SDS-PAGE gel (Figure 2, lower panel).

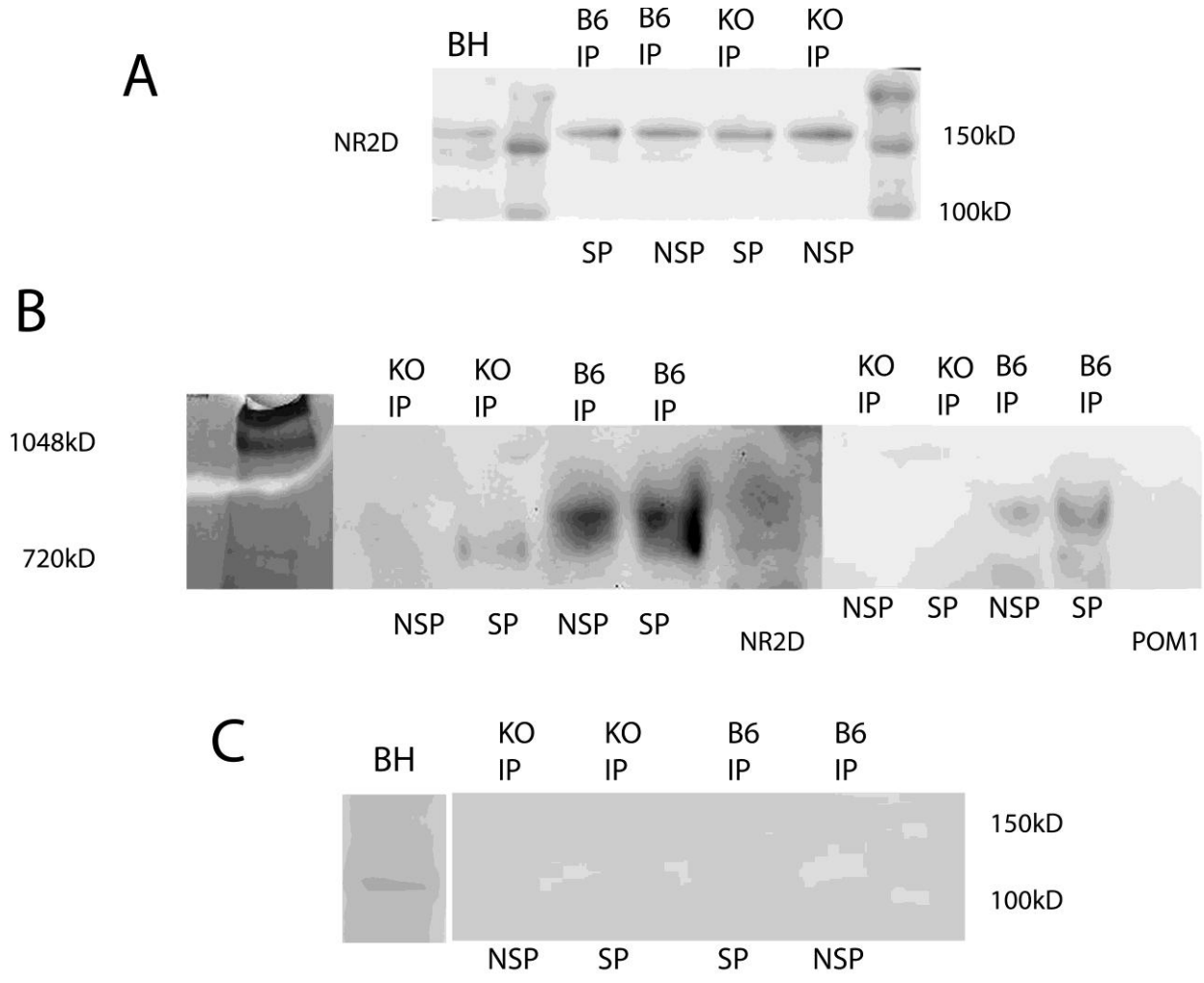


Figure2. Non-specific NR2D (subtype 2D of NMDA receptors) subunit in the IP complex. The upper panel shows an SDS-PAGE immunoblot of the IP complex (as described in Figure 1) stained with a mouse anti-NR2D antibody. In the middle panel immunoblots of a native PAGE gel are shown stained with the same mouse ant-NR2D antibody which was stripped and re-probed with anti-PrP (POM1) antibody. The corresponding native gel was stained with Coomassie and the protein marker lane was scanned. Lower panel shows a blank IP SDS-PAGE blot following a rabbit anti-NR2D staining, despite a positive signal on the brain homogenate lane.

2.2 PrP and cell-cell adhesion. Interaction with NCAM and Vitronectin

Several lines of evidence have pointed to an involvement of PrP in cell adhesion physiology (see Discussion). Chemical crosslinking (1% formaldehyde) in N2a cells has identified Neural Cell Adhesion Molecule (NCAM) as interacting factor of PrP (Schmitt-Ulms et al., 2001). This was further confirmed by an independent research group showing first *in vitro* that PrP co-localizes with NCAM in lipid rafts when neurons were extracted with cold Triton X-100 and second, PrP co-immunoprecipitates with NCAM in a pull-down with specific NCAM antibodies performed *in vivo* (Santuccione et al., 2005).

Likewise, Hajj and colleagues employed overlay assays i.e. coating nitrocellulose membranes with recombinant candidate proteins and tested their binding affinity to recombinant PrP with vitronectin showing the highest affinity, which was also dose-dependent and saturable (Hajj et al., 2007).

Based on this, I sought to examine the association of PrP with NCAM using our own experimental conditions. Staining of POM2 immunoprecipitates from B6 and KO brains with a mouse anti-NCAM antibody gave basically an identical picture as the NR2D staining on the SDS-PAGE gel (**Figure 3a, panel A**), albeit with a slight difference on the native PAGE gel, with the band on the KO lane running at the same height as the B6 lanes, strongly pointing to a non-specific signal (s. Discussion). On the other hand, vitronectin staining yielded bands only

on the unspecific lanes of both B6 and KO SDS-PAGE blots and was completely absent on the native PAGE, again, most likely pointing to non-specific pull down of vitronectin.

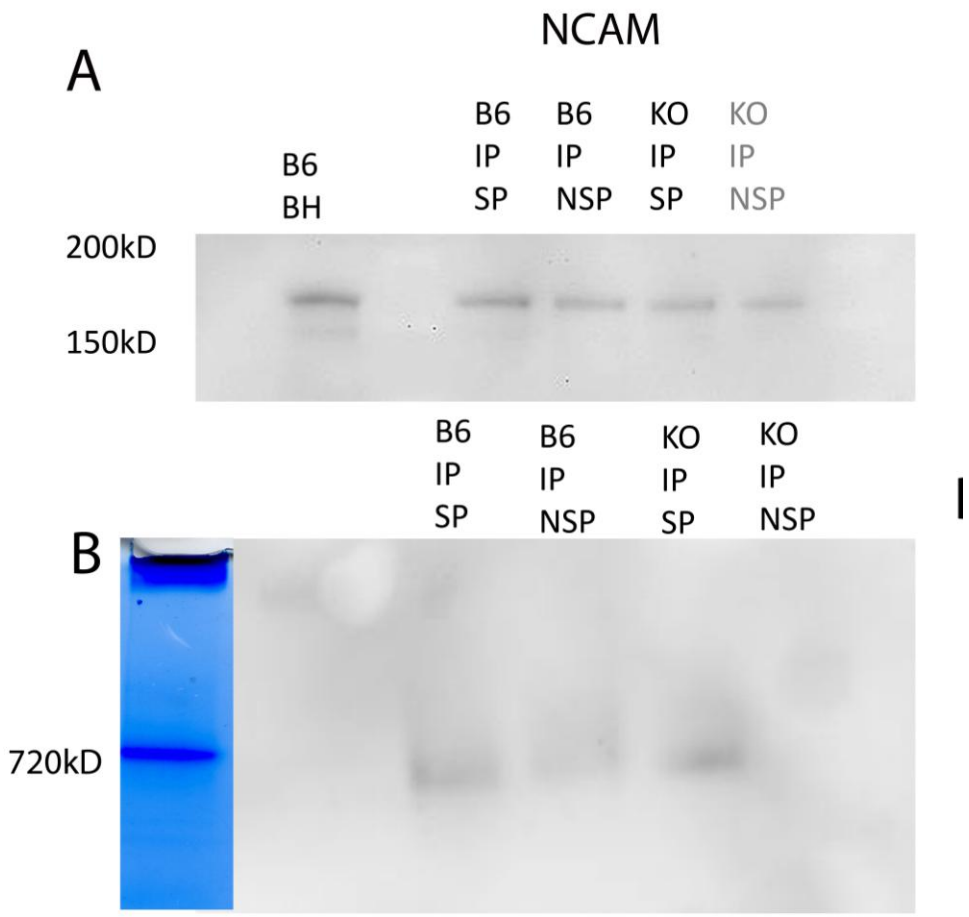


Figure3a. Staining for NCAM in the HMW complex. Immunoblotting with an NCAM antibody revealed bands in all four lanes on the SDS-PAGE gel **(A);** and also a band at roughly 720 kD in the KO lane eluted with specific antibodies alongside the two B6 lanes in a native-PAGE gel **(B).**

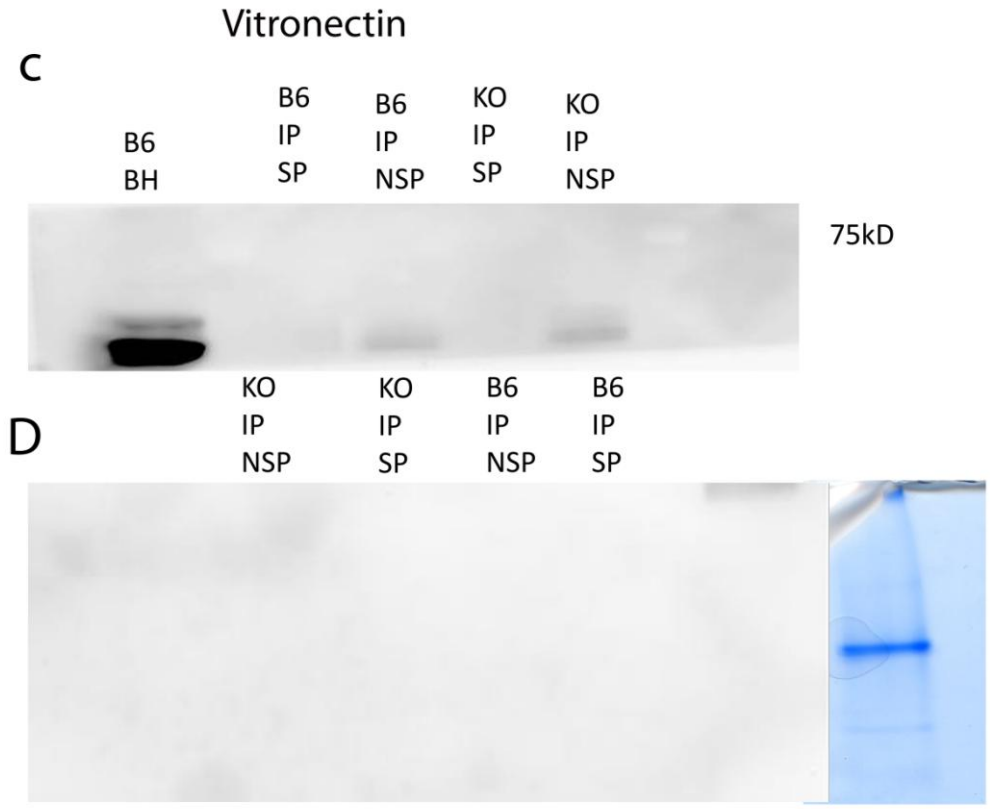


Figure 3b. Staining for Vitronectin in the HMW complex. C shows bands in IPs eluted with non-specific (NSP) peptides on the SDS-PAGE that are absent in the IPs eluted with and specific peptides (SP) on the same gel and absent in all four lanes of the native gel in **D.** The Coomassie band indicates the traditional molecular weight of 720kD.

2.3 PrP and caveolin pathway. Fyn, Shc interaction

Given that PrP is a cell surface protein with several lines of evidence suggesting its involvement in signal transduction, one approach to illuminate how PrP exerts its physiological function was to target it with specific antibodies. Mouillet-Richard and colleagues (2000) treat the neuroectodermal progenitor cell line 1C11 with antibodies recognizing the 142-160 epitope of PrP and observe an activation of fyn kinase, a Src tyrosine kinase, often found in lipid rafts. The authors further report that this activation is mediated by caveolin-1 and caveolin-2 since the two proteins co-immunoprecipitated with PrP (Mouillet-Richard et al., 2000).

This finding indicated that PrP might be involved in the caveolin-mediated endocytotic pathway, which makes an interaction with Fyn and Shc likely. The mass spec analysis already suggested that Shc (Src homology (SH2) adapter protein) is found in the complex. For this reason, I tested their presence in the IP complex, but immunoblotting couldn't confirm it (Figure 4).

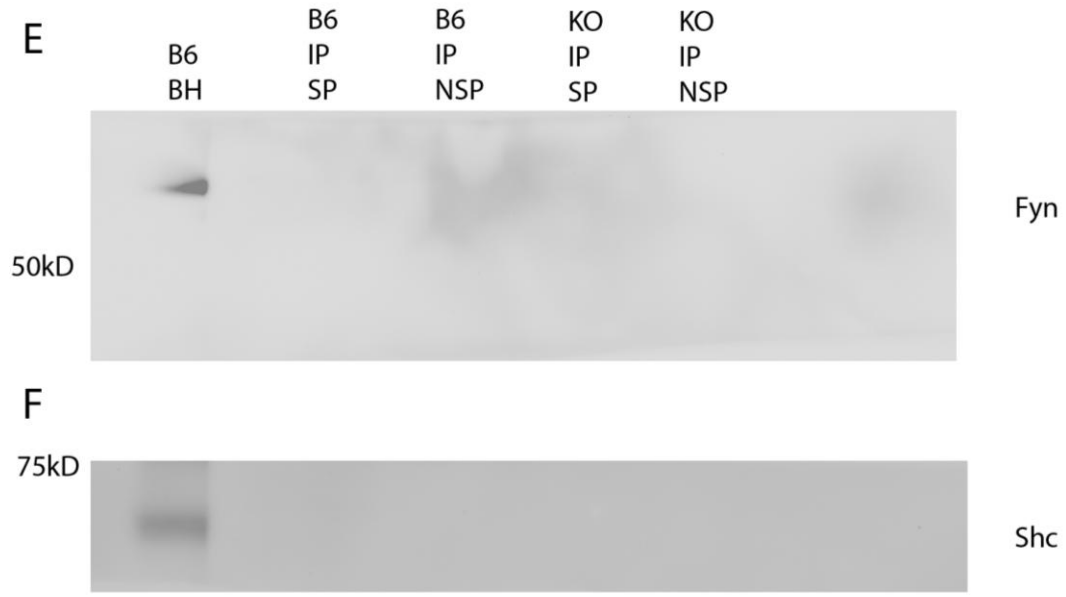


Figure 4. Absence of intracellular signalling molecules in the IP complex. Fyn and Shc immunoblots of POM2 immunoprecipitates from wild-type (B6) and PrP KO brains separated via SDS-PAGE.

Finally I also looked for the presence of the myelination proteins CNPase and Neurofascin but again these resulted in blank blots (data not shown). Myelination proteins are usually non-specifically found following membrane fractionation of the brain (personal communication with Dr. Michael Kiebler, Brain Research Institute, Vienna), so this might also explain their presence in the MS analysis.

2.4 PrP the only protein in the HMW complex?

The inability to verify the presence of other proteins/peptides in the native pull-down raised the suspicion that the complex might be devoid of any other proteins or peptides.

In order to rule out the presence of other proteins in the IP pull I performed a silver staining under denaturing conditions. If the complex were devoid of other proteins than the silver staining in the SDS-PAGE gel should show a single band corresponding to the PrP. And indeed it does, as Figure 5 clearly demonstrates. Also, suggesting that it is the uncleaved, full-length PrP form that predominates in the complex.

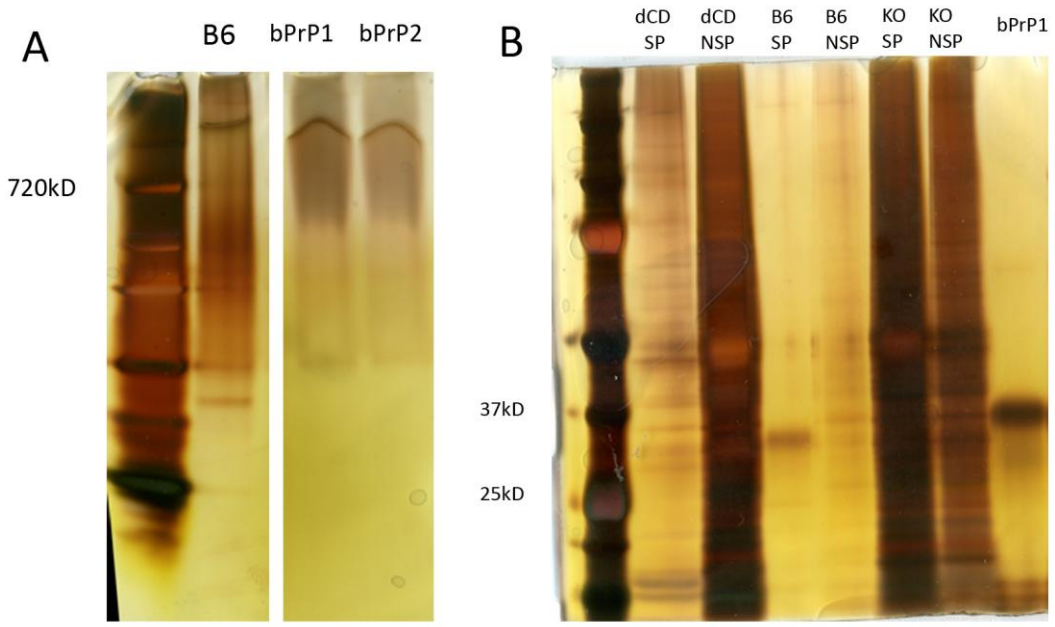


Figure 5. Silver staining points to PrP as the sole protein present in the HMW complex. Silver staining performed on a Tris-Glycine native gel **(A)** shows that the B6 IP band runs similarly as the detergent-free purified and monomeric bovine PrP (bPrP1 and bPrP2). SDS-PAGE silver staining of the denatured PrP complex **(B)** suggests that it consists of PrP only (lane B6/SP).

2.5 PrP organized in multimeric clusters

Next I wanted to see whether this high molecular weight (HMW) complex, potentially consisting of over twelve PrP molecules is not only an aftermath of the IP or it is present physiologically as such. I addressed this by isolating membrane fractions from Tga20 brains and ran them in native gel and stained them with POM1. If there is a multimeric organization consisting uniquely of PrP in the cell membrane, the membrane fractions in the native gel should also yield a HMW band when stained with POM1, running approximately at the same

height as the IP complex. In order to make sure that the resulting gel band isn't merely an artefact originating from the non-ionic detergent, I also performed a dodecyl-maltoside (DDM)-free resuspension of the membranes (see Methods and Materials). The membrane fractions (MF) in the Tris/Glycine did run almost identically as the IP, and there wasn't any visible difference between the detergent and detergent-free MFs; clearly suggesting that this isn't at least a DDM resuspension artefact.

Yet, an artefact arising from the gel running itself wasn't ruled out this way. The thick bands already suggested that there might be electrophoretic obstacles resulting from the aggregate-generating DDM in the sample buffer (further elaborated in the Discussion) which could easily form big protein aggregates impermeable for the acryl-amid pores (Schägger, 2003) and therefore remain perplexed at the top of the gel. On the other hand, it could also be the pH value of the running buffer that initiates the aggregation. The running buffer of Tris/Glycine has a pH of 8.5 and the isoelectric point of PrP is 9.5 suggesting therefore that at this pH the PrP is relatively uncharged. This could facilitate a hydrophobic aggregation and therefore drastically impair the electrophoretic run, hence explaining the poor migration of the MFs in this native gel.

I addressed these questions by first making sure the thick bands that could contain different HMW moieties undergo a proper separation. Doubling the running time didn't dislocate the bands any further (data not shown) potentiating again that their migration is most likely impaired. The lack of DDM in the sample buffer gave bizarre signals not reminiscent protein bands (blots not shown) suggesting that the addition of this non-ionic detergent does favour the aggregation of PrP and the bands from Figure 6a are probably huge aggregates. To rule out the influence of pH in the migration of membrane fractions I ran them in native PAGE gel, displaying a more physiological pH 7.5. POM1 staining after the transfer, unlike the Tris/Glycine, showed a large smear throughout the gel, again suggesting that the previous relatively well confined bands result from pH-induced protein aggregation. Finally, the Coomassie staining of the native gels clearly reveals very distinctive electrophoretic migration patterns of membrane proteins under different pH values, with more confined bands in basic buffers, most likely due to aggregation of proteins with basic isoelectric points as Figure 6A illustrates.

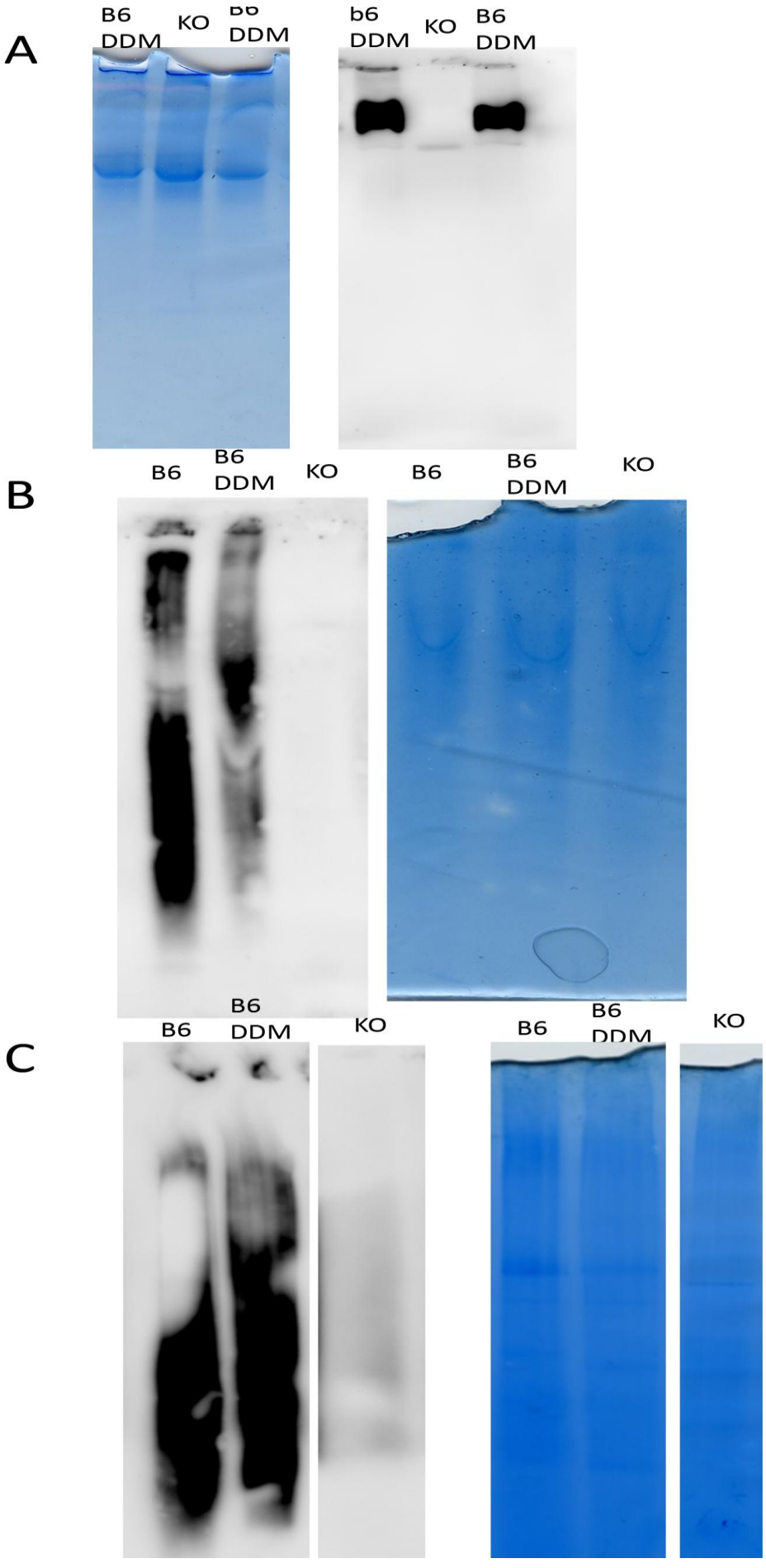
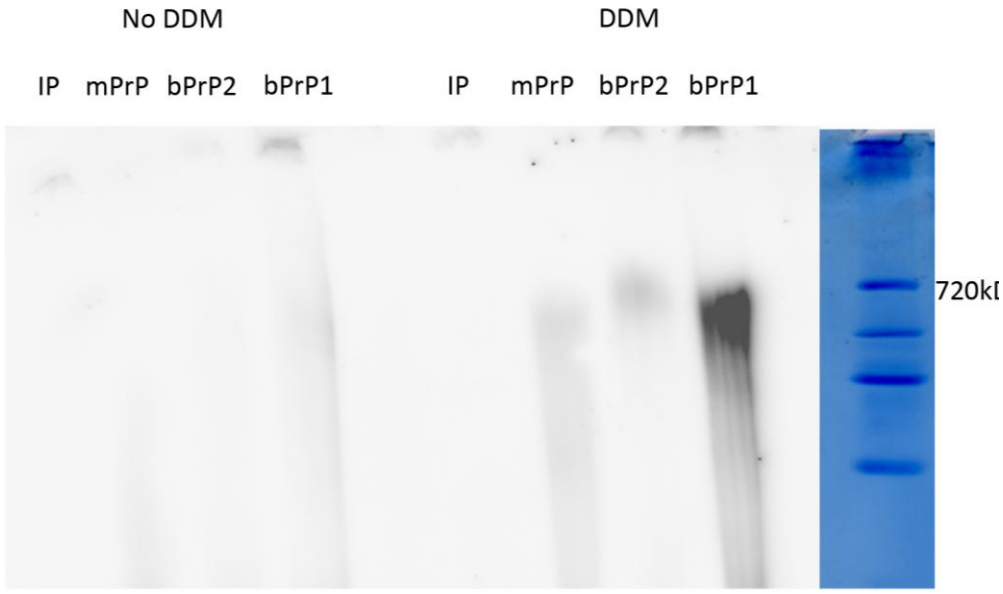
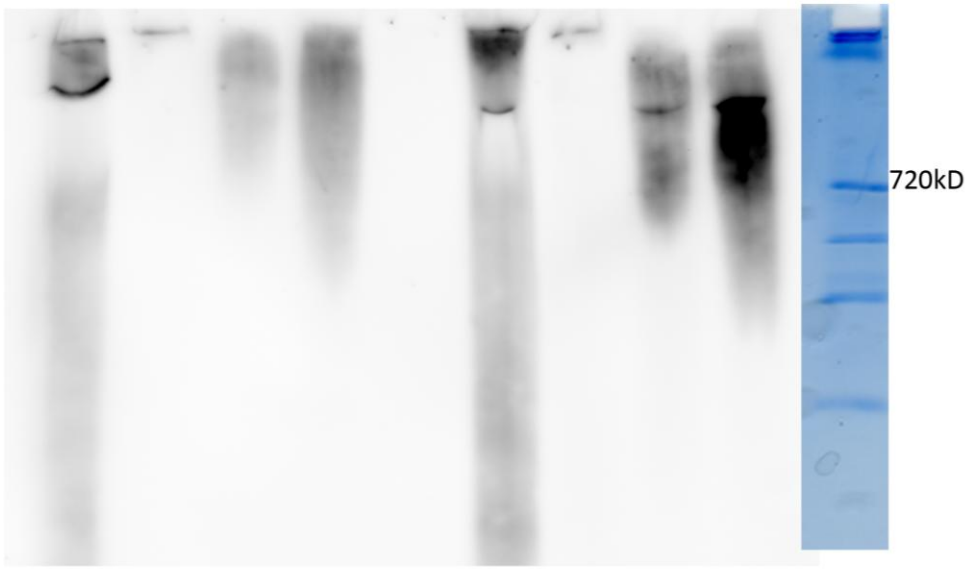

A
B6
DDM
KO
B6
DDM
b6
DDM
KO
B6
DDM
B
B6
B6
DDM
KO
B6
B6
DDM
KO
C
B6
B6
DDM
KO
B6
B6
DDM
KO

Figure 6a. Basic pH facilitates aggregation of PrP and other membrane proteins. Brain membrane fractions run on Tris-Glycine native gel **(A);** colourless native (CN) PAGE **(B)** and Blue Native (BN) PAGE **(C)**. Only the Tris-Glycine native gel (pH 8.5) yields well circumscribed PrP bands on the immunoblot (**A, right**), as well as nicely defined bands on the Coomassie staining of the corresponding native gel **(A, left)**. CN and BN give PrP smears all over the immunoblot (**B** and **C**, left) regardless of the addition of DDM, which is also reflected on the Coomassie staining of the corresponding gels (**B** and **C**, right).

D1


No DDM
DDM
IP
mPrP
bPrP2
bPrP1
IP
mPrP
bPrP2
bPrP1
720kD

D2


720kD

E

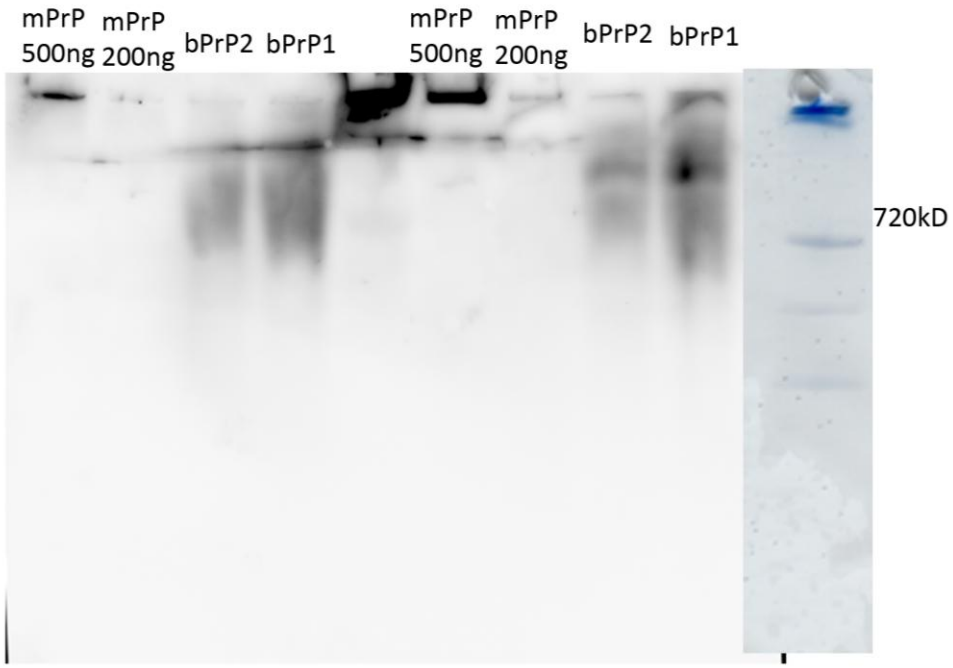

mPrP 500ng
mPrP 200ng
bPrP2
bPrP1
mPrP 500ng
mPrP 200ng
bPrP2
bPrP1
720kD

Figure 6b. The PrP complex band does not arise as a consequence of impaired electrophoretic migration and it likely consists of multiple PrP molecules. Blue native gel **(D1)** reveals that DDM defies the Coomassie G250 negative charge and still impairs the migration of monomeric PrP (bovine **(bPrP)** or recombinant mouse **(mPrP)**), yet it doesn't prevent the disassembly of the IP complex. **D2** provides the evidence that the IP complex is not a consequence of DDM aggregation since it runs at the same height in its absence too, whilst the bovine monomeric PrP disassembles. **E** illustrates that the basic pH of the Tris-Glycine gel can induce the aggregation of the monomeric detergent-free purified bovine PrP.

2.6 HMW band a consequence of impaired electrophoretic migration?

The fact that the immunoprecipitation wasn't able to pull down other proteins except for PrP-, raised the question of whether this procedure pulls down a monomeric PrP that potentially aggregates in native gel or multiple PrP molecules are pulled down with pre-existing distinctive multimeric organization. To test this, I used recombinant mouse PrP and phospholipase C (PI-PLC) cleaved and detergent-free purified bovine PrP as controls. The PI-PLC cleaved bPrP is, unlike the recombinant PrP, post-translationally glycosylated (Hornemann et al., 2004) and both of them exist as monomers. If the IP pulls down PrP present in a multimeric state, this should behave differently than the monomeric controls in the native gel.

The Tris/Glycine native gel surprisingly revealed that the enriched bovine PrP runs almost at the same height as the IP, whereas recombinant mPrP seems to largely stick in the gel slots. This suggests that we might pull down single PrP molecules which aggregate in the course of the native electrophoretic migration and form the HMW band. This raised again the question of whether PrP, a protein with a basic isoelectric point is able to migrate to the anode at all? The influence of sample buffer detergent on the gel running still remained unclear. I addressed these questions by running blue-native PAGE gels of mouse recombinant and the enriched bovine PrP. The negatively charged Coomassie G-250 should facilitate the PrP run towards the anode. Previous data that had shown that the IP complex disassembles in the BN-PAGE additionally inspired me to figure out whether PrP is able to migrate in a native gel.

The gel revealed that when no DDM is added, both the recombinant and the enriched bPrP reach the bottom of the native gel, whereas detergent addition on the other hand results in the appearance of HMW bands, reminiscent of the IP complex band. This virtually identified DDM as a PrP aggregate inducer and raised again the question of whether the IP complex band simply arises from this as well? In addition, it had previously been shown that addition of 1.5% NP40 instead of 0.5% DDM in the sample buffer didn't yield the 720kD band, clearly pointing to the detergent DDM as an artefact generator. Also, I sought to test the impact of the pH value on the electrophoretic running of the complex comparing Tris/Glycine and native PAGE gels.

To address the impact of the sample buffer detergent, I ran colourless native (CN)-PAGE of the IP and the recombinant together with bPrP controls with and without DDM. If the IP band is just a detergent artefact this band shouldn't be present when no detergent is added. Unlike the control bands that virtually disappeared, the IP complex band didn't remain the same. In marked contrast to this, Tris/Glycine showed no difference between the two samples, clearing showing that the alkaline pH value of the running buffer itself can impair the electrophoretic running, probably via hydrophobic aggregation.

This finally rules out the HMW band containing solely PrP, of being merely a gel running artefact. The size exclusion chromatography had already suggested that the 700 kD plus band indeed corresponds to the size of the isolated complex, yet running control experiments with monomeric PrP samples (pro- and eukaryotic) finally demonstrating that the IP complex does behave differently than then monomeric controls, thus ruling out post-extraction aggregation artefacts and-, reinforced the hypothesis that PrP might indeed be physiologically present in a multimeric state.

However this still doesn't rule out homogenization artefacts i.e. artefacts arising from the addition of the ionic CHAPS and non-ionic NP40 detergents bearing in mind the fact that PrP is a GPI-anchored protein, the family of proteins that tend to display detergent resistance. This implies that under certain circumstances the detergent addition gives rise to interactions not necessarily present under native conditions.

2.7 The same complex can be isolated in vitro

Performing the same immunoprecipitation (IP) protocol on the PrP expressing HPL cell line yielded a native gel band strongly resembling the band of the *in vivo* isolated complex (Figure 7A). This again would rule out the fact that the HMW band could be specifically a brain homogenization phenomenon and supports the idea that PrP universally exists as an oligomer.

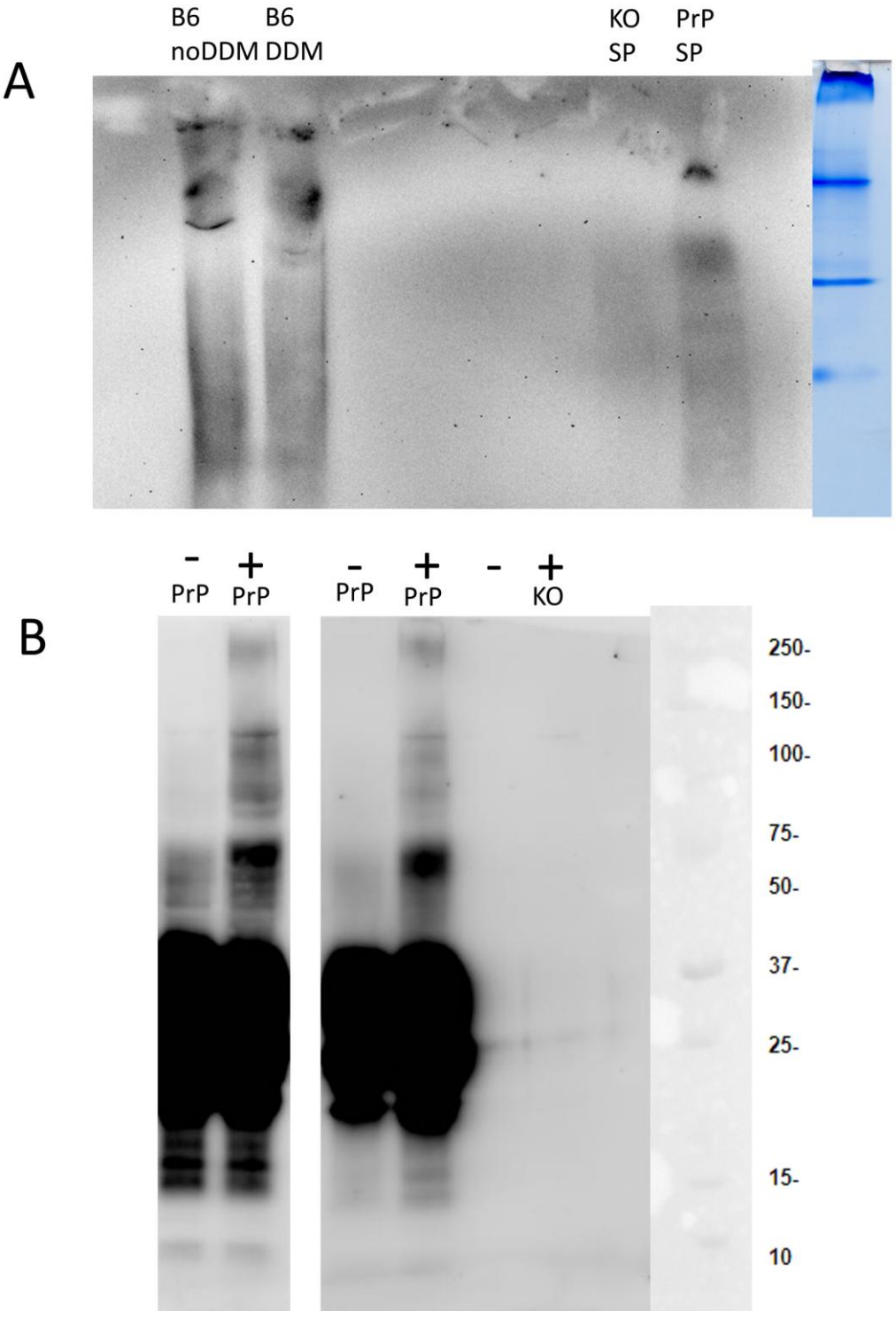


Figure 7. PrP is organized in oligomeric cell surface clusters. A IP performed on PrP expressing HPL cells yields a roughly 720 kD band (lane PrP/SP) when specifically eluted, reminiscent of the *in vivo* bands on native PAGE. **B** represents results from two different experiments. Chemical crosslinking of these cells with membrane impermeable crosslinking agent BS^3 gives rise to band shifts which would correspond to dimeric, tetrameric and multimeric PrP (50-75kD, around 100kD, and roughly 250kD, respectively.

2.8 Chemical crosslinking indicates pre-existence of PrP clusters at the cell surface

The high molecular weight band in the native gel (which size was later confirmed by size exclusion chromatography), vacant of other proteins and behaving differently than monomeric, anchorless bovine prion protein, could be interpreted as a multimeric organization of PrP at the cell surface. To test the hypothesis that PrP molecules interact with each other, or at least are organized in close vicinity of another at the cell surface, I sought to adopt an *in vitro* system where I could test this interaction or the clustering prior to the use of detergents. Hence, I reasoned to apply chemical crosslinking to test the homotypic association of PrP molecules.

On the other hand, as noted above, the IPs from the anchorless deletion mutants showed that the putative homotypic interaction was GPI-anchor dependent. In conjuction with this observation, the evidence presented in the literature concerning other GPI-anchored proteins also demonstrated that they exist as clusters at the cell membrane in a GPI-dependent manner (s. Discussion). Therefore I decided to use a membrane impermeable chemical crosslinker i.e. bis(sulphosuccidimidyl)suberate (BS^3), a membrane impermeable amine to amine crosslinker, to directly address the membrane associated clustering of PrP *in vitro*. Given that PrP molecules are present as clusters at the cell surface-; this should be reflected as molecular weight shifts in SDS-PAGE gels following chemical crosslinking.

As Figure 7B illustrates, indeed the crosslinking led to formation of band shifts that would correspond to dimeric, tetrameric PrP and another band over 200 kD which could represent a higher multimeric organization. Nonetheless, this is not the ultimate evidence for the existence of PrP-only clusters, since I cannot rule out the existence of other proteins of the same size as PrP in its vicinity. In the Discussion part, I present a series of experiments that would give more insights as to whether the band shifts arise from PrP molecules involved in homotypic clustering.

3. Discussion

3.1 Calcium influx pathologies

3.1.1 Prion protein implications in neuronal excitability and excitotoxicity

It still remains enigmatic why prions damage preferentially the central nervous system. Prions are also able to replicate in follicular dendritic cell (FDCs), spleen, muscle (Aguzzi et al., 2007) but these tissues remain unaffected by prion replication and accumulation, unlike the CNS which undergoes severe spongiosis, neuronal death, and astrogliosis. The aetiology of these diseases remains poorly characterized.

Strikingly, the deletion mutants encompassing the central domain of PrP display ultrastructural morphologies typical for non-apoptotic neuronal cell death induced by excitotoxic Ca^{2+} influx (Christensen et al., 2010). Solomon et al., (2010) present electrophysiological data showing that these PrP mutations induce spontaneous cationic currents and the authors speculate that this could occur via formation of PrP cationic channels or through PrP triggered membrane pore formation. Their conclusion is that neurons don't undergo autophagy when undergoing cell death, despite membrane leakage. However, this is not consistent with the *in vivo* evidence from *Lurcher* mice, where a naturally occurring mutation transforms the GRID2 receptor, a glutamatergic receptor, into a constitutively open channel. These mice do undergo a massive neurodegeneration of Purkinje neurons forming numerous autophagic vacuoles independent of depolarization (Selimi et al., 2003).

Consistent with the Lurcher finding and unlike the *in vitro* data from Harris lab, electron microscopic analysis from prion-infected slice cultures reveals autophagy hallmarks, as well (Falsig et al., submitted). The neurotoxicity in this *ex vivo* model is mediated via Ca^{2+} dependent calpain activation and subsequent NOX- dependent ROS generation (Falsig et al., submitted). It is still not clear from where the excessive Ca^{2+} originates, as the prion-infected slices could not be rescued by no NMDAR, AMPAR and some Ca^{2+} channel inhibitors (Falsig

et al., submitted), suggesting that there is NMDAR-mediated Ca^{2+} influx, leaving room for speculation that there still might be a Ca2+ influx from the ECM, or through alternative, unknown pathways. In a nutshell, these data indicate that there might be distinct toxic pathways that cause neuronal damage in prion protein-induced neurotoxicity-, other than glutamatergic excitotoxicity. Given that spongiotic vacuoles contain membrane fragments, unorthodox pore formation could be a conceivable scenario.

On the other hand, a wealth of evidence has been presented, suggesting a physiological engagement of PrP^{C} in regulating neuronal membrane receptors and thus regulating neuronal membrane excitability. It was surprising to link PrP to the regulation of glutamatergic receptors given that several lines of biochemical evidence suggest that PrP usually localizes to the presynapse (Herms et al., 1999; Moya et al., 2000). This would point to a novel mechanism of presynaptic regulation of postsynaptic proteins, clearly a very tantalizing scenario.

The first evidence of PrP's involvement in membrane excitability came from the electrophysiological recordings Collinge and colleagues (1994) conducted in hippocampal slices from PrP knockout mice, which displayed reduced neuronal inhibition and impaired long-term potentiation (LTP) regardless of the strain background. GABA receptor-mediated inhibitory postsynaptic potentials were weaker and inhibitory postsynaptic currents showed a slower rising phase compared to wildtype controls, evoking additional action potentials upon afferent neurons in the CA1 pyramidal cell layer. The authors attribute this to augmented diffusion of the inhibitory transmitter GABA towards the extrasynatic receptors and therefore allowing a hyperactivity of NMDA receptors. Hence, PrP seemed to be directly involved in regulating the neuronal excitability, potentially through a physical interaction with the known receptors or channels responsible for that.

3.1.2 Involvement of NMDA receptors

Prolonged decay times of NMDAR-dependent miniature postsynaptic potentials (mEPSCs) in the cultured knockout hippocampal neurons further reinforced the hypothesis that PrP might be involved in the regulation of NMDA receptor activity (Khosravani et al., 2008). In

order to sort out which NMDARs might be modulated by PrP the authors kept the neurons under voltage clamp, blocked them with TTX, picrotoxin, CNQX, glycine and finally activated them by administering NMDA. The recorded currents showed a slowed deactivation in the knockouts compared to wildtype neurons, indicating that PrP deficiency ignites an activation of NR2D-subtype containing NMDARs, while WT neurons showed current amplitude patterns mediated by NR2B-subtypes. The authors further identify PrP as a negative regulator via a direct protein-protein interaction.

Khosravani and colleagues demonstrated that siRNA silencing of PrP reproduces the KO phenotype and conversely, overexpression of PrP in neurons via transfection rescued the knockout phenotype, virtually excluding the possibility of a genetic artifact. However, they fail to provide data showing specific inhibition of NR2D-subtype receptors that would explain the slowed deactivation kinetics, or a direct involvement of PrP in NR2D-specific modulation. A phenotype rescue by specifically inhibiting the NR2D receptors would have directly proven that PrP is a specific downregulator of NR2D-containing NMDA receptors and ruled out that this arises from a complex intertwining of various pathways.

Not much is known about the function of NR2D containing NMDA receptors. The recombinant receptors showed a prolonged AP decay *in vitro,* but *in vivo* evidence is thus far missing and their low and ubiquitous expression doesn't pinpoint any particular function. The electrophysiological alterations from 12-16 DIV neurons might also be developmentally specific (Khosravani et al. 2008). If nothing else, NR2D receptors are most highly expressed during development. I was not able to verify the presence of NR2D, nor that of AMPA subunits. This would suggest that excitatory alterations reported before do not come about as a result of a direct interaction of PrP with components of glutamate system.

This lack of an *in vivo* interaction of PrP is consistent with the findings from Lledo et al., (1995). Here, the authors could not confirm the results from the previous study, stating that PrP knockout mice don't display any postsynaptic inhibition impairment and have no alterations in synaptic transmission of hippocampal CA1 pyramidal cell layer compared to wildtype mice, in three different genetic backgrounds. Together with my results, this

suggests that PrP at least does not physically interact with factors involved in excitatory pathways.

Whether PrP physiologically facilitates neuronal excitability has been difficult to prove. This notwithstanding, an excessive Ca^2 influx as the proximate cause of neuronal death seems quite likely (Sonati et al., submitted). How this could come about is entirely unknown. Spongiosis might be caused by membrane ruptures, as the engulfed membrane compartments suggest. However, it's been also shown that calcium influx via NMDAR is required to unleash neuronal death signalling, calcium overload itself doesn't suffice (Sattler et al. 1998). PrP in its aberrant forms could interact with these receptors rendering them permeable to extracellular calcium ions.

3.1.3 TRP Channels and PrP

Electrophysiological recordings and behavioural studies from Le Pichon and colleagues have demonstrated that PrP KO mice exhibit impairment in odour discrimination which is restored when PrP is expressed in the neurons of the olfactory bulb. Nevertheless, this doesn't necessarily imply that this is a consequence of a missing protein-protein interaction between the PrP and a protein directly involved in olfactory physiology. Given the ubiquitous expression of PrP in the CNS, weakened olfaction in KO mice could be an artefact of their neural circuit malfunction. A global neuronal malfunction would also explain the numerous phenotypes reported about the PrP deficient mice.

3.2 Cell adhesion and the role of PrP

3.2.1 NCAM interaction

Unlike mammals, zebra fish *(Danio rerio)* expresses two PrP genes *PrP1* and *PrP2,* with PrP1 being expressed in early development. Their knockdown exhibits evident Ca^{2+}-dependent and E-Cadherin-mediated cell adhesion impairment and gastrulation arrest, which is partially reversed by the introduction of *PrP1* (Malaga-Trillo et al., 2009). The fact that the expression

of mouse PrP partially rescued the cell adhesion phenotype suggests that the physiological function of PrP is conserved among vertebrates. In support of a role for PrP in cell adhesion, findings from yeast two-hybrid screens and chemical crosslinking *in vitro* have identified 37-kDa laminin receptor precursor (Rieger et al., 1997) and NCAM (Schmitt-Ulms et al., 2001) as PrP interacting factors respectively.

The NCAM interaction with PrP was shown via *in situ* chemical crosslinking in the mouse neuroblastoma cell line (N2a). The authors show that in the presence of 1% formaldehyde PrP forms a complex of 200-215 kD on an SDS-PAGE gel, co-isolating with caveolin-like domains. Ion-exchange purification and the subsequent mass spectrometry analysis and immunoblotting of the crosslinked complex revealed that all three splice variants of Neural Cell Adhesion Molecule (N-CAM), two transmembrane (N-CAM-180 and NCAM-140) and one GPI-anchored (N-CAM-120) variant, constitute the 200-225 kD gel band together with PrP. This interaction was also reported *in vivo* following chemical crosslinking (this time employing homo-bifunctional BS^3, a membrane impermeable crosslinker) of isolated lipid rafts (Santuccione et al., 2005). Moreover, the authors were also able to co-immunoprecipitate PrP while performing a pull-down with an NCAM specific antibody and failed to do so in the pull-downs from the NCAM -/- mouse brains and they concluded that PrP is important for recruiting and redistributing NCAM to the lipid rafts.

However, the band that shows the shifted signal of NCAM after being crosslinked with PrP is from a stripped membrane that was previously stained with an anti-PrP antibody and was reprobed with an anti-NCAM antibody. Stripping and re-probing of membranes often leads to artifactual signals. Furthermore, in none of the studies was evidence from PrP knockout cell lines (or PrP knockout *in vivo* data) presented where no shifts should be evident upon crosslinking. Schmitt-Ulms and colleagues (2001) present evidence that the reversal of crosslinking does cause an approximately 25kD shift when stained with NCAM antibody thus corresponding to the molecular weight of PrP, however this is not necessarily direct evidence that this potential 25kD NCAM interacting factor is indeed PrP. If there would have been no shift following the chemical crosslinking in the PrP deficient cells when stained with an anti-NCAM antibody and conversely none in the NCAM deficient cells when stained with

an anti-PrP antibody, this would have been direct evidence for a specific interaction between these two proteins.

In addition, Schmitt-Ulms and colleagues fail discuss the appearance of additional bands below 200-225kD which interestingly would correspond to dimeric or tetrameric PrP molecules (roughly 50 and 100 kD). Interestingly, the crosslinking reversal (with excessive NH_2) reveals that these extra bands disappear with increasing incubation time, suggesting again that formaldehyde leads to an *in situ* crosslinking of clustered PrP molecules.

Moreover, PIPLC treatment preceding crosslinking prevents molecular weight shifts. Of note, a growing body of evidence suggests that the clustering of GPI anchored proteins is exclusively GPI dependent (Friedrichson 1998, Sharma 2004), with the transmembrane variants also being unable to cluster. This implies that anchorless and therefore unclustered PrP molecules are not able to crosslink with themselves. In addition, the fact that PIPLC was unable to release monomeric PrP^{C} molecules following crosslinking resembles the PIPLC resistance of the PrP^{Sc} aggregates that solely contain aberrant PrP multimers, potentially suggesting that chemical crosslinking might lead to PrP stabilizatioin of multimeric conformation that sterically hinders the PIPLC accession. But this could also be a result of the aggressive and ubiquitary protein crosslinking achieved by formalin that hinders the access of PI-PLC.

It becomes increasingly evident that NCAM is present in biochemical approaches for isolating PrP and identify its associations with other proteins. But it also remains unclear how specific an NCAM-PrP interaction is. The immunoprecipitations from the PrP KO mice also pulled down NCAM, as illustrated in the SDS-PAGE gel. Nonetheless, the native gel showed only a band in the specific peptide elution, running at the same height as the wildtype, potentially suggesting that POM2 could also recognize NCAM and it can be eluted with peptides mimicking the octapeptide repeats. However, BLAST analysis showed that there are no peptide sequences in other proteins that would be homologous to octapeptide repeats, making this result even more puzzling.

The fact that PrP and NCAM co-localized after detergent extraction maybe indicates that introduction of detergent brings the two lipid raft proteins in closer proximity to one another, thus increasing NCAM's likelihood to be pulled down following a PrP specific immunoprecipitation. Phenotypically, NCAM knockout mice display among other phenotypes, an impaired neurite outgrowth. This has been reported for the PrP-deficient neurons too, but this has not been observed in our lab (Dr. Juliane Bremer, personal communication). PrP's involvement in cell-cell adhesion physiology still remains contested.

3.3 PrP and the caveolin pathway. Fyn, Shc interaction

The activation of fyn kinase following antibody-mediated PrP crosslinking (Mouillet-Richard et al. 2000) could be explained by the reported multimerization of GPI-anchored proteins after short antibody treatment and their redistribution to caveolae (Mayor et al., 1994). The multimerization was induced via crosslinking of fluorescently labelled primary antibodies with unlabelled secondary antibodies thus generating a punctate staining pattern in the confocal microscope. This multimerization was also confirmed by electron microscopy employing gold labelled secondary antibodies, again demonstrating an enrichment of these clusters in the caveolae. So the antibody-induced and caveolin-mediated activation of fyn kinase doesn't necessarily represent a natively occurring PrP signal transduction mechanism.

In the case of an interaction between PrP and fyn kinase, PrP would likely be upstream of fyn kinase which implies that fyn activity would also be affected in PrP deficient mice. The phenotype of fyn kinase knockout mice is fairly pleotropic. Fyn is expressed in many tissues, including the brain where its overexpression has been implicated in glioblastoma development, and the EGF signalling pathway (reviewed in Yeatman, 2004). It is difficult to draw any conclusion about possible PrP-fyn interactions based on the PrP KO phenotype. Pharmacological inhibition of fyn didn't rescue the toxic phenotype in the prion toxicity model (Falsig et al., submitted), nor did it in the antibody-induced toxicity model (Sonati et al., submitted), making a role for fyn in the toxicity execution pathway unlikely.

Fyn activation via antibody crosslinking presented in the Mouillet-Richard (2000) study could be specific for this neuroectodermal progenitor cell line after it has acquired neuronal

characteristics such as the expression of serotonergic or noradrenergic markers. Antibody crosslinking of PrP has also been described to cause severe neurodegeneration when injected intracerebrally in mice (Solforosi et al., 2004). It was believed that the toxicity was triggered through crosslinking of PrP^{C} molecules and it was also suggested that the PrP^{Sc} oligomers are also able to crosslink PrP molecules and hence initiate the devastating neuronal death.

However, the authors didn't provide biochemical evidence that would confirm PrP crosslinking induced by the specific monoclonal antibodies. In addition, data from our lab have shown that only the monoclonal antibodies that target the carboxy terminus of PrP trigger neuronal death; and moreover, the single chain peptides derived from these toxic antibodies are also able to induce the same toxic phenotype, suggesting that PrP crosslinking is indispensable for initiating neuronal death mechanisms (Sonati et al., submitted).

In fact, the preliminary data from our lab show a diffuse signal pattern of the toxic antibodies, whereas the innocuous ones show a more punctuate staining (O'Connor et al., drafted), potentially suggesting that the toxic antibodies might on the contrary, prevent the clustering of PrP molecules and this could account for initiation of toxic events.

3.4 The HMW complex, a biochemical artifact?

Immunoprecipitation performed on HPL cells expressing PrP yielded the same band as the *in vivo* IP. Consistent *in vivo* and *in vitro* data usually tend to suggest non-artefactual evidence; yet the fact that the bands barely run in the native gel is leaves room for greater skepticism and speculations that the IP is an extraction artifact. The complex also has a trypsin-resistant core (Callela et al., unpublished) further fevering speculations that the 700kD is a non-physiological aggregate, most likely caused by detergent administration.

Indeed, it has been reported that non-ionic detergent octylglucoside triggers a β–sheet conformation in PrP (Pergami et al., 1999). The scrapie form of PrP consists largely of β–conformation with a remarkable propensity to aggregate. Hence, NP40, the non-ionic detergent used in our IPs, which structurally resemble octylglucoside might facilitate

aggregation of PrP molecules. DDM in the sample buffer could also have induced by augmenting β conformation. As discussed in the subsequent section, non-ionic detergents are also known to enlarge the size of GPI- anchored protein clusters.

On the other hand, CHAPS an ionic detergent with a steroid backbone, might be too harsh to sustain all existing protein-protein interactions. Numerous interactions of PrP with other proteins have been described, potentially pointing to elusive interactions that could have been missed due to the use of this ionic detergent. CHAPS is also supposed to "compete" with cholesterol in the membrane, and therefore most likely also affects the nativity of the lipid rafts where PrP and its potential interacting partners resides. Thus the pull-down of PrP alone might not be the best method of assessing the native membrane environment.

Ionic detergents tend to restore the α– helical conformation according to Pergami and colleagues (1999), so it remains questionable whether CHAPS could counteract a potential aggregation triggered by NP40. *In vitro* experiments testing the effect of the detergents separately and employing chemical crosslinkers would give more accurate insights on the effects of these detergents on the formation of the HMW complex (see subsequent section).

It has been hypothesized that the PrP protein exerts its function by interacting with other proteins, but this interaction seems not to be manifest by an *in vivo* immunprecipitation method. It should also be noted that interaction with glycosaminoglycans and other high molecular weight molecules found in the extracellular matrix are not ruled out, since they would not enter the conventional SDS-PAGE gel and would not be observed by silver staining.

3.5 PrP as a GPI-anchored protein

3.5.1 The big shift. How does it come about?

The unusual big shift in the native gel devoid of any proteins other than PrP resembled much more an artifactual occurrence and therefore was very difficult to be plausibly interpreted. This immediately raised two questions: How does a small protein natively form such a huge

conformation solely through interactions with itself? Is such clustering still native? However, a journey to the literature in the research field of GPI-anchored proteins provides insights that might help find a plausible interpretation for the isolated PrP complex *in vivo* and *in vitro.*

3.5.2 GPI-anchored proteins, the lonesome mavericks

The original hypothesis of Simons and Ikonen (1997) postulated that lipid rafts are specialized membrane platforms enriched with cholesterol and specialized glycolipids where GPI-anchored proteins tightly associate with each other excluding other proteins. This hypothesis was primarily based on biochemical evidence and was later hotly contested by numerous research groups claiming that these detergent resistant lateral heterogeneities are an artefact of detergent extraction thus questioning the very existence of lipid rafts in cells (Heerklotz et al., 2003; Zurzolo et al., 2003).

Notwithstanding the controversy over the existence of lipid rafts, current evidence, at least in biochemical terms, doesn't argue against a homotypic association of GPI-anchored proteins and the exclusion of other proteins from interacting with them. Friedrichson and Kurzchalia (1998) were the first to show that GPI-anchored proteins do exist in clusters in living cells. They employed chemical crosslinking, using the same chemical crosslinker as I did bis(sulphosuccidimidyl)suberate and observed molecular weight shifts in an SDS-PAGE gel, corresponding to dimeric and tetrameric conformations of GH-DAF, clearly pointing to pre-existing GPI-anchored clusters. Consequently, the transmembrane and anchorless variants of growth hormone, the GPI-anchored protein studied here, were unable to crosslink and hence showed no band shifts, demonstrating again that the GPI anchor is required for clustering.

Conversely, the authors performed a two-dimensional electrophoresis using 3,3'-dithiobis-(sulphosuccinimidyl propionate), a reduceable form of BS^3 to analyse these covalently bound GH-DAF oligomers. The electrophoresis in the first dimension showed the usual shift pattern as BS^3, whereas the second dimension conducted under reducing conditions revealed almost

only GH-DAF monomers and barely any other interacting proteins, thus supporting the hypothesis of their scarce interaction with non-GPI anchored proteins.

Alternative evidence that the molecular weight shifts indeed originate from GPI-anchored monomers and not from an interaction with other proteins-, came from fluorescence resonance transfer (FRET) studies. Varma et al. (1998) virtually confirmed the multimeric organization of GPI-anchored proteins in submicron domains at the cell surface of living cells. In another study from the same research group, lower fluorescence anisotropy values were measured upon exciting fluorescently tagged GPI-anchored with polarized light (Sharma et al., 2004). This increased fluorescence depolarization arises from the energy transfer that occurs between the fluorescently tagged GPI-anchored proteins because they are in close proximity. This same fluorescence depolarization was measured when the proteins were chemically crosslinked with glutaraldehyde, demonstrating that GPI-anchored proteins are indeed very close to each other and thus enabling a homo-transfer of energy.

Consequently, the phospholipase cleavage of the GPI anchor and the transmembrane variants of the fluorescently tagged folate receptor (GFP, YFP and mCherry tags) showed no fluorescence depolarization (i.e. showed the expected fluorescence anisotropy), identifying the GPI anchor as the factor that confers their tight proximity.

Taken together, these two alternative lines of evidence strongly suggest that clusters of different GPI-anchored proteins do exist and this clustering materializes in a GPI-dependent fashion. In light of this evidence, the HMW band obtained from the IPs *in vivo* could be interpreted as an isolated complex of multimeric membrane anchored PrP. Supporting evidence for this observation would be an absence of HMW formation from the anchorless PrP. Alternatively, the disappearance of the band shifts following phospholipase cleavage of PrP's GPI anchor and moreover, an approximately 10 kD shift downwards after complete PNGase digestion would have finally proven that the band shifts upon chemical crosslinking derive from PrP multimers and that the isolated complex occurs natively.

3.5.3 PrP cluster enlargement following detergent treatment

The GPI-anchored proteins are usually isolated in low density complexes as a consequence of their detergent-insolubility (Hooper and Turner, 1988). It was widely believed that these low-density complexes where the GPI-proteins massively deposit represent a purified caveolae and reflects on the native distribution of these proteins. This view has been challenged by immunocytochemistry and more sophisticated live imaging techniques such as FRET, demonstrating that GPI-anchored proteins are rather diffusely distributed throughout the membrane and they cluster in caveolin-like domains only after detergent treatment or antibody cross-linking.

Mayor and Maxfield (1995) use fluorescently (Cy3) labeled monoclonal antibodies directed against two GPI-anchored proteins, decay-accelerating factor (DAF) and folate receptor to show that their diffuse membrane fluorescent signal redistributes into a punctate structure after 30 minutes of cold Triton X-100 treatment of the cells. Consistent with that, the fluorescent analogue of folate (FLP) that binds with high affinity to the GPI-anchored folate receptor, displays the same signal distribution pattern as the two aforementioned proteins when treated with fluorescently-labeled monoclonals antibodies. On the other hand, caveolin staining showed an unaltered punctuate pattern independent on the detergent incubation. This finding demonstrates that GPI-anchored proteins have an inherent detergent resistance while they still remain attached to the cell membrane and this leads to their redistribution into more enriched membrane clusters.

Consistent with this, Friedrichson and Kurzchalia (1998) again employing chemical crosslinking to test the detergent effects on the clustering of GPI-anchored proteins. They observe huge molecular weight shifts (up to 200 kD) of an approximately 30 kD GPI-anchored growth hormone (GH) protein once the MDCK cells were pre-incubated with 0.5% Triton X-114. Under harsher detergent treatment the crosslinked oligomers become much bigger, so that they barely enter the gel. This confirms the effect of detergents (at least the non-ionic ones) on the oligomerization of GPI-anchored proteins.

Hence, the high molecular weight band of the IP complex in the native gel (over 720kD) could be a consequence of PrP detergent insolubility *in vivo*. The fact that the *in vitro* IP yielded the same band in the native gel, testifies to an inherent detergent insolubility of PrP, which like the other GPI-anchored proteins is present in platforms with saturated acyl chain-containing phospholipids, neutral glycolipids and enriched cholesterol (Brown and Rose, 1992). An *in vitro* experiment similar to Friedrichson and Kurzchalia involving detergent treatment of cells prior to chemical crosslinking of PrP alongside crosslinked mouse brains homogenized with and without detergent would finally test PrP's detergent resistance. If the detergent addition in these two experiments yields a high molecular weight shift, this would confirm PrP's detergent insolubility.

3.5.4 Clusters induced by POM2

It should also be noted that beside the detergent, the POM2 binding might also promote a clustering of PrP. In another study, Mayor and colleagues (1994) tested the effects of antibody binding to GPI-anchored proteins and they found that the crosslinking of bound fluorescently labelled primary monoclonal antibodies via binding of unlabelled secondary antibodies translated into a redistribution of the fluorescent signal reminiscent of that induced by detergent incubation. Indeed, POM2 treatment of primary neurons prior to fixation have unravelled a punctate signal pattern of PrP, unlike POM1 have unravelled a punctate signal pattern of PrP, unlike POM1 which shows a more diffuse pattern (O'Connor et al., unpublished), again indicating that the antibody itself might have enlarged the PrP clusters. This in turn could translate into a huge shift on the native gel.

In fact, preliminary live imaging data employing fluorescently conjugated POM2 again showed a punctate signal (data not shown), which on one hand would show again the clustering potential of POM2, but on the other hand the punctate signal might also be a result of binding of multiple POM2 molecules to a single PrP molecule, since it recognizes the octapeptide repeats and hence the punctate signal could originate from clustered POM2 (bound to a single PrP). The use of other fluorescently labelled POMs that recognize single epitopes of PrP and their single chain peptides would help give a clearer picture whether

punctate signal derives from natively clustered PrP molecules. If the single chain peptides reveal punctate signal too this is a strong indication that native PrP clusters are not induced by antibody crosslinking.

3.6 Protein-lipid interactions

The fact that interacting proteins singled out in the mass spectrometry analysis could not be verified by Western blotting and the subsequent confirmation of a PrP-only multiprotein complex broadens the scope of the nature of the physiological interaction. A growing body of evidence reiterates a mandatory GPI-membrane anchoring of PrP for an induction of toxic pathways (reviewed in Aguzzi, 2005). Cell-free assays and cell culture studies also stress the importance of lipid interaction for pathological prion conversions (Baron et al., 2002; Baron et al., 2003). Structural analyses have indicated that the central domain (90-130) of GPI-anchored PrP stands in a close proximity to the cell membrane (DeMarco and Dagett, 2009).

In addition, several independent lines of evidence have recently emerged suggesting a direct interaction of PrP's N-terminal peptides with cell membranes (Baron et al., 2002; Hornemann et al., 2009; Boland et al, 2010). This is in agreement with our cell culture observations (Figure 3a), where recombinant N-terminus binds to cultured Schwann cells derived from PrP knock out mice, while the recombinant anchorless C-terminus doesn't (Küffer et al., unpublished). What is still unknown is the chemical nature of that interaction, i.e. whether it is ionic or hydrophobic?

3.6.1 Ionic protein-membrane interaction

The positively charged cluster next to the hydrophobic core might serve as an interacting domain with an anionic membrane lipid component via ionic protein-lipid interactions and thus transducing a signalling activity, reminiscent of the interaction between a myriad of proteins engaged in vesicle docking and fusion with phosphatidylinositol-4,5 bisphosphate (PIP2) (reviewed in Mclaughlin and Murray, 2005). This raises the question of what might be the usefulness of the first charged cluster, or why the octapeptide region is flanked by two

positively charged clusters. Could this be a structured interaction with a small lipid molecule acting as a second messenger? After all, lipid rafts are believed to be a niche for small lipid signaling (van Rheenen et al., 2005; Golub and Caroni 2005)

In BN-PAGE, the negatively-charged Coomassie G250 disassembles the complex, potentially indicating that the interaction might be ionic. Finally, there is good evidence that lipid rafts sequester proteins *via* so-called small "lipid shells" which expand in response to changing extracellular environments or antibody cross-linking and initiate signaling (Anderson and Jacobson, 2002; Mayor and Rao, 2004). This is certainly an intriguing scenario that could explain the antibody-induced toxicity (Solforosi 2004; Sonati et al., submitted) and once again stresses the importance of clustering or homo-interaction of PrP molecules.

3.6.2 Hydrophobic protein-membrane interaction

To date, three mutations occurring in the central domain, P102L, P105L and A117V have been identified to cause GSS. Hegde and colleagues, show that this domain is protected by the cell membrane, since protease K is unable to digest this domain, in mice expressing the A117V mutation. These mutations show an increased ^{Ctm}PrP conformation (20-30%), the topological variant singled out in the introduction as a proximate cause for some of the prion-related neurotoxic developments.

Hornemann et al., (2009) used NMR chemical shifts to test the interaction of recombinant mPrP and the aforementioned mutants with zwitterionic detergent dodecylphosphocholine, a biomembrane mimetic. The authors conclude that there is a weak predisposition of the central domain to form α helices upon interaction with micelles, which logically, increases in the aforementioned mutants since proline; a "helix breaker" is replaced by leucine. Hence this region interacts with higher affinity with the membranes, regardless of where the mutations occur. In spite of this knowledge, how the tremendous neurodegeneration in these mutants is initiated remains unknown to date. Putative toxic scenarios will be discussed in the subsequent sections.

From this perspective, I could argue that pulling down PrP using a steroid-derived zwitterionic detergent like CHAPS might have evoked an enhanced formation of α helices and therefore an enhanced membrane interaction which makes a pull down of a lipid (membrane)-containing protein complex not unlikely.

The fact is that some of these deletion mutants completely lacking the central domain (ΔCD) and lacking only the hydrophobic core (ΔHC; 112-134), thus exhibiting tremendous neurotoxicity, run differently in the native gel compared to the wild type. One explanation for these lower bands could be that the lack of these two domains leads to a dissociation of the PrP-lipid interaction, making them “lighter” and therefore running further in the gel. The fact that delC, the non-toxic mutant lacking the whole octapeptide repeat region and retaining the central domain, runs equivalently to the wild-type, suggesting that the engulfed lipids are still in the complex. In order to prove the presence of fatty acids in the multiprotein complex, a thin-layer chromatography would need to be performed.

4. Conclusion

Recently, the Aguzzi lab has focused its research work on a biochemical approach to elucidate the role of cellular PrP and has invested a great deal of effort in isolating an *in vivo* PrP immunoprecipitaion complex. My primary assignment was to verify the presence of potential interacting proteins in IPs, as previously identified by mass spectrometry. Membrane channels and receptors dominated the list of mass spectrometry hits, trailed by signaling, cell-adhesion and myelination molecules. Together with the emerging cell biological data suggesting a calpain-mediated toxic pathway in prion pathologies, this data had nurtured expectations the PrP might directly interact with receptors/channels or other molecules participating in Ca^{2+}-initiated signaling. So far, I have been unable to verify by Western blotting a specific binding of any of the proteins singled out in the mass spectrometry analysis to PrP.

The discrepancies between the mass spectrometry and immunoblotting results could be ascribed to their different sensitivity. It is also known that mass spectrometry spectra can be compromised by the use of non-ionic detergents (Cadene and Chait, 2000) which can increase the number of non-specific hits. Parallel cell biological studies i.e. real time expression studies, RNAi silencing, live imaging etc., characterizing the phenotypically overwhelming toxic pathways in deletion mutants or antibody models could help narrow down the molecular pathways PrP is also physiologically involved in. This way one should be able to virtually provide independent evidence of interactions with other proteins thus greatly simplifying the biochemical work.

Nevertheless, the inability to verify the presence of other proteins in the IP complex raised the suspicion that the complex might be devoid of other proteins. This was essentially confirmed by SDS-PAGE silver staining following denaturation of the IP complex, which revealed a single band corresponding to PrP. Further experiments on native gel basically excluded this band as a hydrophobic aggregation artifact, since mouse recombinant PrP and bovine phospholipase-cleaved and detergent-free purified bovine PrP behaved differently on the gel.

One possible scenario that would explain a high molecular weight complex consisting only of a small protein is a distinctive organization of the complex involving clustered PrP molecules. This has already been proposed by Aguzzi and Behrens a decade ago, based entirely on transgenesis since the re-introduction of the wild-type allele blocks the toxicity of PrP deletion mutants, suggesting that the toxicity originates from an open window provided by the inability of PrP molecules to oligomerize. A pre-existing cluster of PrP molecules might also increase the likelihood of aggregation. Sporadic prion diseases, which are by far the most frequent, point to a propensity of PrP to aggregate ("bad-luck" hypothesis (Aguzzi and Glatzel, 2006)), but this aggregation could by be facilitated by the physical vicinity that the PrP clusters provide.

Pre-existing clusters involving GPI-anchored proteins have already been reported in studies also deploying chemical cross-linking or FRET in living cells, pointing to very dynamic structures. This notwithstanding, further experiments are needed to prove the nativity of the PrP complex i.e. as pointed out in the discussion, it needs to be ruled out that the clustering is not a consequence of detergent addition or POM2 itself.

While figuring out the physiological function of PrP might not be the question that moves the prion research field most-, nonetheless, the question of how PrP-mediated toxicity is initiated is the key to understanding the prion pathologies and might eventually help tackling the very upstream events. Numerous efforts to elucidate the physiological function of the prion protein via transgenic mutants have failed. Adding the cluster dimension could extend the context of PrP's physiology and pathology and lead to a better understanding of them. In this case, the open window hypothesis put forward by Aguzzi and Behrens (2002) would be the simplest explanation. This new dimension would link PrP-mediated toxicity to its conformation. The fact that deletions on both termini of PrP render it toxic might also point to its conformational alteration as the toxicity initiator, either providing directly the window or causing that by interacting with other membrane proteins or maybe the membrane lipids themselves.

Several lines of evidence have emerged suggesting a direct interaction of PrP's N-terminal peptides with cell membranes (Baron et al., 2002; Hornemann et al., 2009; Boland et al,

2010), and this is consistent with cell culture observations in the lab, where recombinant N-terminus binds to cultured Schwann cells derived from PrP knockout mice, but not the recombinant C-terminus (Kueffer et al., unpublished). The *in vivo* immunoprecipitations involving the toxic deletion mutant lacking the GPI anchor later revealed that no HMW complex could be formed unlike the membrane anchored variants thus clearly suggesting that membrane anchorage is necessary for a clustering of PrP monomers. However, the role of the lipids in PrP's physiology/pathology is yet to be established.

Further complementary live studies are necessary to show the existence of PrP^{C} clusters, therefore also testing whether PrP functioning in health and disease is indeed encoded into those distinctive spatial organizations. Combining biochemistry, live imaging technologies and cell biology should help deepen the insights the molecular mechanisms in prion pathologies and perhaps other neurodegenerative diseases associated with protein aggregation.

5. Materials and Methods

Antibodies and peptides

Antibodies raised against following proteins (with the corresponding dilution) were commercially acquired and used for immunoblotting: Voltage-gated sodium channel alpha subunit (Chemicon; 1:1000), TRPC2 channel (Chemicon; 1:1000), SK2 channel (Sigma; 1:1000), GluR2 subunit of AMPA Receptor (Cell Signaling; 1:1000), NCAM1 (Cell Signaling; 1:1000), Fyn (Cell Signaling; 1:1000), CNPase (Abcam; 1:10,000), Vitronectin (Chemicon; 1:1000), TREK-1 channel (Chemicon; 1:5000), Neurofascin (Chemicon; 1:10,000), mouse NR2D (Chemicon; 1:10,000) and rabbit NR2D (Abcam; 1:1000). The monoclonal POM antibodies (1:10,000) were generated in the lab (Polymenidou et al. 2008). The following synthetic peptides were used to elute the IP complex: specific P20 (H-WGQPHGGSWGQPHGGSWGQPHGGGW-NH2; JPT Peptide Technologies) and the non-specific SP20 (H-QGHSGHSHGWWGWGHPGHGWPGPGQ-NH2JPT; Peptide Technologies).

Coupling of POM2 to magnetic beads

Tosylactivated M-280 beads (Dynal No. 142.03, Invitrogen) were first resuspended in 0.1 M borate (pH 9.5) coupling buffer, and POM2 antibodies were added (3 ug antibody per 10^7 beads), vortexed and incubated for 24 hours at 1400 rpm at 37 degrees. Thereafter, supernatant was removed, and beads were washed twice with cold PBS (pH 7.4) for 5 minutes at 4 degrees. Beads were subsequently placed on a magnet, and the washing supernatant was removed. The freshly POM2 coupled beads were incubated for 4 hours in 0.2 M Tris (pH 8.5) blocking buffer. To wash the unbound antibody, beads were washed with 1% Triton (in PBS) for 10 min (1400 rpm). Finally, the beads were washed again with cold PBS (pH 7.4) and stored in it at 4 degrees.

Immunoprecipitation

The immunoprecipitation (IP) buffer contained PBS (pH 7.5) with 0.5% of 3-[(3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPS, Roche) a zwitterionic detergent and 0.5% of non-ionic nonyl phenoxypolyethoxylethanol (NP-40, Sigma Aldrich) and protease inhibitors (Roche MiniTablette). PrP wild-type and KO (Büeler et al. 1992) mice had a mixed B6/129 background. Mice were bred and maintained in-house. Mice were sacrificed and their brains were excised and flash-frozen in liquid nitrogen, and finally stored at -80° C. In order to keep the working environment as native as possible and to avoid harsh rotations of the tissue homogenizer that could potentially cause aggregation of PrP, the homogenization of mouse brains was conducted with plastic pestles and syringes with descending needle diameter (18G, 21G and 25G). Finally, the homogenates were diluted down to 10%, incubated for 30 minutes on ice and centrifuged at 16,000 × g for 20 minutes at 4° C. Protein concentration was measured via BCA (s. Immunoblotting section), and 10mg total protein was added to 140 μg POM2-coupled beads. After overnight incubation, supernatant was removed, and the beads were washed twice with 0.5% IP buffer and subsequently with 1% IP buffer to wash non-specifically bound proteins. The bound PrP complex was eluted from the POM2-conjugated beads with 50μg of specific peptides p20 (spanning the octapeptide repeat; s. *Antibodies and peptides*) for 3 hours at 4° C. 50 μg of the scrambled peptide sp20 were used as a control.

Isolation of membrane fractions

Whole mouse brains were homogenized in cold 0.32 M sucrose, 20mM Tris–HCl and 5mM EDTA at pH 7.5 containing protease inhibitors using plastic pestle and syringes with ascending needle diameter (18G, 21G and 25G). The 10% homogenates were then centrifuged at 1000g for 10 minutes at 4° C (all the centrifugation steps were performed at 4° C) to pellet the nuclear fraction. To obtain the membrane fraction, the supernatant (post-nuclear fraction) was then ultracentrifuged for 1 hour at 100,000 × g. This crude membrane pellet was washed in homogenization buffer and spun again at 100,000 × g for one hour. The pellet was finally resuspended in 20mM Tris-HCl with or without 1% n-Dodecyl-ß-maltoside

(DDM) and incubated for 30 minutes at 4° C. Finally, this was centrifuged at 4,500 × g, the supernatant was collected and native gels were run (s. next section).

Immunoblotting of native and SDS-PAGE gels.

Protein concentration was measured using bicinchoninic acid (BCA) reagent (PerkinElmers) and 10-20 μg protein were loaded on 4-12% gradient SDS-PAGE gel. The gradient gel was run at 115V for (upper third) and then at 130V and was transferred onto nitrocellulose membrane at 50V for at least 2 hours. The membrane was then blocked with 5% bovine serum albumin (BSA) for 1 hour and was incubated (O/N) with diluted (from 1mg/ml initial concentration s. *Antibodies and peptides)* primary antibodies or 1:10,000 POM1 for PrP staining. Likewise, the native gels: NativePAGE™ Bis-Tris (Invitrogen) and Novex® 4-20% Tris-Glycine gels were run for approximately 5 hours at 100V on ice to avoid thermic protein denaturation. The native gels were then transferred onto polyvinylidene difluoride (PVDF) membranes. The subsequent steps are identical for both gels. Next day, the membrane was washed three-times with 1X PBS-Tween (0.01%) and incubated for 1h with secondary (goat anti-mouse) antibodies (1:10,000). Afterwards, the membrane was washed again with 1X PBS-Tween (0.01%) and incubated for 5 minutes with ECL (enhanced chemiluminescence reagent West Dura) and exposed using a phosphorimager (XStella). Loading control was performed via staining against actin (Chemicon mouse anti-actin antibody, 1:5000).

Cell culture and chemical crosslinking

The HPL cell line is derived from PrP knockout mice and HPL-PrP are the cells that have been stably transfected with PrP. Cells were first carefully thawed from 1ml vials and resuspended in 9ml OPTI-MEM Complete media, and this media was changed after 2-4 hours. Cells were usually split when they reached 70-80% confluency. For crosslinking, cells were first washed twice with ice-cold PBS and then crosslinked for 45 minutes at 4° C with 0.5 mM bis(sulfosuccinimidyl)suberate (BS^3), a cell-impermeable, amine-to-amine crosslinker, as described in Friedrichson and Kurzchalia (1998). The crosslinking was stopped

for 15 minutes with 50 mM glycine to saturate *N*-hydroxysulfosuccinimide (NHS) ester, the crosslinking functional group with NH_2. Cells were washed twice with cold PBS and lysed with lysis buffer containing 10mM Tris-HCl, 150mM NaCl pH 8.0; 1mM EDTA, mild non-ionic detergent 1% Triton X-100 and protease inhibitors. The lysates were then gently sonicated to get rid of the DNA, and proteins were precipitated with acetone (4-5 volumes) (O/N) at 4° C. Thereafter, the precipitates were centrifuged at 12-13,000 × g for 20 minutes at 4° C. Pellets were resuspended in 1% SDS, boiled for two minutes and diluted 1:5 (v/v) in digestion buffer (20 mM Hepes (pH 7.2)), 1.5% CHAPS and protease inhibitors (Roche MiniTablette), as described in Schmitt-Ulms et al., 2001) and were loaded on an SDS-PAGE gel.

Silver staining

SDS-PAGE gels were first fixed with 50% methanol and 10% acetic acid, whereas native gels were fixed with 8% trichloracetic acid for 10min at RT. 0.03% of glutaraldehyde was added for 10min as silver-ion sensitizer using the Invitrogen kit, LC6 100). Gels were then washed with ddH_20 and were stained with 0.1% silver nitrate (Solution A) and 30% sodium hydroxide (Solution B) for 15 minutes. Gels were washed twice with ddH_20 and developed with 37% formaldehyde in 3% sodiumcarbonate for 3-15 minutes at RT. The reaction was stopped by directly adding 5ml of 1% anhydrous citric acid-containing solution.

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