Cellular form of prion protein (PrP^C) associates with contactin associated protein (Caspr) and inhibits its serine protease reelin-mediated shedding on the neuronal surface in mouse (*Mus musculus*, Linnaeus, 1758)

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Vasudharani Devanathan

Aus Chennai, Indien

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Jörg Ganzhorn

Professor Dr. Jörg Ganzhorn
Leiter des Departments Biologie

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Abstract

1 Abstract

The brain and spinal cord are composed of neurons that do different functions such as controlling movements, processing sensory information, and making decisions. The growth of a neuron during its development is a complex process involving the construction of the cytoskeleton and the insertion of new membrane. As neurites grow out, the process of elongation is regulated by the external environment. This process of neurites growing out is called neuritogenesis.

Cell adhesion molecules (CAMs) and extracellular matrix proteins (ECM) are involved in the process of neuritogenesis. The cellular prion protein, PrP^{C} (from here on mentioned as PrP) is a normal cellular protein found on the membranes of neuronal cells and anchored to the membrane by a GPI anchor. PrP regulates neuronal survival, neurite outgrowth and synapse formation. Misfolding of this protein results in the formation of its scrapie form (PrP^{Sc}), which is the causative agent of neurodegenerative prion diseases. Hence, understanding the mechanisms by which PrP regulates cellular functions is an important issue in the field of neurobiology.

This study identifies a novel role for Caspr as inhibitory cue that interferes with neurite outgrowth of the central nervous system neurons. Brain homogenate analysis shows that the levels of full length Caspr are regulated by the cellular form of prion protein, which directly binds to Caspr. Prion protein inhibits reelin-mediated shedding of Caspr from the cell surface thereby increasing surface levels of Caspr and potentiating the inhibitory effect of Caspr on neurite outgrowth in cerebellar neurons. PrP deficiency results in reduced levels of Caspr at the cell surface, enhanced neurite outgrowth in cerebellar neurons maintained in vitro and more efficient regeneration of axons in vivo following spinal cord injury. Thus this study reveals a previously unrecognized role for Caspr and PrP in inhibitory modulation of neurite outgrowth in central nervous system neurons

2 Introduction

2.1 Neuritogenesis

During development, neurons become assembled into functional networks by growing out axons and dendrites (collectively called neurites) which connect synaptically to other neurons. The outgrowth of neurites proceeds by migration of growth cones -- specialized structures at the tip of growing neurites. Growth cone migration elongates the neurite, while growth cone splitting creates a branch point. Promotion or inhibition of axonal growth is mediated by interactions of cell surface receptors on neuronal growth cones with guidance cues in the local environment.Many proteins are involved in the process of neuritogenesis. The cell adhesion molecules such as NCAM, L1, PrP and Contactin (F3) are known to play a key role in the process of neuritogenesis.

2.1.1 Role of cell adhesion molecules and extracellular matrix molecules in neuritogenesis

During development of the nervous system, neurons extend axons over considerable distances in a highly stereospecific fashion in order to innervate their targets in an appropriate manner. This involves the recognition, by the axonal growth cone, of guidance cues that determine the pathway taken by the axons. These guidance cues can act to promote and/or repel growth cone advance. The directed growth of axons is partly governed by cell adhesion molecules (CAMs) on the neuronal growth cone that bind to CAMs on the surface of other axons or nonneuronal cells. It was proposed long ago that precise axonal targeting and formation of specific synaptic contacts in the CNS are driven by changes in adhesivity of individual growth cones. A plethora of CAMs has subsequently been identified that are capable of both “mechanically” regulating cell adhesion and triggering intracellular signaling cascades, resulting in responses such as neurite outgrowth. All CAMs are expressed in the developing CNS and PNS, here they participate in neuronal cell migration, myelination, neurite outgrowth, and axonal guidance and fasciculation.The neurite promotive properties demonstrated for CAMs (Doherty et al., 1996; Riehl et al., 1996) were proven to be dependent on homophilic interactions between corresponding molecules. However, neurite outgrowth induced by heterophilic interactions of L1 and axonin-1 has also been demonstrated.*In vitro* assays have established the importance of the CAMs (NCAM, N-cadherin, and L1) in promoting axonal growth over cells. Compelling evidence implicates the fibroblast growth factor receptor tyrosine kinase as the primary signal

transduction molecule in the CAM pathway. In mature neurons, CAMs are important constituents of synapses, and appear to play important and diverse roles in regulating synaptic plasticity associated with learning and memory.

An interesting feature of some of the Immunoglobulin superfamily members (contactin, TAG-1, Axonin) is that they can modulate neurite outgrowth by acting both as a neuronal receptor and as a substratum ligand (Doherty and Walsh., 1989). This is likely to be the case for the GPI-anchored molecules F3/F11 and TAG-1/axonin-1. For example, TAG-1/axonin-1 used as a substrate stimulates neurite extension of DRG neurons (Kuhn et al., 1991; Furley et al., 1990) and, conversely, can be part of a neuronal receptor mediating Ng-CAM effect (see below; Buchstaller et al., 1996) (Fig 2.1). Similarly, F3 expressed at the surface of cerebellar neurons mediates the inhibitory effects of a tenascin-R substrate (Pesheva et al., 1993; Xiao et al., 1996) while F3 presented as a substrate inhibits neurite outgrowth of cerebellar neurons (Buttiglione et al., 1996) or stimulates neurite outgrowth of DRG neurons (Gennarini et al., 1991; Durbec et al., 1994).

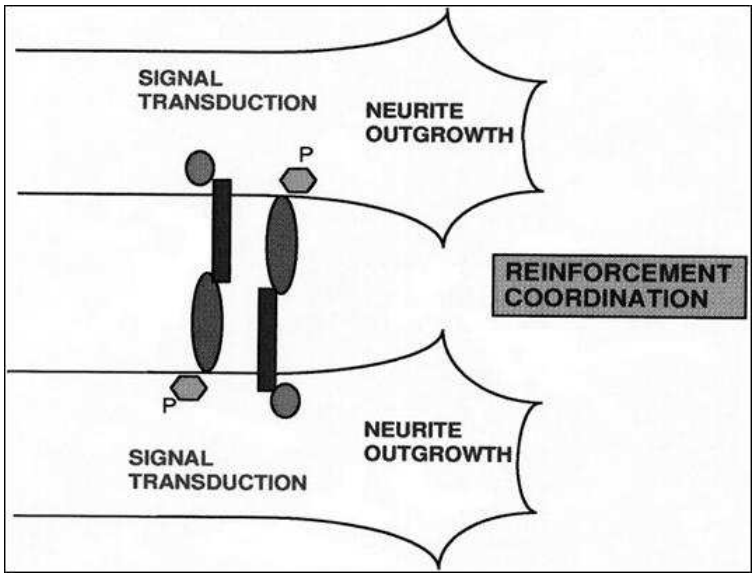

Figure 2.1: Schematic representation of GPI anchored molecules and transmembrane cell adhesion molecules involved in neuritogenesis. Bidirectional interactions occurring between axons growing in the same fascicle. Two axons are expressing the couple ligand and receptor on the same membrane. F3/F11 or TAG-1/axonin-1 (oval) counteract molecule (L1/Ng CAM or Nr-CAM/Bravo, rectangle). Similar signal transduction pathways (hexagon, small oval) are activated in each neuron. Double interactions might reinforce fasciculation and synchronize growth (Faivre-Sarrailh., 1997).

A transmembrane protein tyrosine phosphatise RPTPβ has also been identified as a F3 ligand. The interaction is mediated via the carbonic anhydrase domain of the phosphatase. This domain, used as a coated substrate, mediates F3/contactin-dependent neurite extension of tectal neurons (Peles et al., 1995). In yet another transmembrane protein Amyloid precursor protein (APP), a

heparin-binding domain that interacts with heparin sulfate proteoglycans supports a role of APP in neurite outgrowth through specific interactions with extracellular matrix molecules (Schubert et al., 1989; Koo et al., 1993; Small et al., 1994).

Negative extracellular signals that physically direct neurite growth have also been described. The latter include the myelin inhibitory proteins, Nogo, myelin-associated glycoprotein, and oligodendrocyte-myelin glycoprotein. Potentiation of outgrowth-promoting signals, together with antagonism of myelin proteins or their convergent receptor, NgR, and its second messenger pathways, may provide new opportunities in the rational design of treatments for acute brain injury and neurodegenerative disorders.

Taken together, the results suggest that transmembrane molecules may be often a part of an adhesion/transduction system involved in cell-cell and/or cell matrix interactions.

2.2 The prion protein

Prions are defined as proteinaceous infectious particle that are devoid of nucleic acid and composed exclusively of PrP^{Sc}. Prion is a short form for proteinaceous infectious particle (-on by analogy to virion)

2.2.1 Prion diseases or transmissible spongiform encephalopathies

Prion diseases (PDs) or transmissible spongiform encephalopathies (TSEs) are rare fatal neurodegenerative disorders of humans and animals, encompassing scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt-Jakob disease (CJD) in humans (Fig 2.2) (Aguzzi et al., 2001). They are usually characterized by neuronal loss and spongiform degeneration of the brain accompanied by the appearance of activated astrocytes and microglia. The striking phenomenon observed in these disorders is the accumulation of PrP^{Sc} (prion protein-scrapie form), an abnormally folded form of the normal cellular prion protein (PrP) in the central nervous system, which is responsible for prion diseases like CJD, TSE and BSE (Fig 2.2).

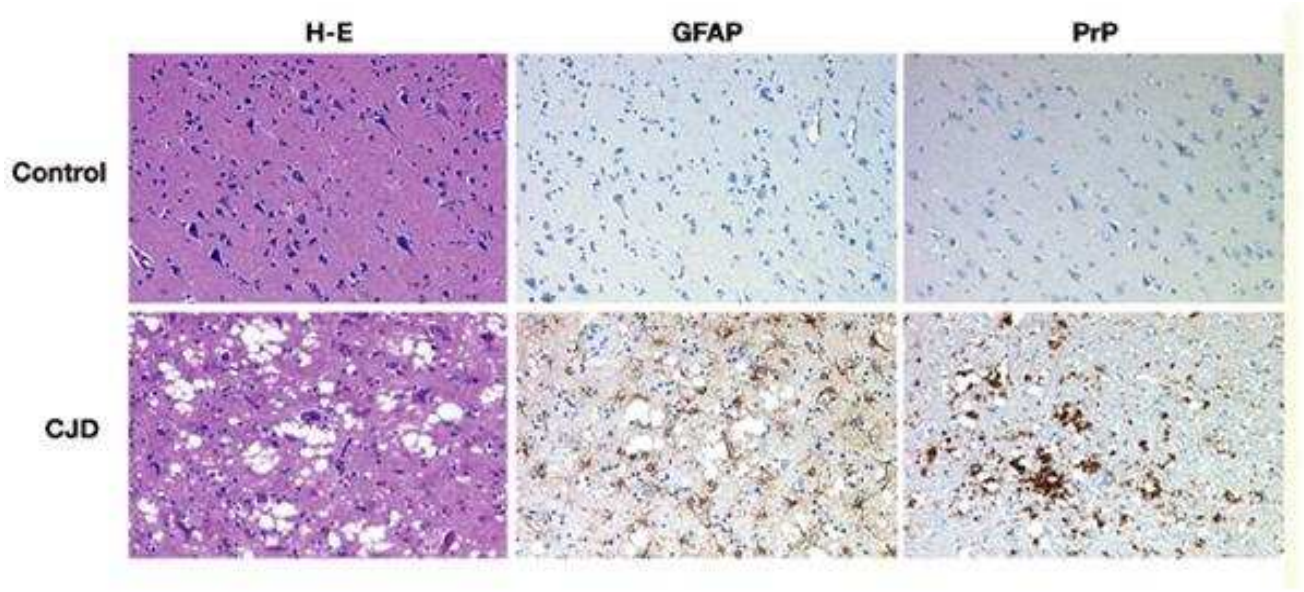

Figure 2.2: Histological and immunohistochemical analysis of frontal cortex samples from the brain of a patient who died of non-cerebral causes (upper row) and of a patient suffering from Creutzfeldt–Jakob disease (CJD; lower row).

Brain sections were stained with haematoxylin-eosin (H-E, left panels), with antibodies against glial fibrillary acidic protein (GFAP, middle panels) and with antibodies against the prion protein (PrP, right panels). Neuronal loss and prominent spongiosis are visible in the H-E stain. Strong proliferation of reactive astrocytes (gliosis) and perivacuolar prion protein deposits are detectable in the GFAP and PrP immunostains of the CJD brain samples (Aguzzi et al., 2001)

PrP^{Sc} demonstrate properties of classic amyloid form β-sheet rich, insoluble protein deposits that exhibit birefringence after staining with the amyloid dye Congo red (Fig 2.3). Therefore, TSEs are often considered as another type of amyloid disease such as Alzheimer's or Huntington's disease except that it is transmissible. Much evidence suggests that the abnormal form of PrP may be critical in the transmission and pathogenesis of TSEs. The normal prion protein, known as cellular PrP (Fig 2.3A), contains mostly alpha-helical structures (the spiral-shaped brown ribbons) and a small portion of flattened beta sheet. The increase in beta sheet upon conversion into the prion form is illustrated in (Fig 2.3B)

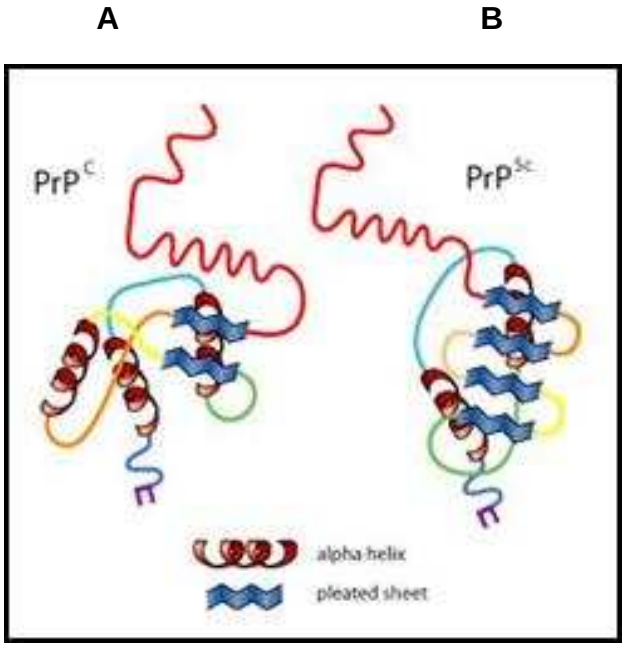

Figure 2.3: Comparison of the three-dimensional structures of the normal prion protein (A) with a model of the pathological form (B).

2.2.2 The cellular form of the prion protein (PrP) and its disease-associated isoform (PrP^{Sc})

The non pathogenic cellular prion protein, PrP, is a glycoprotein that contains a single disulfide bond. It is N-glycosylated and attached to the plasma membrane by a C-terminally linked glycosylphosphatidylinositol anchor. The primary structure of PrP is dominated by alpha helix, whereas the pathogenic PrP^{Sc} (from here on PrPSc) isoform is rich in beta sheets (Fig 2.3).

PrP comprises of three α-helices (amino acids 144-154, 175-193 and 200-219) and a small antiparallel β-sheet (amino acids 128–131 and 161–164). The C-terminal part (i.e. amino acids 126–231) contains the complete globular part of the structure, whereas the N-terminus (i.e. amino acids 23–125) is flexible. Close to the small β-sheet, a disulphide bridge (yellow colour thread) connects helix 2 and helix 3 (Fig 2.3). PrP consists of 253 aminoacids with a molecular weight 27663 Da. The gene encoding the cellular isoform of the prion protein is located on chromosome 20 in humans and on chromosome 2 in mice (Fig 2.4). It has been cloned and co termed Prnp in mouse and PRNP in humans (Basler et al., 1986).

Examining the prion protein sequence for structural clues to its function indicates an unusually 'split' molecule (Fig 2.4). The C- terminal half of the protein contains amino acid sequences of mixed character, with early secondary structure predications indicating at least two long stretches of α-helix. The presence of these α-helices was subsequently proved correct; a protein fragment encoding the C-terminal half of the protein provided the first three-dimensional structure of any prion related sequence by NMR spectroscopy in 1996. The domain is a tightly folded globular structure made up of three α-helices, two of which are held together by a single disulphide bond, and two short sections of β-sheet (Fig 2.3). Following an initial 'signal peptide' region, there is a short series of hydrophobic amino acids followed by an eight amino acid motif, the octarepeat. As its name implies this motif is repeated perfectly four times and includes residues typical of those that interact with metal ions. Following the octarepeat region a second hydrophobic stretch of amino acids occurs before the beginning of the well characterised C-terminal domain (Fig 2.4)

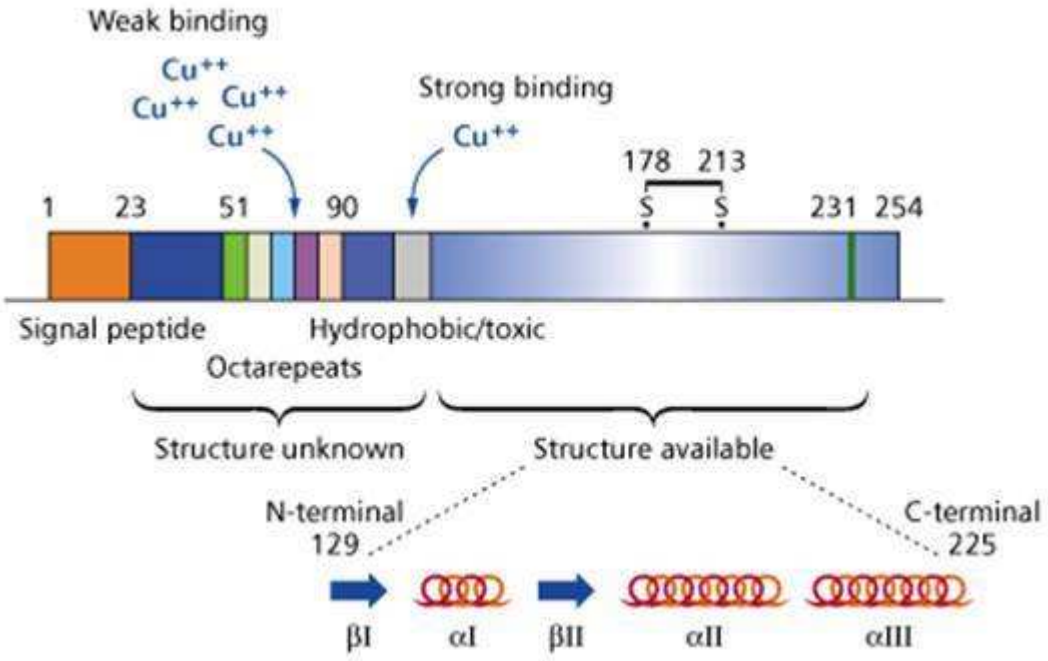

Figure 2.4: Structure of prion protein. Adapted from: http://www.chemsoc.org/chembytes/ezine/2002/jones_apr02.htm

2.2.3 Expression of PrP

PrP is expressed during early embryogenesis and is found in most tissues in adults (Manson et al 1992). Highest levels of expression of PrP are seen in the central nervous system, in particular in association with synaptic membranes (Herms J., et al, 1999). PrP is expressed in kidney, bone

marrow, dendritic cells, T-cells, B-cells, monocytes and Schwann cells.PrP is also widely expressed in cells of the immune system (Dodelet & Cashman 1998). It is also expressed by other neural cell lineages like astrocytes and oligodendrocytes (Moser et al., 1995).

2.2.4 Biosynthesis of PrP

Like other membrane proteins, PrP is synthesized in the rough endoplasmic reticulum (ER) and transits the Golgi on its way to the cell surface. During its biosynthesis, the N-terminal hydrophobic signal sequence of 22 amino acids guides PrP to the membrane of the ER, where it is cleaved off. After translocation, 23 amino acids are removed from the C-terminus and a glycosylinositol-phospholipid (GPI) anchor is added (Stahl et al., 1987). In the ER, high mannose glycans are attached to the 25 kDa polypeptide at two asparagines residues and processed to hybrid or complex glycans during the passage through the Golgi apparatus (Bolton et al., 1985). The mature PrP is transported within secretory vesicles to the external cell surface and it is anchored to the surface by the GPI-moiety. PrP does not remain on the cell surface after its delivery but, rather, constitutively cycles between the plasma membrane and an endocytic compartment (Caughey et al., 1989; Harris et al., 1996).

2.2.5 The physiological role of PrP and binding partners of PrP

The physiological function of PrP remains elusive. Much hope was pinned on the use of PrP knock-out mice to unveil the function of the protein, but no obvious phenotype was observed (Büeler et al., 1992). Even postnatally induced Prnp ablation does not elicit any phenotype (Mallucci et al., 2002). Aging mice show demyelination in the peripheral nervous system, albeit without clinical symptoms (Nishida et a, 1999). A number of subtle abnormalities like abnormalities in synaptic physiology (Collinge et al., 1994) and in circadian rhythms and sleep (Tobler et al., 1996) have been described in PrP deficient mice, but their molecular basis is undefined. The only definite phenotype of PrP -/- mice is their resistance to prion inoculation (Büeler et al., 1993). Several physiological roles for PrP have been proposed, in particular cell adhesion, signalling, neuroprotection and metabolic functions related to its copper-binding properties.

Cell culture experiments revealed a constitutive internalization process of PrP from the plasma membrane into endocytic organelles, yet most of the protein recycles back to the membrane without degradation (Shyng et a, 1993). The existence of a recycling pathway suggests that one physiological role of PrP might be to serve as a receptor for uptake of an extracellular ligand. It has been shown that copper ions rapidly and reversibly stimulate endocytosis of PrP from the

cell surface (Pauly and Harris, 1998). Moreover, it has been reported that PrP can bind copper ions and possesses superoxide dismutase activity (Brown and Besinger, 1998; Brown et al., 1997). Additionally, amino-proximal truncated PrP seemed to depress endogenous dismutase activity (Sakudo et al., 2003), suggesting a role for copper binding of the N-terminal octapeptide-repeat segment.

Given the localization of PrP as a GPI-anchored protein in rafts or caveolae, it has been proposed that PrP, as other GPI-anchored proteins, could be involved in signal transduction (Jacobson and Dietrich, 1999). Crosslinking of PrP with F (ab)'2 antibody fragments have been reported to activate Fyn tyrosine kinase (Mouillet-Richard et al., 2000). However, it was reported that PrP-mediated Fyn activation in cerebellar granule neurons might be responsible for neurite outgrowth rather than neuronal survival (Chen et al., 2003). PrP is thought to interact with components of signal transduction pathways, such as Grb2, an adaptor protein (Spielhaupter and Schatzl, 2001). PrP mediated activation of the cAMP/ protein kinase A PKA) pathways have a cytoprotective effect in nervous tissue (Chiarini et al., 2002). These studies clearly support a neuroprotective role for PrP through signal transduction events. PrP has been shown to interact with the 37 kDa/67 kDa laminin receptor precursor (Gauczynski et al., 2001; Hundt et al., 2001; Rieger et al., 1997) and heparan sulfate (Warner et al., 2002), supporting a possible role in cell adhesion and/or signalling.

It was shown that PrP can bind to laminin in a high-affinity, specific and saturable fashion which suggests that PrP plays a role in neuronal differentiation evidenced by its participation in neurite outgrowth promotion by laminin, both in cell lines and rat hippocampal neurons (Graner et al 2000). Moreover, with the aid of synthetic peptides they demonstrated that PrP recognizes a carboxy-terminal domain on the laminin γ-1 chain, previously shown to stimulate neurite outgrowth, to modulate electrical activity of neocortical pyramidal neurons and to be highly expressed in rat neocortical and hippocampal neurons (Liesi et al., 1989).

Furthermore, comparison of the neuritogenesis induced by laminin or its γ-1-derived carboxyterminal peptide alone, using primary cultures of hippocampal neurons from either wild-type mice or mice in which the PRNP gene has been ablated (Büeller et al., 1992) indicate strongly that PrP is the main cellular receptor for that particular laminin domain. The PrP–laminin interaction is involved in the neuritogenesis induced by NGF plus laminin in the PC-12 cell line and the binding site resides in a carboxy-terminal decapeptide from the γ-1 laminin chain. Neuritogenesis induced by laminin or its γ-1-derived peptide in primary cultures from rat or either wild type or PrP null mouse hippocampal neurons, indicated that PrP is the main

cellular receptor for that particular laminin domain. These results point out to the importance of the PrP–laminin interaction for the neuronal plasticity mechanism and neuritogenesis.

PrP is involved in neurite outgrowth and neuronal survival via different signal transduction pathways (Chen et al., 2003). It is conceivable that different receptors are involved in the signal transduction mechanisms mediating neurite outgrowth, on the one hand, and neuronal survival, on the other. In addition, the concentration at which PrP enhances neurite outgrowth and neuronal survival appears to be critical, arguing for the specificity of the phenomenon. This dose-dependent effect has been observed for other neural recognition molecules involved in neuritogenesis, such as L1 and NCAM (Chen et al., 1999). The authors have also shown an inhibition of neurite outgrowth and neuronal survival that they observed with the polyclonal antibody R340 directed against PrP confirming the specificity of the interaction between PrP-Fc and neurons. Lima et al in 2007 showed that PrP, STI1 (stress inducible protein 1), and laminin interactions are important for neuronal differentiation and also showed the importance of PrP and STI 1 in the neuronal survival.

Multiple PrP interacting partners have been identified in the recent years: the antiapoptotic protein Bcl-2 (Kurschner and Morgan, 1995), caveolin (Gorodinsky and Harris, 1995; Harmey et al., 1995), NCAM (Schmitt-Ulms et al., 2001, Santuccione at al, 2005), neurotrophin p75 receptor (Della-Bianca et al., 2001), plasminogen (Fischer et al., 2000), caveolin (Gorodinsky and Harris, 1995; Harmey et al., 1995), Grb2 (spielhaupter and Schatzl HM, 2001), laminin receptor protein (LRP) and cadherins (Kurschner and Morgan, 1995).

It has also been shown that PrP can interact with several other proteins invivo like amyloid precursor like protein 1, amyloid precursos like protein 2, dynamin 1 (a GTPase responsible for endocytosis), Adam 23 (ADAM metallopeptidase domain 23),myelin oligo dendrocyte glycoprotein and few others (Schmitt-ulms et al., 2004).

2.3 Contactin associated protein (Caspr)

2.3.1 Structure of Caspr

Caspr (contactin-associated protein), which has been suggested to be a coreceptor with contactin, is a transmembrane protein (Peles et al., 1997). Caspr has recently been localised to the paranodal axolemma in myelinated fibers of PNS and CNS (Peles et al., 1997; Menegoz et al., 1997; Einheber et al., 1997). Caspr is a type I integral membrane protein with a molecular mass of 190 kDa that is highly expressed in the CNS especially in brain. In brain it is expressed in myelinated nerve fibers and is predominantly found in paranodal axoglial junctions. It is also

present at reduced levels in several extraneuronal tissues like ovary, pancreas, colon, lung, heart, intestine, testis (Peles et al., 1997). It copurifies with contactin when the carbonic anhydrase domain of the receptor protein tyrosine phosphataseβ is used as an affinity ligand. Contactin and Caspr coimmunoprecipitate, suggesting that they are constitutively complexed.

Figure 2.5: Schematic representation of Contactin associated protein (Caspr) showing various domains.

FA58C: F5/8 type C domain, Lam G: laminin G domain, EGF: Epidermal growth factor-like domain, FBG: Fibrinogen-related domains (FReDs), EGFL: EGF like domain, TM: Transmembrane domain, B41: Band 4.1 homologues.

The extracellular domain of caspr contains a series of laminin G–like and EGF-like domains characteristic of the neurexins; accordingly, caspr is a member of the neurexin superfamily (Fig 2.5). The cytoplasmic segment of caspr contains potential binding sites for SH3 domain–containing proteins and band 4.1 proteins but lacks the carboxy-terminal motif found in other neurexins that binds to PDZ domains. This could allow caspr to participate in signaling pathways within the axon. Of interest, the Drosophila homologue of caspr, neurexin IV (Baumgartner et al., 1996), which has a similar extracellular domain organization and a 30% amino acid identity (Peles et al., 1997 a; Littleton et al., 1997), is expressed by glial cells and is a component of their septate junctions that compose the blood–nerve barrier (Baumgartner et al., 1996).

2.3.2 Binding partners of Caspr and their functional implications

Caspr is a relatively newly discovered protein and much has not been explored. It is speculated to be involved in cell to cell signalling between axons and myelinating glial cells. It is also a major protein involved in saltatory conduction of nerve impulses in myelinated axon. It forms a demarcation between the axo-glial junctions.

The cytoplasmic domain of Caspr contains a proline rich sequence with atleast one canonical SH3 domain binding site. A reasonable hypothesis, therefore, would be that the cytoplasmic domain of Caspr can serve as binding site for SH3 domains of signalling molecules which will

transmit the signal initiated by RPTPβ binding to the contactin Caspr complex. The cytoplasmic domain of Caspr associated with c-src upon transient transfection; however co immunoprecipitation experiments did not show the same (Peles et al., 1997).

In 2003 Gollan and colleagues showed that neurofascin 155 (NF155) binds directly to contactin (Fig 2.6) and that surprisingly, expression of Caspr inhibits this interaction. This inhibition reflects the association of Caspr with contactin during biosynthesis and the resulting expression of a low molecular weight (LMw), endoglycosidase H-sensitive isoform of contactin at the cell membrane, which remains associated with Caspr but is unable to bind NF155. Accordingly, deletion of Caspr in mice by gene targeting results in a shift from the LMw- to HMw-contactin glycoform.

These results demonstrate that Caspr regulates the intracellular processing and transport of contactin to the cell surface, thereby affecting its ability to interact with other cell adhesion molecules. Gonnan and colleagues in the same year showed that when transfected alone in neuroblastoma N2a cells, caspr is retained in the endoplasmic reticulum (ER).Using chimerical constructs, they showed that the cytoplasmic region does not contain any ER retention signal, whereas the ectodomain plays a crucial role in caspr trafficking. A series of truncations encompassing the extracellular region of Caspr was unable to abolish ER retention. They also showed that N-glycosylation and quality control by the lectin-chaperone calnexin are required for the cell surface delivery of Caspr.

Cell surface transport of F3 and Caspr is insensitive to brefeldin A and the two glycoproteins are endoglycosidase H-sensitive when associated in complex. Both proteins are recruited into the lipid rafts, and expressed on the cell surface. This indicates a Golgi-independent trafficking pathway for the paranodal cell adhesion complex that may be implicated in the segregation of axonal subdomains.

The Caspr–F3 complex interacts with NF155 *in trans*, and is an example of axoglial molecular connection at the paranode (Girault and Peles, 2002) (Fig 2.6). It was shown that blocking interaction between NF155 and the Caspr-contactin complex inhibits myelination (Charles et al., 2002) In 2003 Nie and colleagues showed that Nogo-A associates specifically with the Caspr–F3 complex and this Nogo-A-Caspr complex in turn interacts with potassium channels (K^+). Their results showed that Nogo A may complement Caspr in regulating the localisation of Kv1.1.

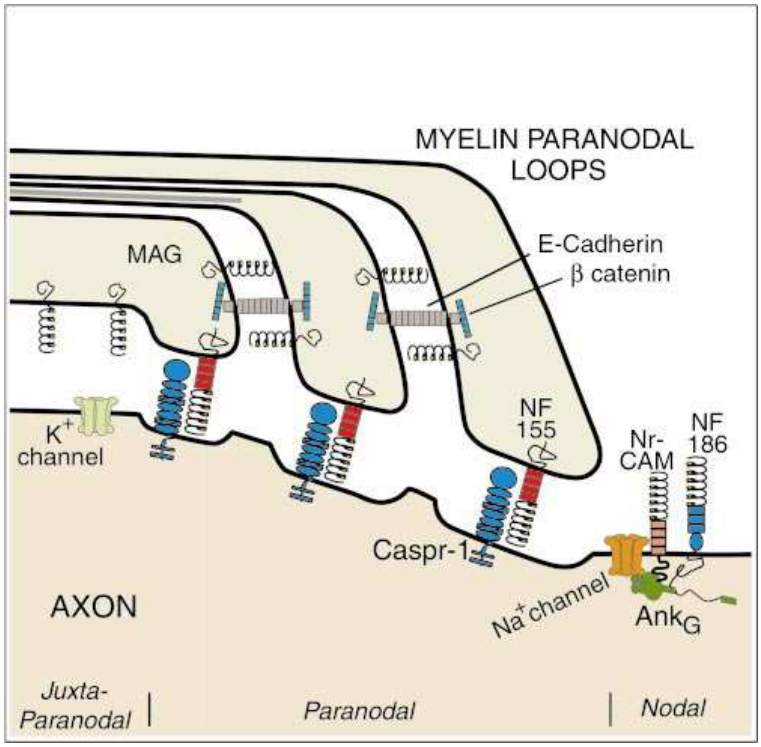

Figure 2.6: A schematic of nodal region in longitudinal orientation.

Three regions can be distinguished based on ultrastructure and molecular composition. The juxtaparanodal axon membrane contains high concentration of K1 channels. The adaxonal myelin

membrane and paranodal loops (PNS only) contain MAG. The paranodal loops are connected to each other by adherens junctions that contain E-cadherin and b catenin and to the axon by septate-like junctions that contain caspr-1 and neurofascin 155 Na+ channels cluster in the nodal axolemma and bind to the skeletal protein ankyrinG. Neurofascin 186 and NrCAM also bind ankyrinG and may help target Na+ channels to the node (Trapp BD and Kidd GJ, 2000)

2.3.3 Role of Caspr in disease

It was shown that paranodal junctions on some myelinated axons in the borders of lesions of patients with chronic progressive multiple sclerosis are no longer intact. Previous work (Wolswijk and Balesar, 2003) has reported a diffuse distribution of Caspr in multiple sclerosis tissue, only at the border of the plaque, whereas no Caspr immunoreactivity was detected in the plaque. Later it was also shown that there was a diffused Caspr immunoreactivity along naked axons in the plaque (Coman et al., 2006) This diffused distribution of Caspr leads to impairment in saltatory impulse conduction and leads to further myelin loss, thereby contributing to disease progression in multiple sclerosis. Caspr on myelinated axons in control and normal-appearing tissue surrounding the multiple sclerosis lesions was present in the form of distinct, cylindrical structures flanking the nodes of ranvier with bundles of neurofilaments passing through their

centre; peripheral axons were also found to harbour Caspr-positive structures (Einheber et al.,1997; Menegoz et al.,1997; Peles et al., 1997 b; Rasband and Trimmer., 2001). It is evident that Caspr plays a significant role in multiple sclerosis although the mechanism is yet to be explored.

2.3.4 Caspr deficient mice

Caspr deficient mice (also called as NCP1 mutant) survive but exhibit tremor, ataxia, and significant motor paresis. Normal paranodal junctions fail to form in these mice and the organization of the paranodal loops is disrupted. The Caspr mutant mouse weighs approximately half the weight of the wild type littermate (Fig 2.7).

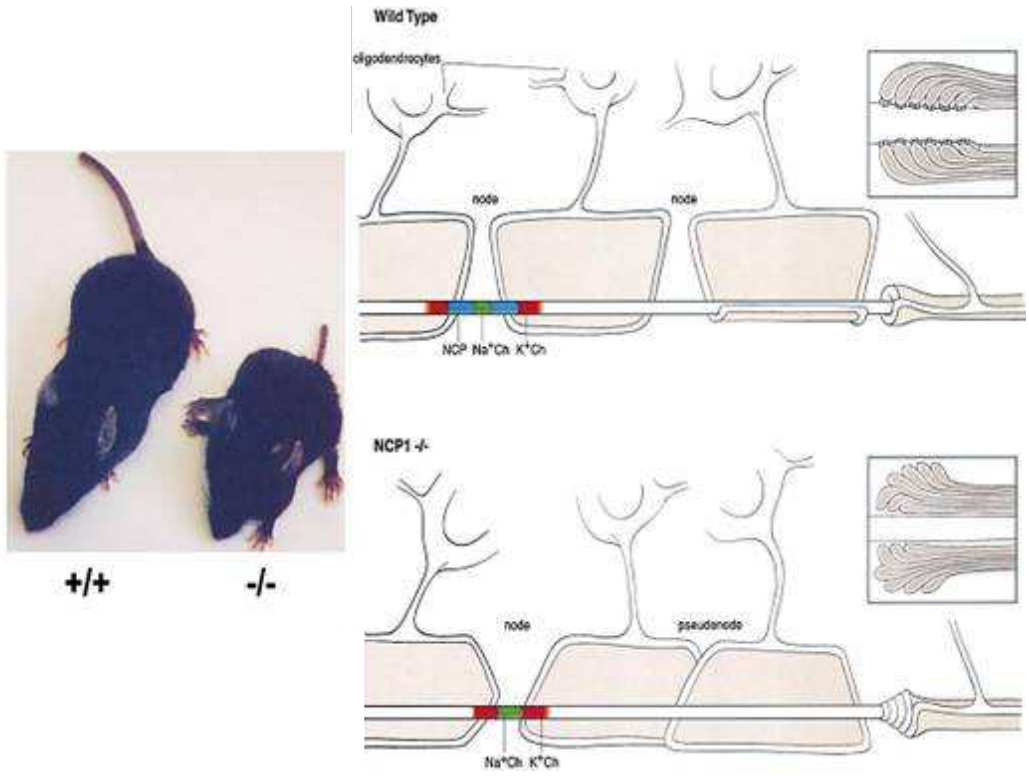

Figure 2.7: Phenotype of Caspr mutant mice. A) Phenotype of Caspr mutant Mice. Wild-type (on left) and Caspr mutant (on right) littermates at day 35 are shown; the Caspr mutant mouse is approximately half the weight of the wild-type mouse and also exhibits a characteristic wide base in the hind limbs. B) Diagram showing myelin sheaths elaborated by oligodendrocytes associated with axons in wild-type and Caspr mutant mice. In wild-type mice, the myelin sheaths have a trapezoidal shape when unrolled; the sheaths are wider in their proximal region, which forms the outer myelin lamellae. In the mutant mice, the trapezoidal shape is inverted and the distal region is wider. Corresponding insets show the normal organization of the paranodal junctions in the wild type mice and the everted arrangement of paranodal loops in the knockout mice (Bhat et al., 2001).

Contactin is undetectable in the paranodes in these mice, and potassium channels are displaced from the juxtaparanodal into the paranodal domains. Loss of Caspr results in decrease in peripheral nerve conduction velocity (Bhat et al., 2001). Other abnormalities in the Caspr mutants include segments that are wider in the middle of the sheath, which would give rise to a double row of paranodal loops, or adjacent segments that overlap in the presumptive nodal region resulting in "occluded pseudonodes". The potassium (K1) channels are mislocalized in the mutant mice. (Fig 2.7)

2.4 Serine proteases or serine endopeptidases

They are a class of peptidases (enzymes that cleave peptide bonds in proteins) that are characterised by the presence of a serine residue in the active site of the enzyme. Serine proteases are grouped into clans that share structural homology and then further subgrouped into families that share close sequence homology. The major clans found in humans include the chymotrypsin-like, the subtilisin-like, the alpha/beta hydrolase, and signal peptidase clans. Serine proteases participate in a wide range of functions in the body, including blood clotting, immunity, and inflammation, as well as contributing to digestive enzymes in both prokaryotes and eukaryotes.

Serine proteases are best characterized for their roles in the blood coagulation and fibrinolytic systems, but there is considerable evidence concerning the central functions of proteolytic cascades mediated by these enzymes in the nervous system (Gingrich and Traynelis, 2000). Established or proposed roles of serine proteases and their endogenous serpin inhibitors include: cell migration; neurite outgrowth and path finding; synaptic remodelling; Seizure activity, cell excitability; and both glial and neuronal cell survival (Moonen et al., 1982; Monard, 1988; Seeds et al., 1990; Liu et al., 1994a, b; Houenou et al., 1995; Tsirka et al., 1995, 1997; Davies B et al., 2001). These events are mediated, in part, by the ability of serine proteases to cleave, thereby activating growth factor precursor proteins, to degrade components of the extracellular matrix, and to bind to cell surface receptors, activating intracellular signalling cascades.

The enzymatic activity of serine proteases is tightly regulated, afforded in part by a series of specific endogenous serpin inhibitors. Imbalances between proteases and their inhibitors, due to injury or disease, have been shown to result in CNS pathogenesis, including neuronal degeneration (Tsirka et al., 1995).

2.4.1 Reelin and its Expression

Reelin (D'Arcangelo et al., 1995; De Silva, et al., 1997) is an extracellular protein essential for the development of laminated cortical brain structures in vertebrates (Rice DS and Curran T., 2001). Reelin is expressed in the adult brain, notably in the cerebral cortex, where it might play a role in synaptic plasticity. The mechanism of action of reelin at the molecular level has been the subject of several hypotheses.

The mouse reelin sequence (D`Arcangelo et al., 1995) encompasses 3461 amino acids and possesses a signal peptide followed by a domain with 28% sequence identity with F-spondin (as assessed by Ψ-Blast software)**,** a protein secreted by floor plate cells and promoting cell adhesion and neurite growth (Klar A et al., 1992). This region is followed by a unique region with no sequence homology, and then by eight internal repeats of 350-390 aa, each repeat containing two related subdomains flanking a cystein rich sequence similar to the epidermal growth factor (EGF)-like motif. The carboxy terminus region contains many positively charged amino acids required for secretion (D'Arcangelo et al., 1997)**.** Human reelin (De Silva et al., 1997) is 94.8% identical to the mouse protein at the amino acid level, indicating strong functional conservation.

2.4.2 Reelin Knockout

Loss of reelin in reeler mutant mice results in wide spread neuronal ectopia and ataxic behavior. Reelin is secreted during embryonic brain development by cells that are positioned near the pial surface of the cortical structures where neuronal radial migration terminates and cellular layers are formed. Genetic studies in combination with biochemical studies led to the discovery of some components of reelin signal transductions that are essential for cortical layer formation (D'Arcangelo et al., 1999; Hiesberger et al., 1999).

2.4.3 Functions of reelin

Several findings suggest that the reelin signal transduction involves binding to the very low density lipoprotein receptor (VLDLR) and to apoE receptor 2 (ApoER2) followed by intracellular activation of the adapter protein disabled-1 (Dab1) (Hiesberger et al., 1999; D'Arcangelo et al., 1999; Trommsdorff et al., 1999). Other possible reelin signal transduction pathways may involve interaction with the α3β1 integrin receptor (Dulabon et al., 2000; Anton et al., 1999) and with cadherin-related neuronal receptors (CNRs) (Senzaki et al., 1999).

Reelin interacts with Dab1 (The Disabled-1 (Dab1) gene encodes a key regulator of reelin signalling). This reelin–Dab1 signalling pathway plays a key role in neuronal cell migration and in the positioning of neurons within laminated structures (Katyal et al., 2007). Previous studies have demonstrated a role for Dab1 and reelin in dendrite formation. For example, a reduction in the density of amacrine dendrites has been observed in reelin$^{-/-}$ and Dab1$^{-/-}$ mice (Rice et al., 2001). Reelin and Dab1 phosphorylation have also been shown to be important for dendritic outgrowth of hippocampal pyramidal cells and dentate granule cells. Thus reelin has some positive effects on the neuritogenesis. On the other hand reelin also acts an inhibitory molecule.

Several hypotheses have been suggested regarding the function of reelin: i) Reelin may act as an attractant molecule for migrating neurons; ii) it may act as a repulsive molecule, or iii) Reelin may interrupt the association between migrating neurons and radial glia (Hoffarth et al., 1995; Dulabon et al., 2000), thus allowing migrating neurons to switch from a “gliophilic” to a “neurophilic” state (Anton et al., 1999).

It was shown that the hippocampal neurons grown on reelin substrate showed inhibited neurite outgrowth (Hoareau et al 2006). Excess of APP cytoplasmic domain internalized by a cell permeable peptide was able to antagonize the neurite outgrowth inhibition of reelin. The difference in the behavioural pattern of reelin as a promoting and an inhibitory molecule is attributed to the difference in coating reelin. In the studies of Hoareau and colleagues (Hoareau et al., 2006) neurons were cultured on a substrate including reelin bound to the dishes coated with polyornithine and laminin. It is interesting to note that the presence of laminin in the substrate can switch the response of growing neurites from attraction to repulsion in response to a given factor (Hopker et al., 1999). This raises the possibility that the stimulation of neurite outgrowth by reelin in the absence of laminin may be converted to neurite outgrowth inhibition in presence of laminin, and this may explain the divergent results obtained here and those reported by others.

Moreover, reelin has been recently shown to be expressed in several adult neuronal cells, including glutamatergic cerebellar granule neurons and specific GABAergic interneurons of the cerebral cortex and hippocampus (Pesold et al., 1999), and in the adult mammalian blood, liver, pituitary pars intermedia and adrenal chromaffin cells (Pesold et al., 1998; Smalheiser et al., 2000). The cellular function of reelin in the adult organism is unknown. Evidence is accumulating for involvement of reelin in human diseases such as autosomal recessive lissencephaly (Hong et al., 2000); schizophrenia (Guidotti et al., 2000) and autistic disorder (Persico et al., 2001).

2.4.4 Reelin is a serine protease of the extracellular matrix

There are enough evidences for reelin to be a serine protease namely: (i) Reelin contains the sequence G-K-S-D-G (aa 1280-1284 of human reelin) (De Silva et al., 1997), corresponding to the serine hydrolase consensus sequence G-X-SX-G; this sequence is 100% conserved among mouse, chicken and human reelin; (ii) Reelin shows significant structural similarities with serine hydrolases, such as the extracellular serine protease precursor (EC 3.4.21) of Serratia marcescens, and the probable ubiquitin carboxyl-terminal hydrolases FAM and FAF-Y (EC 3.1.2.15); (iii) Reelin contains eight EGF-like repeats (Fig 2.8); EGF-like repeats are observed in serine proteases, for example coagulation factors VII, IX and X, and protein C, Z (Kalafatis et al., 1997), calcium-dependent serine proteinase (CASP) (Kinoshita et al., 1989) which degrades extracellular matrix proteins, and complement C1s and C1r components (Kusumoto et al., 1988); (iv) several serine hydrolases, such as lipoprotein lipase and the urokinase-type plasminogen activator, bind VLDLR and ApoER2 (Cooper and Howell, 1999; Gliemann 1998).

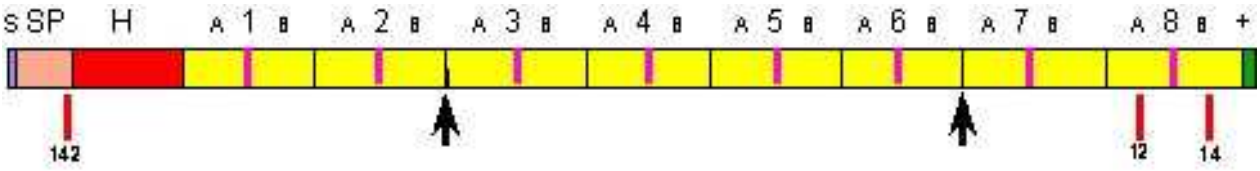

Figure 2.8 Structure of reelin protein

Reelin is cleaved invivo at two sites. The arrow marks indicate the cleavage sites in vivo. The G10 and CR 50 epitopes are close to 142. S-signal peptide, SP spondin similarity region , H unique region, 1 to 8 reelin repeats, composed of an EGF motif (pink)flanked by tow subrepeats A and B. Adapted from http://www.dene.ucl.ac.be/images/reln_csf.png.

In 2001 Quattrocchi et al showed that reelin is a serine protease of the extracellular matrix (ECM). Converging biochemical and cellular evidence shows that reelin is a serine protease, and that its enzymatic activity is important for the modulation of cell adhesion. CR-50 is a monoclonal antibody known to inhibit reelin function both *in vitro* and in vivo. This antibody also blocks the proteolytic activity of reelin (Fig 2.8); further supporting the hypothesis that proteolytic activity is of fundamental importance for the function of reelin. These findings appear interesting in view of the fact that serine proteases, such as tissue plasminogen activator (tPA), are already known to be important modulators of cell migration and axon growth.

3 Aim of the project

Initial studies using cross-linking approach have shown that PrP and Caspr interact with each other. Further analysis revealed a reduction in the levels of full length Caspr and a proportional increase of its degradation product in PrP -/- brains.

The aims of the present studies are:

- To analyse the binding between PrP and Caspr by other methods like coimmunoprecipitation and to verify direct binding between these molecules by ELISA.
- To investigate the role of PrP in proteolytic processing of Caspr in the central nervous system.
- To analyse the role of PrP in cell surface expression of Caspr.
- To identify the class of protease involved in the processing of Caspr.
- To analyse the functional role of this interaction in neurite outgrowth in developing neurons and axonal regeneration following spinal cord injury.

4 Materials and methods

4.1 Materials

4.1.1 Laboratory equipments

Gel documentation	Herolab, Germany
Spectrophotometer	BioTeK Instruments Inc, USA
Eppendorf 5804R	Eppendorf, Hamburg,Germany
Refrigerated Centrifuge	Beckman Instruments GmbH, Munich, Germany
Ulracentrifuge L8-70M	Beckman Instruments GmbH, Munich, Germany
Sorvall ultracentrifuge	Kendro, Hanau, Germany
Beckman XL-80 Ultracentrifuge	Beckman Instruments GmbH, Munich, Germany
SW28, 80Ti, 45Ti	(Beckman Instruments GmbH, Munich, Germany)
Table top centrifuge, Eppendorf centrifuge 5415 C	Eppendorf, Hamburg,Germany
PAGE and western blotting equipments	Bio-Rad laboratories, Hercules, CA, USA
Western blot developing Casette	Amersham pharmacia, Buckingamshire, UK
Western blot developing films	Kodak, Rochester, NY, USA
Axiophot microscope	Carl Zeiss Inc,Germany
Laser Scanning Microscope	Leica Inc, Germany
Shakers Heidolph promax 2020	Heidolph instruments, Schwabach
Red rotor	Hoefer pharmacia biotech, San francisco USA

Table top mixer Vortex genie 2	Scientific industries Inc, USA

4.1.2 Chemicals, reagents and kits

β-Mercaptoethanol	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
2-propanol	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
Acetic acid 100%	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
Acrylamide /Bis solution 29:1 30 % w/v	SERVA Electrophoresis GmbH, Heidelberg, Germany
Ammonium persulphate (APS)	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
Aprotinin	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
BC (bicinchoninic acid) Assay protein Quantification Kit	Uptima, INTERCHIM, France
B-PER® Bacterial Protein Extraction Reagent	Pierce, PerBio Science Deutschland GmbH, Bonn, Germany
Biotrace polyvinylidene fluoride transfer membrane (PVDF)	VWR International GmbH, Hannover, Germany
Bovine serum albumin (BSA)	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
Bromphenol blue	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
Calcium chloride CaCl2	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany

dATP, dCTP, dGTP, dTTP	Invitrogen GmbH, Karlsruhe, Germany
N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT)	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
Di Methyl Sulfoxide (DMSO)	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
Dulbecco modified Eagle's Medium (DMEM)	Invitrogen GmbH, Karlsruhe, Germany
Enhanced chemiluminescence (ECL) western blotting reagents	Amersham Pharmacia Biotech Europe GmbH, Frieburg, Germany
Ethanol absolute	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
Ethidium bromide	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
Ethylene diamine tetracetic acid (EDTA)	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
Glasgow MEM medium	Invitrogen GmbH, Karlsruhe, Germany
Glucose	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
Glycerol	Merck Biosciences GmbH, Bad Soden, Germany
Glycine	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
GM 6001	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
Hydrochloric acid (HCl)	Merck Biosciences GmbH, Bad Soden, Germany

Hank's balanced salt solution (HBSS)	Invitrogen GmbH, Karlsruhe, Germany
Lipofectamine	Invitrogen GmbH, Karlsruhe, Germany
Lipofectamine™2000	Invitrogen GmbH, Karlsruhe, Germany
Magnesium chloride (MgCl2)	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
MagnaBind Streptavidin beads	Pierce, Rockford, IL, USA
Methanol	Th. Geyer Hamburg GmbH & Co. KG
Neurobasal medium	Invitrogen GmbH, Karlsruhe, Germany
N,N,N',N'-Tetramethylethylenediamine (TEMED)	SERVA Electrophoresis GmbH, Heidelberg, Germany
NiNTA Agarose beads	QIAGEN GmbH, Hilden, Germany
Opti-MEM® I medium	Invitrogen GmbH, Karlsruhe, Germany
Orange G	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
Pepstatin	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
Phenylmethylsulfonyl flouride (PMSF)	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
Phenanthroline	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
Phospho creatine disodium salt hydrate enzymatic	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
Plus reagent	Invitrogen GmbH, Karlsruhe,

	Germany
Polyethylene glycol 300 (PEG 300)	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
Potassium chloride (KCl)	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
Potassium L-glutamate	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
Protease inhibitor cocktail EDTA free	Roche Diagnostics GmbH, Mannheim, Germany
Protein A/G plus agarose	Santa Cruz Biotechnology Inc., Santa Cruz, CA USA
Protran® nitrocellulose membranes	VWR International GmbH, Hannover, Germany
PureLink™HiPure Plasmid Filter Maxiprep Kit	Invitrogen GmbH, Karlsruhe, Germany
QIAfilter Plasmid Maxi kit	Qiagen GmbH, Hilden, Germany
QIAquick gel extraction kit	Qiagen GmbH, Hilden, Germany
QIAquick rapid PCR purification kit	Qiagen GmbH, Hilden, Germany
Restriction enzymes	New England Biolabs GmbH, Frankfurt am Main, Germany,
Shrimp Alkaline Phosphotase (SAP)	New England Biolabs GmbH, Frankfurt am Main, Germany.
Sodium azide (NaN3)	Merck Biosciences GmbH, Bad Soden, Germany
Sodium chloride (NaCl)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium Dodecyl Sulfate (SDS)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium fluoride (NaF)	Sigma-Aldrich Chemie Gmbh,

	Deisenhofen, Germany
Sodium orthovanadate (Na3VO4)	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
Sodium phospate dibasic (Na2 HPO4)	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
Sodium phosphate monobasic (NaH2PO4)	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
Sucrose	Sigma-Aldrich Chemie Gmbh, Deisenhofen, Germany
Sulfo-NHS- LC-biotin	Pierce, Rockford, IL, USA
SuperSignal West Dura	Perbio Science Deutschland GmbH, Bonn, Germany
SuperSignal West Pico	Perbio Science Deutschland GmbH, Bonn, Germany
T4 Taq polymerase	New England Biolabs GmbH, Frankfurt am Main, Germany
Tris (2-Amino-2-(hydroxymethyl)propane-1,3-diol)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Trypsin-EDTA	Invitrogen GmbH, Karlsruhe, Germany
Tween® 20 (polyoxyethylene (20) sorbitan monolaurate)	Merck Biosciences GmbH, Bad Soden, Germany

4.1.3 Molecular weight markers

Protein markers: Bench mark prestained protein ladder (Invitrogen biotechnologies, Carlsbad, CA, USA) and Precision plus Protein all blue standard (Bio-Rad Laboratories, Hercules, CA, USA)

4.1.4 Buffers and solutions

Blocking solution (For Immunoblotting)	3-5 % skimmed milk powder in phosphate buffered saline
Blocking buffer for antibody purification	200 mM Glycine 500 mM NaCl, pH 8.0
Coupling buffer	100 mM $NaHCO_3$ 500 mM NaCl, pH 8.3 adjusted with 100 mM Na_2CO_3, 500 mM NaCl
De-staining solution (For coomassie staining)	40 % (v/v) ethanol 10% (v/v) acetic acid
Dialysis buffer	50mM Tris HCl 150mM NaCl pH 7.2
DNA-sample buffer (5x) (For Agarose gels)	20% (w/v) glycerol in TAE buffer 0.025% (w/v) orange G
dNTP-stock solutions (PCR)	20 mM each dATP, dCTP, dGTP, dTTP
Elution buffer for affinity chromatography	50mM ethanolamine 150mM NaCl 0.2% CHAPS pH 11.5
Ethidium bromide solution (For agarose gels)	10 µg/ml ethidium bromide
Homo buffer (For whole brain homogenization)	320 M sucrose 1 mM $MgCl_2$ 1 mM $CaCl_2$ 1 mM $NaHCO_3$ 5 mM Tris-HCl

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Neutralization solution	1M Tris/HCl pH 6.8
PBS (Phosphate buffered saline)	10 mM Na_2HPO_4 2.5 mM NaH_2PO_4 150 mM NaCl 3 mM KCl
PBST (Phosphate buffered saline PBST)	10 mM Na_2HPO_4 2.5 mM NaH_2PO_4 150 mM NaCl 3 mM KCl 0.2% Tween
PBS with Ca^{2+} and Mg^{2+} for cell surface biotinylation	10 mM Na_2HPO_4 2.5 mM NaH_2PO_4 150 mM NaCl 0.2mM $CaCl_2$ 2mM $MgCl_2$
Radio Immunoprecipitation assay (RIPA) buffer for immunoprecipitation	50 mM Tris 150 mM NaCl 2 mM EDTA 1 mM Na_3VO_4 1 mM NaF 1% Nonidet P-40 100 µM PMSF Protease inhibitor cocktail-EDTA free. Used according to the manufacture's instructions.
Regeneration buffers for the Affinity columns	0.1 M Tris/HCl,

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Buffer A	500mM NaCl
	pH 8.5
	0.1 M Na Acetate
Buffer B	500 mM NaCl
	pH 7.2
	PBS + Sodium Azide 0.02%
Buffer C	
	0.312 M Tris-HCl pH 6.8
	10% (w/v) SDS 5 % (w/v)
Sample buffer for SDS-PAGE (5x)	5% (w/v) β-Mercaptoethanol
	50% (v/v) Glycerol
	0.13% (w/v) Bromphenol blue
	192 mM glycine
SDS-PAGE running buffer	25 mM Tris
	0.1% SDS
	40% (v/v) ethanol
Staining solution (SDS-PAGE)	10% (v/v) acetic acid
	0.1% (w/v) Brilliant blue R250
Swelling solution forAntibody purification	1 mM HCl
	192 mM glycine
Transfer buffer (For electrophoretic transfer of proteins)	25 mM Tris
	0.1% SDS
	20% methanol
Tris EDTA (TE) buffer 10x (Solvent for DNA)	0.1 M Tris-HCl, pH 7.5
	10 mM EDTA
Tris EDTA acetate (TAE) buffer 50X	2 M Tris-Acetate, pH 8

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(Buffer for running DNA agarose gels)	100 mM EDTA 10 mM Tris-HCl, pH 8.0 150 mM NaCl
TSS buffer in Luria-Bertani broth	10 % w/v PEG 300 30 mM $MgCl_2$ 5 % v/v DMSO Filter sterilize with 0.22 μm filter.
Washing buffer A for affinity purification of brain homogenates	25mM Tris-HCl 150 mM NaCl 5mM EDTA,pH 7.4 25mM Tris
Washing buffer B for affinity purification of brain homogenates	150mM NaCl 1% Triton-X-100 0.02% Sodium Azide, pH 7.4 25mM Tris
Washing buffer C for affinity purification of brain homogenates	500mM NaCl 0.1% Triton-X-100 0.02% Sodium Azide, pH 7.4
Washing buffer A for antibody column	100 mM Sodium Acetate 500 mM NaCl pH 4.0
Washing buffer B for antibody column	100 mM $NaHCO_3$

	500 mM NaCl, pH 8.0
Washing buffer C for antibody column	PBS
X-1 medium for primary cultures-cerebellar neurons.	See page 28 for components

4.1.5 Plasmids

Contactin in pcDNA3 vector	For expression of full length Contactin in mammalian cells	Kind gift of Dr.Gianfranco Gennarini, University of Bari, Bari, Italy
pcDNA3	Mammalian expression vector for transfection.	Invitrogen GmbH, Karlsruhe, Germany
Caspr in pcDNA3 vector	For expression of full length Caspr in mammalian cells	Kind gift of Dr. Elior Peles, Weismann institute, Rehovot, Israel.
PrP in pcDNA3 vector	For expression of full length Prion protein in mammalian cells	PrP plasmid was kind gift from Dr. Sylvain Lehmann (Institut de Génétique Humaine du CNRS, Montpellier, France)

4.1.6 Primary and secondary antibodies

Mouse monoclonal antibody 8H4 against PrP^C, a generous gift from Dr. Man Sun Sy (Case Western Reserve University, Cleveland, OH, USA), was used for immunocytochemistry. Rat monoclonal antibody 555 against L1 (Appel et al., 1993) was used for immunocytochemistry and immunoblotting. Rabbit polyclonal antibodies against the extracellular domain of Caspr (L23, L17), a kind gift of Catherine Faivre Sarrailh (Institut Jean-Roche, Marseille, France), were used for immunocytochemistry and neurite outgrowth. Polyclonal goat antibodies against the N-terminus of Caspr (N15) from Santa Cruz Biotechnology (Santa Cruz, CA, USA) were used for immunocytochemistry, Western blot and neurite outgrowth. Goat polyclonal antibodies

against PrP^C (M20) from Santa Cruz Biotechnology were used for immunoprecipitation and Western blot. We also used for Western blot polyclonal rabbit antibodies against the FGF receptor (Flg) from Santa Cruz Biotechnology, polyclonal rabbit antibodies against CHL1 (Buhusi et al., 2003), Polyclonal rabbit antibodies against actin from Sigma (St. Louis, MO, USA), polyclonal goat antibodies against F3/F11/contactin from R&D Systems (Wiesbaden-Nordenstadt, Germany), mouse monoclonal antibody against reelin from Millipore (Billerica, MA, USA), rabbit polyclonal antibodies against MAG (Hu et al., 2003), and mouse monoclonal antibody against GAPDH from Millipore. Secondary antibodies against goat, rabbit, rat, and mouse Ig coupled to Cy2, Cy3, Cy5 or HRP were from Dianova (Hamburg, Germany).

4.1.7 Primary cell culture media

For 100ml X-1 medium the following components have been mixed and filter sterilised. Final concentrations are given in brackets.

MEM 97ml; 7.5% NaHCO3-1.5ml (2.2mg/ml); 100x Penicillin/streptomycin-1ml; 10% Bovine serum albumin-1ml (0.1%); 10mg/ml Insulin-100µl (10µg/ml); 4µm L-thyroxine-100µl (4nM); 50mg/ml transferrin holo-200µl (100µg/ml); 5.4 TIU/mg aprotinin-100µl (0.027 TIU/ml); 30µm sodium selenite-100µl (30nm)

4.1.8 Bacterial and mammalian cell culture medium

Bacterial media was autoclaved and antibiotics added later to warm media.

LB-medium	10 g/l Bacto-tryptone 10 g/l NaCl 5 g/l yeast extract
LB ampicillin medium	100 mg/l ampicillin in LB-Medium
LB ampicillin plates	20 g/l agar in LB-Medium 100 mg/l ampicillin
LB kanamycin medium	25 mg/l kanamycin in LB-Medium
LB kanamycin plates	20 g/l agar in LB-Medium 25 mg/l kanamycin
CHO cell medium	Glasgow MEM (G-MEM) (with L-Glutamine),

10% foetal calf serum

20 ml/l penicillin/ streptomycin solution (100x)

10 ml/l MEM non essential amino acids solution (100X)

1 mM sodium pyruvate MEM

0.4 mM L-glutamic acid , 0.45mM aspartic acid.

4.1.9 Animals

PrP-/- mice were as described (Santuccione et al., 2005). Caspr-/- mice were kindly provided by Dr. Elior Peles (Weizmann Institute of Science, Rehovot, Israel). Six to ten week old animals were used for biochemical experiments.

4.1.10 Bacterial strains and cell lines

CHO-K1	Chinese Hamster Ovary for ectopic mammalian protein expression. Dehydrofolatereductase deficient	ATCC (American Type Culture Collection) CCL 61
DH5α	Escherichia coli strain for cloning and sub-cloning.	New England Biolabs GmbH, Frankfurt am Main, Germany,

4.2 Methods

4.2.1 Molecular biology

4.2.1.1 Maintainence of bacterial strains

Strains were stored as glycerol stocks (LB-medium, 25% (v/v) glycerol) at –80°C.

An aliquot of the stock was streaked on an LB-plate containing the appropriate antibiotics and incubated overnight at 37°C. A single colony was picked up and inoculated into 2 ml of LB

broth with the selection antibiotic and incubated at 37°C either for 4 hours for a starter culture or 8 hours for isolating plasmids (Sambrook et al 1989).

4.2.1.2 Production of competent bacteria

Competant cells were made as according to protocol (Inoue et al., 1990) described briefly below. An innoculum of DH5α or BL21 bacteria from a glycerol stock was streaked on a LB plate to obtain a single colony. This single colony was used to obtain a 5ml overnight culture in LB medium. This culture was diluted 100 fold in LB medium. The diluted culture was grown to an OD_{600} of 0.3 - 0.5. The culture was spilt into two 50ml falcon tubes and incubated on ice for 10 min. All subsequent steps were carried out at 4°C on ice or in the cold room. The cells were pelleted down by centrifuging at 5000 g for 10 minutes at 4°C. The supernatant was discarded. The pellet of cells was resuspended in chilled 5 ml of TSS buffer (refer to materials). The pellet was resuspended thoroughly by vortexing gently to ensure that there are no lumps. The resuspended cells are centrifuged again at 5000 g for 10 minutes at 4°C and the pellet resuspended in 5 ml of pre chilled TSS buffer. Aliquots of 100 µl of the resuspended cells were made in pre-chilled eppendorfs and immediately frozen in liquid nitrogen.

4.2.1.3 Transformation of bacteria

To 100 µl of thawed competent DH5α or BL21 on ice, either 50-100 ng of plasmid DNA or 20 µl of ligation mixture were added and incubated for 30 min on ice. After a heat shock (90 seconds, 42°C) and successive incubation on ice (3 min), 800 µl of LB-medium without antibiotics was added to the bacteria and incubated at 37°C for 40 min. If the transformation was with a circular plasmid, then 100 µl of the transformation mixture was plated on LB plates with the selection antibiotic. If a ligation mixture was used for the transformation, then cells were centrifuged (5000 g, 1 min., RT) and the supernatant removed. Cells were then resuspended 100 µl LB medium and plated on LB plates containing the appropriate antibiotics. Plates were incubated at 37°C overnight (Sambrook 1989).

4.2.1.4 Plasmid isolation

All plasmid isolations were done as per requirement using kits mentioned in materials. Protocols followed were according to the manufacturer's instructions.

4.2.1.5 Determination of DNA concentration and purity

DNA concentrations were determined spectroscopically. The absolute volume necessary for measurement was 10 µl. For determining the concentration of DNA in case of large-scale

plasmid preparations, the DNA was diluted appropriately with distilled water before taking the reading. Concentration was determined by measuring the absorbance at 260 nm and 280 nm. Absorbance at 260 nm had to be higher than 0.1 but less than 0.6 for reliable determinations. The ratio of the absorbance of DNA solution and the absorbance of the dissolvent was measured at 260 nm wave length by BioSpec-mini spectrometer (Shimadzu, Duisburg, Germany). The concentration of DNA was calculated from the absorbance at 260nm (50 µg/ml of double stranded DNA has an absorbance of 1 at 260nm). A ratio of A_{260}/A_{280} between 1.8 and 2 indicated sufficient purity of the DNA preparation for either transformation or further enzymatic modifications.

4.2.1.6 DNA gel electrophoresis

DNA fragments were separated by horizontal electrophoresis in DNA electrophoresis chambers (BioRad) using agarose gels. Agarose gels were prepared by heating 1-2 % agarose (w/v) (depending on the size of DNA fragments) in 1xTAE buffer till all the agarose is dissolved and then poured onto DNA gel trays. The gel was covered with 1xTAE buffer and the DNA samples were pipetted into the sample pockets. DNA sample buffer was added to the samples and the gel was run at constant voltage (10 V/cm gel length) until the orange G dye had reached the end of the gel. Afterwards, the gel was stained in an ethidium bromide staining solution for 20 min. The gels were documented using the E.A.S.Y. UV-light documentation system (Herolab, Wiesloh, Germany).

4.2.2 Cell culture

4.2.2.1 Maintainence of CHO cells

CHO cells were either cultured in GMEM with 10 % FCS (fetal calf serum) and 2% Penicillin/Streptomycin (P/S) 37°C, 5 % CO2 and 90 % relative humidity in 75 cm^2 flasks (Nunc) with 15 ml medium or in six-well plates (d = 35 mm; area = 9.69 cm^2) with 2 ml medium. Cells were passaged when they were confluent (usually after 3-4 days). Medium was removed and cells were detached by incubation with 4 ml trypsin EDTA for 5 min at 37°C. Cells were resuspended in 10 ml fresh medium and were split 1:10 for maintenance or seeded in six- well plates for transfection (300 µl per well).

4.2.2.2 Transient transfection of CHO cells

CHO cells were transfected with plasmids using Lipofectamine along with Plus™reagent as per manufacturer's instructions. The transfected cells are maintained for 48 hours for transfected gene expression by adding fresh GMEM every 20 hours.

4.2.2.3 Lysis of CHO cells

After maintenance of CHO cells in 35 mm-culture dishes, the medium was removed and washed thrice with ice cold PBS. The cells were lysed in 0.5 to 1 ml of RIPA buffer per 35 mm well with constant agitation (1 h, 4°C). Cells were scraped of the wells and transferred into a 1.5 ml eppendorf tube. In case of analysis of supernatants for shedding product, supernatants are collected 48hrs after transfection and the cells are lysed and lysate collected as above for further analysis by western blot.

4.2.2.4 Processing of coverslips for primary neuronal cultures – PLL coating of coverslips

Coverslips were incubated in acetone at 4°C overnight under gentle shaking, washed 5 times with sterile distilled water, washed twice for 10 min each with ethanol, and sterilized for 2h at 160°C. Further steps were done under sterile hood.

When coverslips cooled down to room temperature, they were coated with PLL by incubating them in 10% PLL in distilled water or PBS overnight at 4°C under continuous and gentle shaking. Coverslips were washed three times with distilled water, dried on tinfoil under the hood, incubated for 30 min under UV light under the hood at room temperature, and stored sterile until use. Before use, they were again sterilized under UV for 15 min.

4.2.2.5 Preparation of dissociated cerebellar cultures

For preparation of dissociated cerebellar cultures, mice of postnatal day 6 – 7 were used. Cerebellum was prepared as followed:

4.2.2.5.1 Dissection

Mice were decapitated and cerebellum removed from skull. The decapitated head was dipped into a petriplate containing 70% ethanol for sterilisation. Then the cerebella were carefully dissected out from the whole brain using a tissue forcep. The cerebella were placed on the sides of falcon tube and crushed using a forcep. The cerebella were washed three times using HBSS before trypsin digestion.

4.2.2.5.2 Digestion

Cerebella were washed twice with ice cold HBSS solution and treated with Trypsin and DNAse I for 15 min at RT. Digestion solution was removed by allowing cerebellar pieces to settle down. Cerebella were washed three times with HBSS.

4.2.2.5.3 Dissociation

Cerebella were resolved in dissection solution containing DNAseI. Titration with pasteur pipettes having successively smaller diameters dissociated cerebella to homogeneous suspensions.

4.2.2.5.4 Removal of cell debris and plating of cells

By subsequent centrifugation (80 x g, 15 min, and 4°C) and resuspension in dissection buffer, cell debris was removed. Cells were counted in a Neubauer cell chamber and plated to provide a densitity of 1.000 cells/mm^2.

4.2.2.5.5 Neurite outgrowth assay

Cell were maintained in 12-well plates (Nunc, Roskilde, Denmark) on glass coverslips (15 mm in diameter; Hecht, Sondheim, Germany) substrate-coated with poly-l-lysine (PLL; Sigma) followed, when indicated, with Caspr antibodies (1µg/ml), laminin (Sigma; 1µg/mL) or PrP-Fc (1µg/ml), and cultured in chemically defined serum-free medium (Chen et al., 1999). When indicated, aprotinin (1µM) was added to the medium. Cells were fixed in 22h with 2.5% glutaraldehyde in PBS and stained with toluidine blue/trypan blue. The total length of all neurites per neuron was measured by the KS image analysis system (Kontron, Zeiss, München, Germany). At least 3 coverslips for each group were taken in each experiment, and at least 100 cells form one coverslips were analysed. Experiments were reproduced three times with similar effects.

4.2.2.6 Immunocytochemistry

Indirect immunofluorescence staining was performed as described previously (Sytnyk et al., 2002; Leshchyns'ka et al., 2003). Neurons on coverslips were washed in PBS, pH7.4, and fixed in 4% formaldehyde in PBS for 15min at RT. Neurons were then washed three times with PBS, permeabilized with 0.25% Triton X-100 in PBS applied for 5min, blocked in 1% BSA in PBS and incubated with primary antibodies diluted in 1% BSA in PBS for 1h. Primary antibodies were detected with corresponding secondary antibodies applied in 1% BSA in PBS for 30min. To detect Caspr at the cell surface, antibodies against Caspr extracellular domain were applied

to live neurons for 20min in the culture medium in a CO2 incubator, followed by secondary antibodies applied for 15min in the culture medium in a CO2 incubator. In cocapping experiments, antibodies against Caspr extracellular domain and PrP were applied together in the culture medium for 15min, followed by secondary antibodies applied in the culture medium for 15min in a CO2 incubator. Coverslips were embedded in Aqua-Poly/Mount (Polysciences, Inc, Warrington, PA, USA). Images were acquired at RT using a confocal laser scanning microscope TCS-SP2 (Leica, Wetzlar, Germany) using 63x objective. The images were exported using LSM510 software (version 3; Zeiss, Jena, Germany)

Analysis of Caspr shedding to the culture medium

Inhibitors (aprotinin (1μM, Sigma), pepstatin (1μM, Sigma), phenanthrolamine (10μM, Sigma), GM6001 (1μM, Merck, Darmstadt, Germany)) or PrP-Fc (1μg/ml) were applied 6h after plating. Cells were maintained for 5 days with refreshing medium and inhibitors each second day. Supernatants were then collected. Proteins in the supernatants were then concentrated by Amicon columns with 10kDa molecular weight cutoff (Millipore) and used for Western blot.

4.2.2.7 Spinal cord injury.

Mice were anesthetized by intraperitoneal injections of ketamine and xylazine [100 mg of Ketanest (Parke-Davis/Pfizer, Karlsruhe, Germany) and 5 mg of Rompun (Bayer, Leverkusen, Germany), per kilogram of body weight]. Laminectomy was performed at the T7–T9 level with mouse laminectomy forceps (Fine Science Tools, Heidelberg, Germany). A mouse spinal cord compression device was used to elicit compression injury (Curtis et al., 1993). Compression force (degree of closure of the forceps) and duration were controlled by an electromagnetic device. The spinal cord was maximally compressed (100%, according to the operational definition of Curtis et al., 1993Go) for 1 s by a time-controlled current flow through the electromagnetic device. The skin was then surgically closed using 6-0 nylon stitches (Ethicon, Norderstedt, Germany). After the operation, mice were kept in a heated room (35°C) for several hours to prevent hypothermia and after that singly housed in a temperature-controlled (22°C) room with water and standard food provided. During the postoperative time period, the bladders of the animals were manually voided twice daily.

4.2.2.8 Analysis of motor function.

The recovery of ground locomotion was evaluated using the Basso, Beattie, Bresnahan (BBB) rating scale (Basso et al., 1995), modified for mice (Joshi and Fehlings, 2002), and a novel single-frame motion analysis (Apostolova et al., 2006). This method includes evaluation of four

parameters in three different tests: beam walking (foot-stepping angle and rump-height index), voluntary movements without body weight support (extension–flexion ratio), and inclined ladder climbing (number of correct steps). Assessment was performed before, and at 1, 3, and 6 weeks after the injury. Values for the left and right legs were averaged.

4.2.2.9 Preparation of tissue samples and immunohistochemistry

Tissue preparation and immunohistochemical staining were performed as described previously (Irintchev et al., 2005). The mice were anaesthetized with sodium pentobarbital (Narcoren®, Merial, Hallbermoos, Germany, 5 µl g-1 body weight, i.p.) and perfused transcardially with saline (0.9% NaCl) followed by fixative (4% formaldehyde and 0.1% CaCl2 in 0.1 M cacodylate buffer, pH 7.3, 15 minutes at room temperature). Brains were removed from the skulls and post-fixed overnight (18 - 22 hours) at 4°C in the fixative solution supplemented with 15% sucrose, followed by immersion in 15% sucrose solution in 0.1 M cacodylate buffer, pH 7.3, for an additional day at 4°C. The tissue was frozen for 2 minutes in 2-methyl-butane (isopentane) pre-cooled to -30°C in the cryostat and stored in liquid nitrogen until sectioned. Serial coronal sections of 25 µm thickness were cut in a caudal-to-rostral direction on a cryostat (Leica CM3050, Leica Instruments, Nuβloch, Germany). Sections were collected on SuperFrost®Plus glass slides (Roth, Karlsruhe, Germany), air-dried for at least 1 hour at RT and stored in boxes at -20°C until stainings were performed.

The following antibodies were used at optimal dilutions: anti-PrP^C (8H4, mouse monoclonal antibody) and anti-Caspr (rabbit polyclonal antibody raised in our lab). Prior to the staining, antigen de-masking using 0.01 M sodium citrate solution (pH 9.0) was done in a water bath (80°C, 30 min) (Jiao et al., 1999). Blocking of non-specific binding sites was performed for 1 hour at RT using phosphate-buffered saline (PBS, pH 7.3) containing 0.2% Triton X-100, 0.02% sodium azide and 5% normal serum from the species in which the secondary antibody was produced. Incubation with the primary antibody, diluted in PBS containing 0.5% lambda-carrageenan (Sigma) and 0.02% sodium azide, was carried out at 4°C for 3 days. After wash in PBS (3 x 15 min at RT), the appropriate secondary antibody conjugated with Cy3 (Jackson ImmunoResearch Laboratories, Dianova, Hamburg, Germany) diluted 1:200 in PBS-carrageenan solution was applied for 2 hours at RT. Finally, after a subsequent wash in PBS, cell nuclei were stained for 10 min at RT with bis-benzimide solution (Hoechst 33258 dye, 5 µg ml^{-1} in PBS, Sigma aldrich) and sections were mounted under coverslips with anti-fading medium (Fluoromount G, Southern Biotechnology Associates, Biozol, Eching, Germany).

Materials and Methods

Double staining for PrP and Caspr was performed by mixing the primary antibodies at optimal dilutions and using a Cy3-conjugated anti-mouse and a Cy2-labeled anti-rabbit secondary antibody pre-absorbed with rabbit and mouse serum proteins, respectively (multiple-labeling grade antibodies, Jackson immunoResearch). For a given antigen, all sections were stained in the same solution kept in screw-capped staining plastic jars (capacity 35 ml, 10 slides, Carl Roth). Specificity of staining was tested by omitting the first antibody or replacing it by variable concentrations of normal serum or IgG.

4.2.3 Protein biochemistry

4.2.3.1 Determination of protein concentration by Bicinchoninic Acid assay method (BCA).

The protein concentration of cell lysates was determined using the BCA kit (Pierce). Solution A and B were mixed in a ratio of 1:50 to give the BCA solution. 20 µl of the cell lysate were mixed with 200 µl BCA solution in microtiter plates and incubated for 30 min at 37°C. A BSA standard curve was co-incubated ranging from 100 µg/ml to 2 mg/ml. The extinction of the samples was determined at 568 nm in a microtiter plate reader.

4.2.3.2 SDS-Poly acrylamide gel electrophoresis (SDS-PAGE)

Protein samples were subjected 7.5-16 % (depending on the molecular weight of the protein of interest) SDS-PAGE under reducing conditions using the Mini-Protean II system (Bio-Rad) using standard protocols (Laemmli, 1970). Gel composition is given under materials. After polymerization of the gels, the chamber was assembled as described by the manufacturer's protocol. Samples were diluted with appropriate amount of 5X SDS sample buffer followed by boiling for 10 minutes at 100ºC and 10 µg total protein of each sample was loaded per well. The assembly was filled with 1X SDS running buffer and the gel was run at a constant 60V for approximately 15 min and then at 100V for the remainder. The run was stopped when the bromophenol blue running front had reached the end of the gel. Gels were either stained or subjected to Western blotting.

Resolving gel composition for SDS-PAGE	375 mM Tris-HCl pH 8.8 0.1% SDS 0.025% APS 0.001% TEMED Acrylamide /Bis solution 29:1 (according to % of gel required)
Stacking gel composition for SDS-PAGE	125 mM Tris-Hcl pH 8.8 0.1% SDS 0.06% APS 0.025% TEMED 5% Acrylamide /Bis solution 29:1 (according to % of gel required)

4.2.3.3 Production of the polyclonal antibodies against the intracellular domain of Caspr

Polyclonal antibodies against the intracellular domain of Caspr were generated by immunizing rabbits with a peptide derived from the Caspr intracellular domain (H-APGPRDQNLPQILEESRSEC-OH). Imject Maleimide Activated McKLH Kit (Pierce) was used for conjugating the peptide to the carrier protein for animal injections. Animals were sacrificed after 5 boosts and the blood was collected and processed to collect the antiserum. Antiserum thus obtained was purified further by affinity chromatography using Protein G Sepharose 4 fast flow column. Bound antibodies were eluted using 0.1M glycine (pH 2.7-3.0). Eluted material was neutralized with 1M Tris/HCl (pH 8.8) and dialyzed extensively against phosphate-buffered saline (PBS). Purified antibodies were used for biochemical and cytochemical studies.

4.2.3.4 Purification of Caspr

Caspr was purified from brain homogenates using polyclonal antibodies against the intracellular domain of Caspr using BioRad Econosystem (BioRad, Hercules, CA, USA). Antibodies were coupled with CNBr-activated Sepharose according to the manufacture instructions (Amersham

Pharmacia). Brains from adult mice were homogenized in buffer containing 5mM Tris, pH7.5, 0.32M sucrose, 1mM $CaCl_2$, 1mM $MgCl_2$ and 1mM $NaHCO_3$, NP40 0.5% and EDTA-free complete protease inhibitor cocktail (Roche) and centrifuged at 2000g for 15min at 4^{o}C. The supernatant was then collected, and lysed overnight at 4^{o}C by adding N-octyl glucoside and Triton X-100 to the final concentration of 1% and 0.5%, respectively. The lysate was centrifuged at 50000g for 1h at 4^{o}C. The resulting supernatant was collected and applied to the column containing immobilized polyclonal antibodies against Caspr. Bound proteins were eluted with 50mM ethanolamine (pH11.5), 150mM NaCl, and 0.2% CHAPS, and immediately neutralized with 1M Tris HCl (pH 6.8). Protein-containing fractions were collected, dialysed overnight at 4°C with TBS and verified for the presence of Caspr by SDS-PAGE followed by silver staining

4.2.3.5 Coomassie staining of polyacrylamide gels

After SDS-PAGE, the gels were stained in staining solution (1h, RT) with constant agitation. The gels were then incubated in de-staining solution until the background of the gel appeared nearly transparent.

4.2.3.6 Electrophoretic transfer of proteins

Proteins were transferred after SDS-PAGE (see above) onto a Protran® nitrocellulose or Biotrace PVDF membrane using a MINI TRANSBLOT-apparatus (Bio-Rad). After equilibration of the polyacrylamide gel in transfer buffer for approximately 5 min, a blotting sandwich was assembled as described in the manufacturer's protocol. Proteins were transferred at 4°C in blot buffer at constant voltage (80V for 2 h or 35V overnight).

4.2.3.7 Immunochemical detection of electrophoretically transferred protein

After electrophoretic transfer, membranes were removed from the sandwiches and placed protein-bound side-up in glass vessels. Membranes were washed once in PBST for 5 min and were subsequently blocked for 1 h in PBST with 3% skimmed milk powder under gentle shaking at room temperature. Incubation with an appropriate antibody dilution in PBST with 3% skimmed milk powder, was performed either for 90 minutes at room temperature or overnight at 4°C. The primary antibody solution was removed and membranes were washed 5x10 min with PBST under constant shaking. The appropriate horseradish peroxidase (HRP)-conjugated secondary antibody was applied at a concentration varying from 10 ng/ml to 1 ng/ml in PBST with 5% skimmed milk powder, for 90 minutes at room temperature. The membrane was washed six times by incubating with PBST with constant shaking and changing the PBST at five

minute intervals. Immunoreactive bands were visualized using the chemiluminescence detection reagents on BIOMAX film (Sigma-Aldrich). The membrane was soaked for 5 min in detection solution (1:1 mixture of kit-supplied reagent I and II of enhanced cheminuminisence detection system). The detection solution was drained off and the blot was placed between plastic sheets. The membrane was exposed to BIOMAX ML (Kodak) for several time intervals, starting with a 1-min exposure to 12 hours. In case of weak signal detection of the desired protein, enhanced chemiluminiscence was used to amplify the signal.

4.2.3.8 Immunological detection using enhanced chemiluminescence

The antibody bound to the membrane was detected using the enhanced chemiluminescence detection system (SuperSignal West Dura or SuperSignal West Pico). The membrane was soaked for 5 min in detection solution (1:1 mixture of solutions I and II). The solution was removed and the blot was placed between transparent plastic sheets. The membrane was exposed to BIOMAX ML film (Kodak) for several time periods, starting with a 30 sec exposure to 24 hours.

4.2.3.9 Densitometric evaluation of band intensity

Chemiluminescence was quantified using the image processing software, TINA 2.09 software (University of Manchester, UK). The developed film was scanned and the digitized picture was exported to the image processing program. The quantified data was analyzed using Microsoft Excel software.

4.2.3.10 Stripping and re-probing of Western blots and dot blots

For detection of an additional protein on the immunoblot, the Nitrocellulose membranes were stripped from bound antibodies by shaking blots in stripping buffer for 10 min at room temperature. Blots were neutralized by incubation for 2x5 min in 1MTris-HCl (pH 7.5) and again subjected to blocking as described above.

4.2.3.11 Protein interaction detection methods

4.2.3.11.1 Chemical crosslinking

All steps for chemical cross-linking were performed under light protection. PrP-Fc (1mg) was incubated with sulfosuccinimidyl-2-[6-(biotinamido)-2(*p*-azidobenz-amido) hexa-noamido]ethyl-1,3'-dithiopropionate (sulfo-SBED; Perbio, Rockford, IL, USA) (6µl) dissolved in dimethyl sulfoxide (12µl) for 30 min at room temperature (RT). The sample was then dialyzed overnight using a Slide-a-lyzer mini dialysis unit (Perbio). Protein-A magnetic beads

(500 µl, Dynal, Hamburg, Germany) were added, and the mixture was incubated for 1 h at RT. To further stabilize the complex, BS3 (2mg, Pierce Chemicla Co.) was added and the mixture was incubated for 1 h at RT. Magnetic beads were washed six times with phosphate buffered saline (PBS), pH7.4, using a magnet. Beads containing PrP-Fc bait were then incubated overnight at 4°C with axolemma fraction, transferred to a 40X10 cm cell culture dish, placed on ice and exposed to UV light for 15 min in a UV cross-linker (Amersham, Buckinghamshire, UK) at an energy setting of 365. Beads were then washed three times with PBS, two times with PBS containing 0.5% Triton X-100 and two times with PBS containing 1% SDS. Bait/prey protein complexes were eluted from beads by 10 min incubation in elution buffer (0.1M glycine (pH2.3) and 0.5M NaCl). Following removal of the beads with a magnet, the solution containing the bait/prey complexes was neutralized with neutralization buffer (1.5M Tris-HCl, pH 8.0) and subsequently boiled in sodium dodecyl sulfate (SDS) sample buffer containing dithiothreitol to separate bait from prey. Biotinylated prey proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and detected with horseradish peroxidase (HRP)–coupled streptavidin. The identity of Caspr was confirmed by Western blot analysis with polyclonal antibodies against Caspr.

4.2.3.11.2 Immunoprecipitation

Homogenates (1mg of protein) were lysed for 30min in 50mM Tris-HCl buffer (pH 7.5), containing 150mM NaCl, 0.5% Triton X-100, 1% octyl-D-glucopyranoside, 1mM sodium fluoride, 2mM $NaVO_4$, 0.1mM PMSF and EDTA-free protease inhibitor cocktail (Roche). The lysis buffer containing this combination of detergents completely solubilizes lipid rafts and has been used in a number of studies involving coimmunoprecipitation of lipid raft components (Trupp et al., 1999; Paratcha et al., 2001; Santuccione et al., 2005). Samples were then centrifuged for 15min at 20000g and 4°C. Supernatants were cleared with protein A/G-agarose beads (Santa Cruz Biotechnology, Inc.) for 3h at 4°C and incubated with monoclonal antibodies against PrP^C (overnight at 4°C), followed by precipitation with protein A/G-agarose beads applied for 3h at 4°C. The beads were washed three times with lysis buffer, once with PBS, and analyzed by immunoblotting (Leshchyns'ka et al., 2003).

4.2.3.11.3 Enzyme-linked immunosorbent assay (ELISA)

L1-Fc (2µg/ml, Chen et al., 1999), PrP-Fc (2µg/ml, Chen et al., 2003), and BSA (2µg/ml) were immobilized on polyvinylchloride surface in 96-well microtiter plate in PBS overnight at 4°C. Non-absorbed proteins were removed and wells were washed five times for 5min at RT with PBS containing 0.05% Tween-20 (PBS-T). Wells were blocked for 1h at RT with 1% BSA in

PBS and subsequently incubated with different concentration of Caspr (2µg/ml to 0µg/ml) in PBS-T containing 3% of BSA for 2h at RT. Wells were then washed five times for 5min each at RT with PBS-T and bound Caspr was detected with Caspr antibodies followed by HRP-conjugated secondary antibodies. Protein binding was visualized by detecting HRP with ABTS reagent that resulted in colored product that was quantified using an ELISA reader at 562 nm.

4.2.4 Isolation of subcellular organelles by biochemical fractionation

4.2.4.1 Isolation of myelin

Myelin from mouse brain was purified essentially as described by (Norton and Poduslo,1973). In brief, tissue homogenates of 5 % (w/v) were prepared from brain in 0.32M sucrose. Some of the homogenized brain was stored at –80°C. The rest was layered over 0.85M sucrose, centrifuged at 75000g for 30min. The crude myelin was collected from the interface, suspended in water and centrifuged at 75000g for 15min. The resultant pellet was osmotically shocked in deionized water to remove contaminants from within myelin vesicles, and centrifuged twice at 12000g for 15min. The pellets were suspended in 0.32M sucrose, layered over 0.85M sucrose, and centrifuged at 75000g for 30min. The myelin was again collected from the interface, resuspended in deionised water and pelleted at 75000g for 15min to remove residual sucrose. The myelin was resuspended in H2O and stored at –80°C.

4.2.4.2 Lipid rafts preparation

Membrane fractions were isolated from brains of adult mice (Kleene et al., 2001), and rafts were prepared according to Brown and Rose (1992) with modifications. Membranes were lysed on ice for 20 min in four volumes of ice-cold 1% Triton X-100 in TBS and mixed with an equal volume of 80% sucrose in 0.2 M Na_2CO_3. A 10–30% linear sucrose gradient was layered on top of the lysate and centrifuged for 18 h at 230,000 g at 4°C (SW55 Ti rotor; Beckman Coulter).Raft fractions were collected as previously described (He and Meiri, 2002). Nerve growth cones were isolated as previously described (Pfenninger et al., 1983).

4.2.5 Cell surface biotinylation

Surface biotinylation was carried out as described by Schmidt and colleagues (1997) with modifications. Briefly, 48h after transfection cells were washed twice with ice-cold PBS supplemented with 0.5mM CaCl2 and 2mM MgCl2 (PBSCM). Surface proteins were biotinylated by incubating cells with 0.5mg/ml Sulfo-NHS- LS-biotin (Pierce, Rockford, IL,

USA) in PBSCM for 10 min at 4°C. Biotinylation was terminated by incubation with 20mM glycine in PBSCM at 4°C for 10min followed by extensive washing with PBSCM. Biotinylated cells were then lysed for 30min in 50mM Tris-HCl buffer (pH 7.5), containing 150mM NaCl, 0.5% Triton X-100, 1mM sodium fluoride, 2mM NaVO4, 0.1mM PMSF, 1 mM Na2P2O7, 1mM EDTA and EDTA-free protease inhibitor cocktail (Roche) and centrifuged for 15 min at 4°C at 700g. The supernatants were collected and protein concentrations were determined using the BCA kit (Pierce). The amounts of surface-localized proteins were determined by precipitating biotinylated proteins with streptavidin coupled agarose beads (Pierce) applied to the supernatants at 4°C overnight. Agarose beads were pelleted by centrifugation and washed twice with the lysis buffer. Precipitated proteins were solubilized by addition of 2X Laemlli buffer to the agarose beads and analyzed by Western blot.

4.2.6 *In vitro* proteolytic processing of Caspr by reelin

Caspr purified from mouse brain (1µg) was incubated at 37°C for 2h with reelin containing medium from reelin transfected CHO cells or medium from CHO cells transfected with pcDNA3 for control. Samples were then resuspended in sample buffer, boiled for 8min and analyzed by Western blot.

5 Results

5.1 Caspr is a novel binding partner for PrP

5.1.1 Cross linking experiment shows that the cellular form of Prion protein (PrP^C) interacts with Caspr

In order to identify new binding partners of the cellular form of the prion protein (hereafter denoted PrP) we used PrP fused to the human Fc (PrP-Fc) as bait in chemical crosslinking experiments. In these experiments, the trifunctional biotin-carrying crosslinker sulfo-SBED was bound to PrP-Fc and the resulting conjugate was immobilized to protein-A magnetic beads. These magnetic beads carrying the immobilzed PrP-Fc/sulfo-SBED conjugated complex were applied to axolemma-enriched fractions (Sapirstein et al., 1992).

After UV-crosslinking of the proteins located in the vicinity of PrP-Fc and thus possibly bound to PrP-Fc, magnetic beads were washed under high stringency and then boiled in the presence of SDS and the reducing agent dithioerythritol. This procedure cleaves the disulfide bridge within the biotin-containing sulfo-SBED resulting in the detachment of the bound proteins from the immobilized PrP-Fc and in a concurrent transfer of the biotin group to the bound proteins. Eluted material was then analyzed by western blot using peroxidase-conjugated streptavidin that revealed a biotinylated protein band of approximately 190 kDa and several additional protein bands between 80 and 50 kDa (Fig 5.1A). By analyzing the material cross-linked to PrP-Fc by Western blot with the antibodies against proteins enriched in axolemma, we found that 190 kDa band was also recognized by an antibody against the intracellular domain of Caspr (Fig 5.1B), suggesting that Caspr and PrP form a complex.

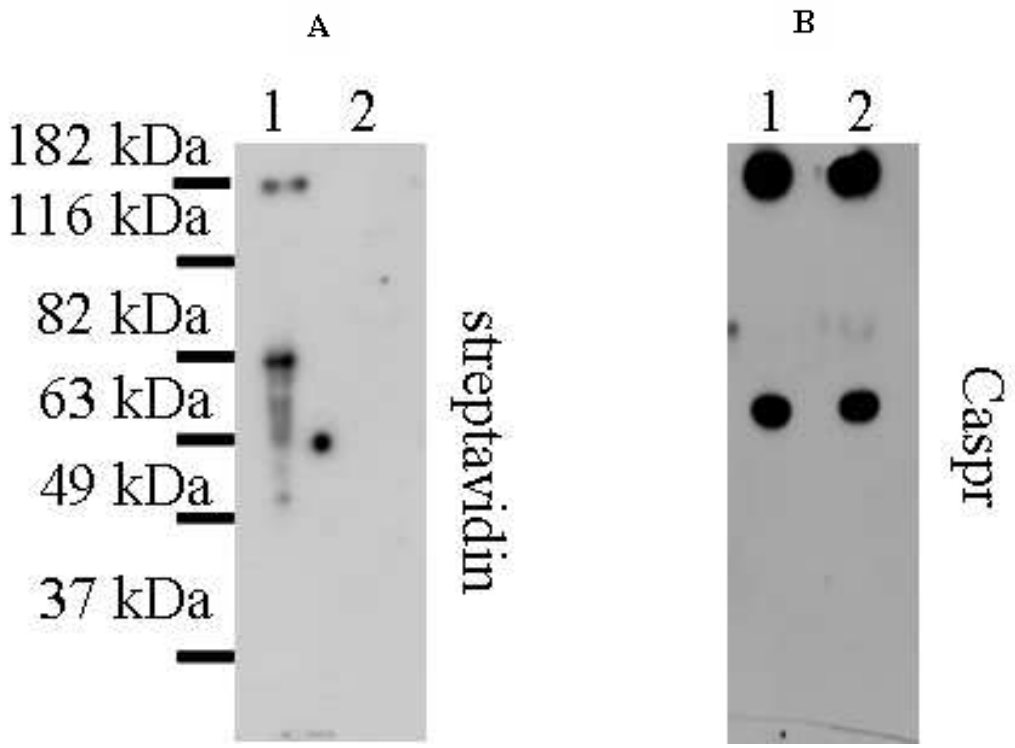

Figure 5.1: PrP directly interacts with Caspr

Proteins, that were cross-linked to PrP in axolemma fraction and concurrently bitotinylated (lane 1), and axolemma fraction (lane 2) were probed by Western blot with streptavidin (A) and antibodies against Caspr intracellular domain (B). Note, that the Caspr positive band at approximately 180 kDa overlaps with the band representing a protein that was cross-linked to PrP.

5.1.2 ELISA binding assay shows that Caspr and PrP interact directly *in vitro*

Since co-immunoprecipitation experiments only suggest direct binding but do not exclude a possibility that the two proteins are linked by intermediate binding partners, we analyzed whether Caspr directly binds to PrP in ELISA. Caspr from mouse brains bound to PrP-Fc in a concentration-dependent manner (Fig 5.2) indicating that the two proteins can interact directly. In contrast, Caspr did not bind to BSA or L1-Fc analyzed in parallel (Fig 5.2).

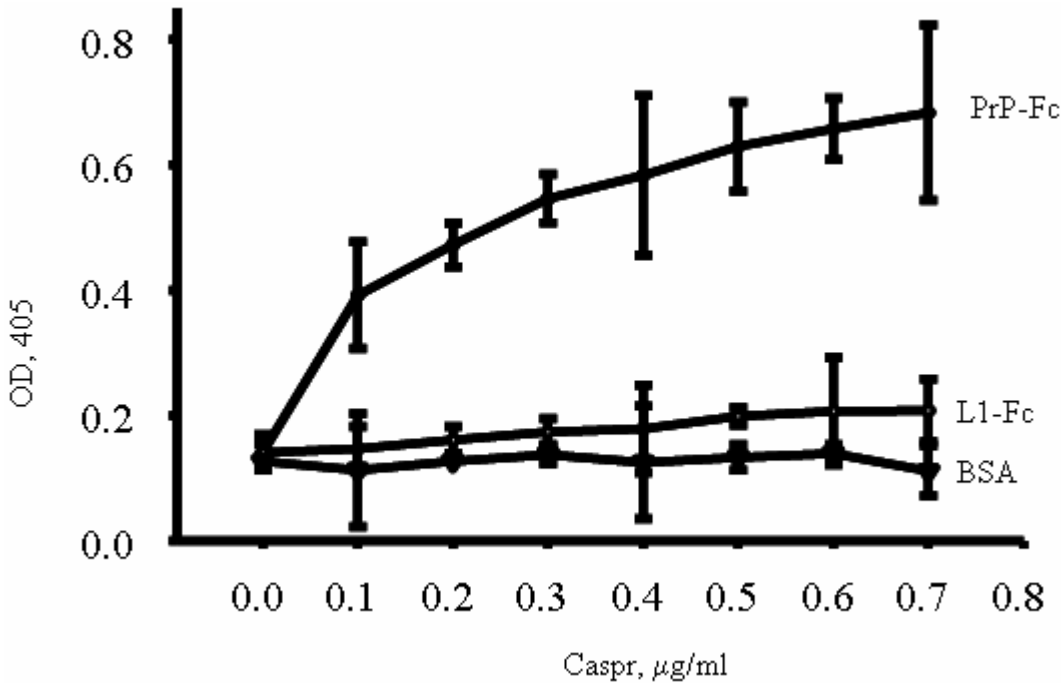

Figure 5.2 Caspr directly binds to PrP.

PrP-Fc, L1-Fc or BSA were immobilized on plastic and assayed for their ability to bind increasing concentrations of Caspr purified from mouse brains. Mean values ± SEM of the optical density (OD) is shown. PrP-Fc but not L1-Fc or BSA bound Caspr in a concentration dependent manner.

5.1.3 Coimmunoprecipitation from brain homogenate confirms the interaction between PrP and Caspr

In order to confirm the binding between Caspr and PrP we performed a coimmunoprecipitaion experiment. We immunoprecipitated PrP from the brain homogenates of PrP+/+ and PrP-/- mouse brains and labelled for antibodies against Caspr. Confirming chemical crosslinking data, Caspr co-immunoprecipitated with PrP that was immunoprecipitated from brain lysates (Fig 5.3), indicating that the two proteins are associated in the brain. Mock immunoprecipitation from PrP-/- brain lysates served as immunoprecipitaion specificity control. Another GPI-anchored immunoglobulin superfamily recognition molecule, F3/contactin, did not coimmunoprecipitate with PrP (Fig 5.3), underscoring the specificity of Caspr interaction with PrP.

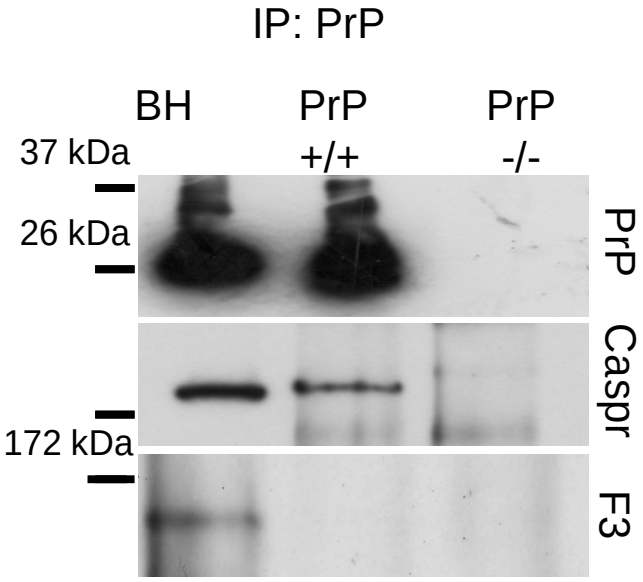

Figure 5.3: Caspr coimmunoprecipitates with PrP.

PrP+/+ brain lysates and PrP immunoprecipitates (IP) from PrP+/+ brain lysates were probed by Western blot with the antibodies against PrP, Caspr intracellular domain and F3. Note, that Caspr, but not F3 co-immunoprecipitated with PrP. Mock immunoprecipitation from PrP-/- brain lysates was performed to control IP specificity.

5.1.4 Caspr and PrP colocalise in the cerebellar neurons cultured in vitro

Since PrP is highly expressed in the developing brain playing a role in regulation of neurite outgrowth rates (Graner et al., 2000; Lopes et al., 2005; Lima et al., 2007; Santuccione et al., 2005), we analyzed the distribution of PrP and Caspr in cerebellar neurons maintained in culture for 24 hours, i.e. at the time of the active neurite outgrowth. Indirect immunofluorescence labeling of neurons showed that PrP partially colocalized with Caspr along neurites and both proteins were highly expressed in growth cones where they also partially colocalized (Fig 5.4 A). Furthermore, when Caspr was clustered at the neuronal surface with the antibodies against the extracellular domain of Caspr applied to live neurons, PrP partially redistributed to Caspr clusters (correlation coefficients between the distributions of PrP and Caspr were 0.2 ± 0.1 and 0.5 ± 0.6 for non-clustered and clustered Caspr, respectively) suggesting that Caspr associates with PrP at the neuronal surface.(Fig 5.4B) In contrast, clustering of Caspr did not induce redistribution of tubulin (correlation coefficients between the distributions of Caspr and tubulin were for non-clustered and clustered Caspr respectively Fig 5.4C)

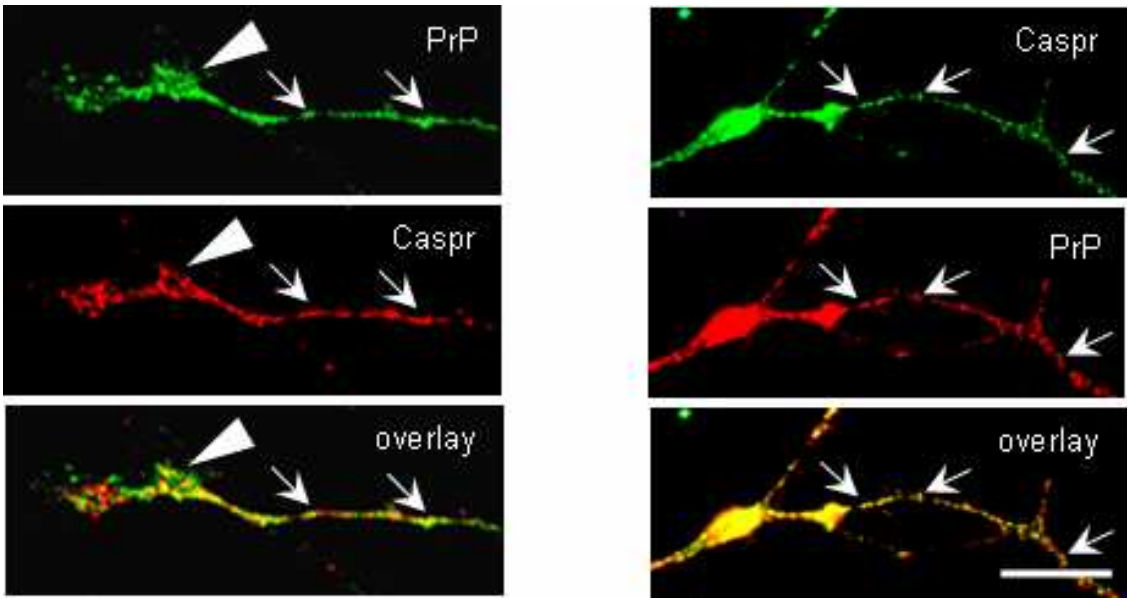

Figure 5.4 (A and B): Caspr and PrP colocalise in the cerebellar neurons. Cultured cerebellar neurons were fixed and labeled with the antibodies against PrP and Caspr extracellular domain (A). Alternatively, labeling with these antibodies was done on live neurons to cluster Caspr and PrP, and then neurons were fixed (B). In both cases, neurons were then co-labeled with the antibodies against tubulin after permeabilization of the surface membrane with detergent. Representative neurites are shown. Note, that PrP and Caspr partially co-localize with each other along neurites and both proteins accumulate in growth cones (arrows show overlapping accumulations of both proteins). Note also that clustering of proteins induced redistribution of Caspr and PrP to overlapping clusters (arrows) suggesting that they are associated.

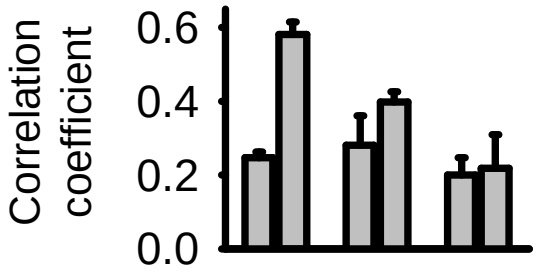

Figure 5.4 C Graphical quantification shows mean ± SEM correlation coefficients between the distributions of PrP, Caspr and tubulin in fixed and live-labeled neurons. *P<0.05, t-test.

5.1.5 PrP and Caspr colocalize in the cerebellum

Colocalization of Caspr and PrP in cultured cerebellar neurons suggests that both proteins may associate in intact brain. This idea is supported by the data showing certain similarities in mice deficient for PrP and Caspr: Caspr knockout mice exhibit ataxia (Bhat et al, 2001) which is also a typical symptom in prion deficient mice, although its onset is delayed and hence age dependent (Moore et al, 1999). To verify our idea that PrP and Caspr associate in the intact brain, we analyzed whether they colocalize in mouse cerebellum.

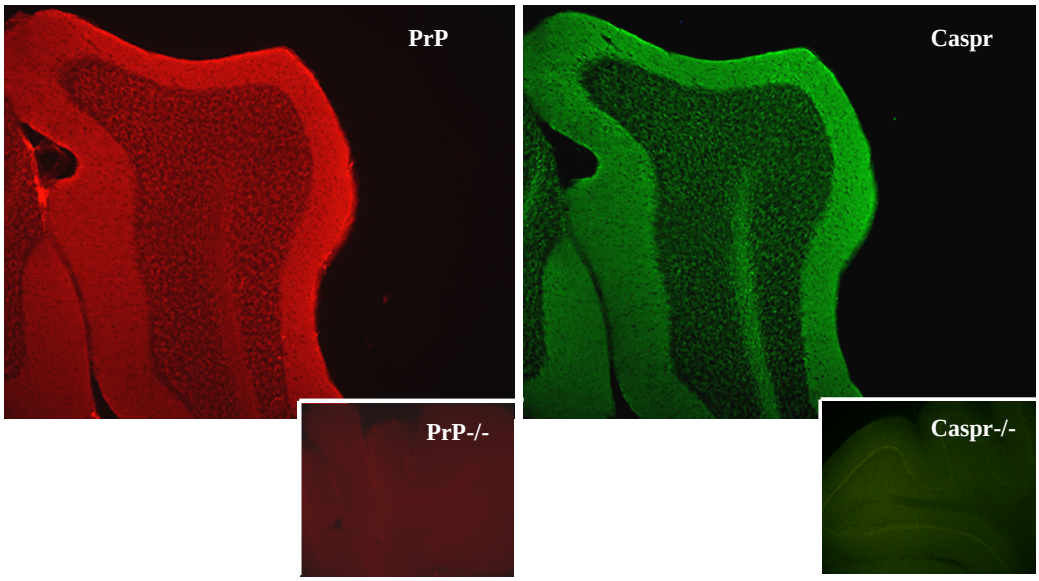

Figure 5.5: Cerebellum sections labelled with antibodies against PrP^C and Caspr are shown.

Both Caspr and PrP show similar distribution patterns being present in the molecular layer and purkinje cells.

Results

To this aim, cerebellum sections obtained from wild type mice were labeled with antibodies against Caspr and PrP. Cerebellum sections from Caspr and PrP deficient mice served as controls of the antibody specificity. Immunolabeling using specific antibodies showed that PrP and Caspr have similar expression pattern in the cerebellum (Fig 5.5).The labeling was absent in the respective knockout mice. (Fig 5.5 inset)

Since Caspr and PrP have similar expression pattern we also analysed the localisation pattern of PrP and Caspr. In cerebellar sections obtained from wild type mice, PrP and Caspr are expressed in the molecular layer of the cerebellum and the overlay of the images shows Caspr and PrP colocalize in the molecular layer of the cerebellum (Fig 5.6). This is in accordance with the data from our cerebellar neuronal culture:

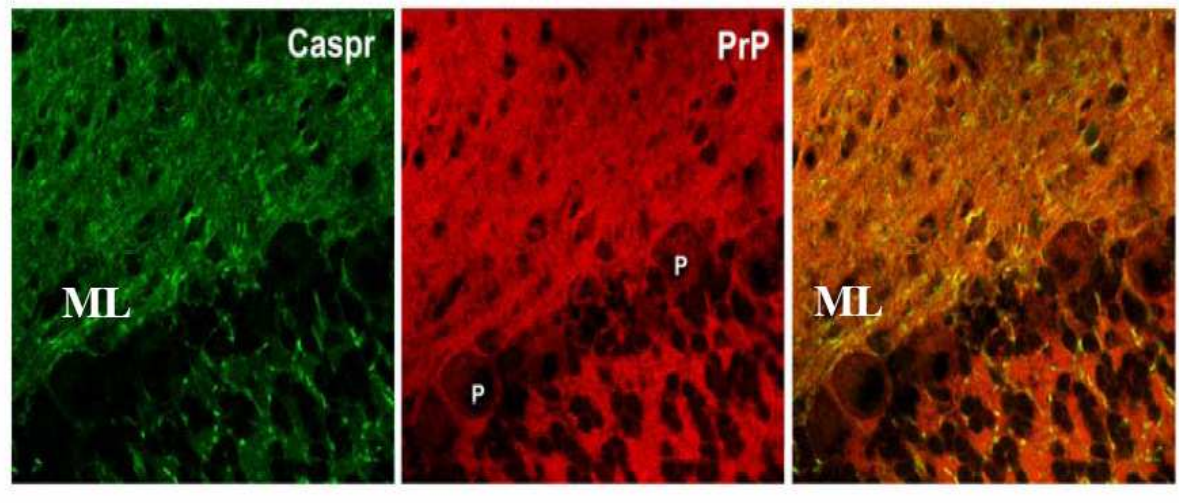

Figure 5.6: Cerebellum sections were co-labelled for Caspr and PrP^{C}.

Cerebellar sections were labelled using antibodies agains Caspr (green) and antibodies against PrP (red). The overlay image shows that the two proteins co-localize in the molecular layer and the purkinje cell layer of the cerebellum.

5.2 Caspr proteolysis is enhanced in the brains of PrP deficient mice

5.2.1 Analysis of total brain homogenates shows that levels of full length Caspr are reduced in the absence of PrP

To analyze whether PrP deficiency affects expression of Caspr, we analyzed the levels of Caspr protein in homogenates of the brains from wild type (PrP+/+) and PrP deficient (PrP-/-) mice. Western blot analysis with the antibodies against the intracellular domain of Caspr showed that the levels of the full length Caspr with the molecular weight of approximately 190 kDa were approximately twofold lower in PrP-/- versus PrP+/+ brain homogenates (Fig.5.7). Strikingly, a reduction in the full length Caspr protein levels in PrP-/- brain homogenates was accompanied by a concomitant increase in the levels of the degradation product of Caspr with the molecular weight of approximately 50 kDa (Fig.5.7).

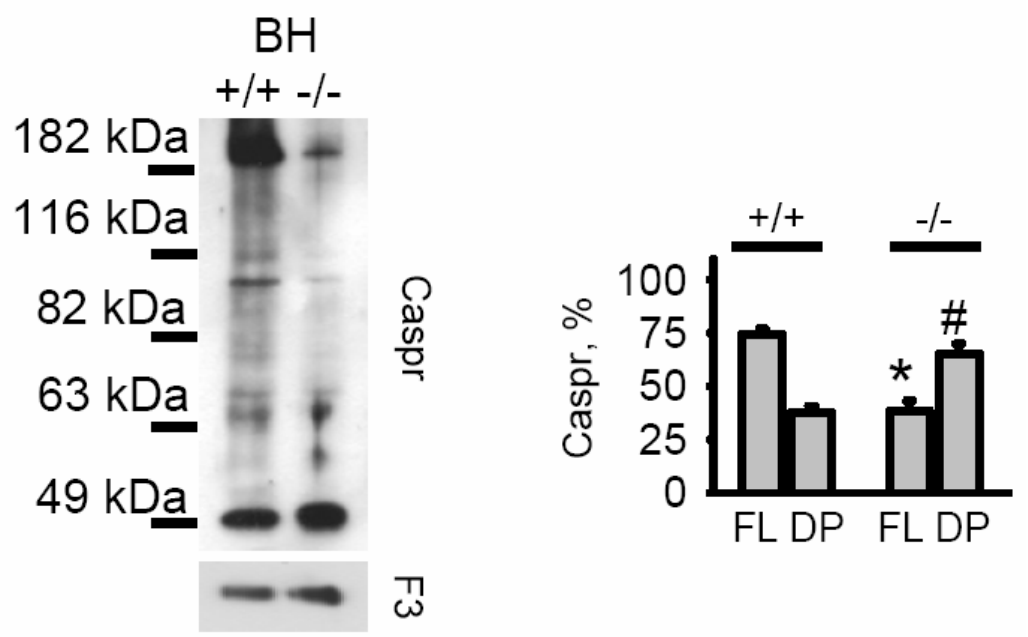

Figure 5.7: The levels of full length of Caspr is reduced in the absence of PrP and there is a proportional increase in the degradation product.

PrP+/+ and PrP-/- Brain homogenates were probed by Western blot with the antibodies against Caspr intracellular domain. Note that the levels of the full length 180 kDa Caspr are reduced, while the levels of the 50 kDa degradation product of Caspr are increased in PrP-/- probes. Graphs show mean optical densities ± SEM of the full length and 50 kDa degradation product of Caspr from n≥5 experiments normalized to the total Caspr level defined as a sum of the full length and 50 kDa degradation product levels. Labeling for F3.

5.2.2 Levels of full length Caspr are reduced in the myelin fractions and lipid rafts fractions isolated from PrP+/+ and PrP-/- brains.

PrP, as a GPI anchored protein accumulates in lipid rafts. By recruiting transmembrane proteins such as NCAM in lipid rafts PrP enhances neurite outgrowth (Santuccione et al., 2005). Like Caspr is also a transmembrane protein, hence it was also important to analyse if Caspr was present in lipid rafts. Lipid rafts were isolated from PrP+/+ and PrP-/- brains and western blot analysis of these lipid raft fractions showed a reduction in the levels of full length of Caspr, a phenomenon similar to that of the brain homogenate. Similar to the brain homogenates, the levels of the full length Caspr were decreased while the level of the 50 kDa degradation product was proportionally increased in the PrP-/- versus PrP+/+ lipid rafts (Fig 5.8).

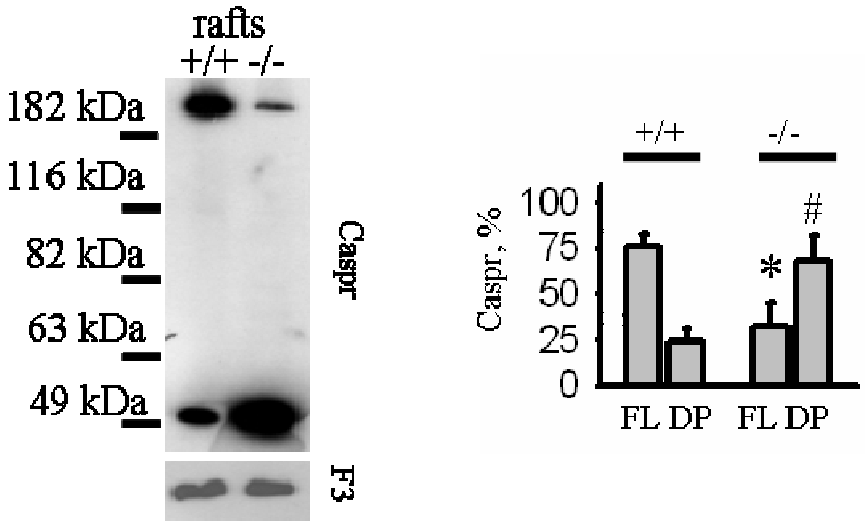

Figure 5.8: Lipid raft fractions from the adult brains of PrP +/+ and PrP -/- labeled for Caspr.

PrP+/+ and PrP-/- lipid rafts were probed by Western blot with the antibodies against Caspr intracellular domain. Note that the levels of the full length 180 kDa Caspr are reduced, while the levels of the 50 kDa degradation product of Caspr are increased in PrP-/- probes. Graphs show mean optical densities ± SEM of the full length and 50 kDa degradation product of Caspr from n≥5 experiments normalized to the total Caspr level defined as a sum of the full length and 50 kDa degradation product levels. Note that the levels of contactin are unaltered, which served as a loading control in the lipid rafts. *P<0.05, paired t-test.

Since Caspr is involved in myelination (Salzer et al, 2000), we isolated myelin fractions from brains of PrP+/+ and PrP-/- adult mice. We observed a phenomenon similar to that of lipid rafts and brain homogenates also in the myelin fractions. Hence, our observations indicate that PrP deficiency results in enhanced proteolytic processing of Caspr in the brain. (Fig 5.9)

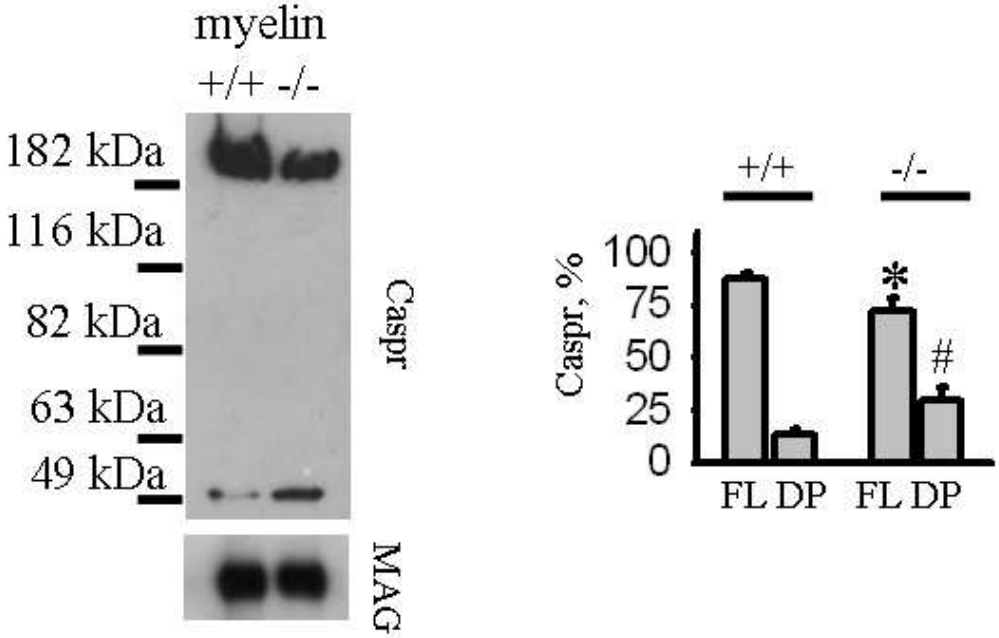

Figure 5.9: Myelin fractions isolated from PrP+/+ and PrP-/- brains shows enhanced degradation of Caspr in the absence of PrP.

PrP+/+ and PrP-/- myelin fractions were probed by Western blot with the antibodies against Caspr intracellular domain. Note that the levels of the full length 180 kDa Caspr are reduced, while the levels of the 50 kDa degradation product of Caspr are increased in PrP-/- probes. Graphs show mean optical densities ± SEM of the full length and 50 kDa degradation product of Caspr from n≥5 experiments normalized to the total Caspr level defined as a sum of the full length and 50 kDa degradation product levels. Labeling for MAG served as a loading control. *P<0.05, paired t-test.

5.3 PrP inhibits Caspr shedding from the cell surface

5.3.1 Antibody against the extra cellular domain of Caspr recognizes both full length and degradation product

The molecular weight of Caspr intracellular domain is approximately 10 kDa. Hence the cleavage product of approximately 50 kDa recognized by the antibodies against the C-terminal part of the Caspr intracellular domain should include the whole intracellular domain and a transmembrane and membrane adjacent portions of Caspr molecule. The presence of this degradation product, therefore, suggests that the extracellular domain of Caspr should be also released. To verify this idea, we analyzed Caspr+/+ and Caspr-/- brain homogenates by western blot with the antibodies against the N-terminal extracellular part of Caspr. Unexpectedly, labeling with these antibodies also revealed a degradation product of Caspr with the molecular weight of approximately 50 kDa (Fig. 5.10).

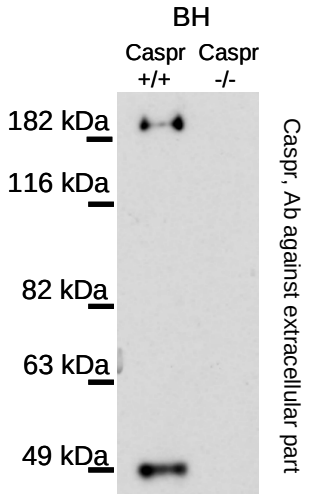

Figure 5.10: Antibody against the extracellular domain of Caspr recognises full length and the shedding product of Caspr +/+ and not in Caspr -/- brain.

Caspr+/+ and Caspr-/- brain homogenates were probed by Western blot with the antibodies against Caspr extracellular domain. Note that the full length 180 kDa product and the 50 kDa degradation product of Caspr were recognized in Caspr+/+ but not in Caspr-/- brain homogenates.

5.3.2 Degradation product of Caspr is shedded to the culture medium from cerebellar neurons

Since a single degradation product of approximately 50 kDa could not contain two epitopes located at the distal parts of the 190 kDa full length Caspr protein, the only explanation for this phenomenon could be that the Caspr processing included the cleavage of this protein at several sites (at least two) within its extracellular domain. Such cleavage could then result in the release of Caspr fragments with the molecular weights of approximately 50 kDa. Cleavage of the cell surface Caspr within its extracellular domain should result in the release of its extracellular domain pieces to the extracellular space resulting in the Caspr shedding from the cellular surface. To verify that this indeed could occur, culture medium from cultured cerebellar neurons was analyzed by Western blot with the antibodies against the extracellular domain of Caspr. Indeed, the labeling revealed that culture medium contained a Caspr degradation product with the molecular weight of approximately 50 kDa that was specifically recognized by Caspr antibody in the culture medium from Caspr+/+ neurons (Fig.5.11). The signal was not present in the culture medium from Caspr-/- neurons (Fig.5.11) indicating the specificity of the labeling. Importantly, the levels of the Caspr degradation product were increased in the culture medium from PrP-/- versus PrP+/+ neurons (Fig. 4.11).

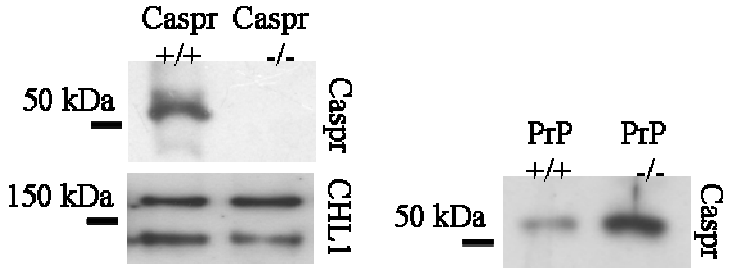

Figure 5.11: Caspr is cleaved and shedded in the cell culture supernatant.

Culture medium from Caspr+/+ and Caspr-/- (left upper panel) or PrP+/+ and PrP-/- (right panel) cerebellar neurons was analyzed by Western blot with the antibodies against Caspr extracellular domain. Note, that these antibodies recognize the 50 kDa degradation product of Caspr in the culture medium from Caspr+/+ but not Caspr-/- neurons. The level of this product is increased in the culture medium from PrP-/- versus PrP+/+ neurons.

5.3.3 Surface labelling of cerebellar neurons shows a reduction in the amount of Caspr on the surface of PrP-/- neurons

Live cerebellar neurons from PrP+/+ and PrP-/- neurons were surface labeled for Caspr. L1 was used as a control. The levels of Caspr, but not L1, were reduced at the surface of PrP-/- versus PrP+/+ neurons that were live labeled by indirect immunofluorescence against the extracellular domain of Caspr or L1 (Fig. 5.12C and 5.12D).

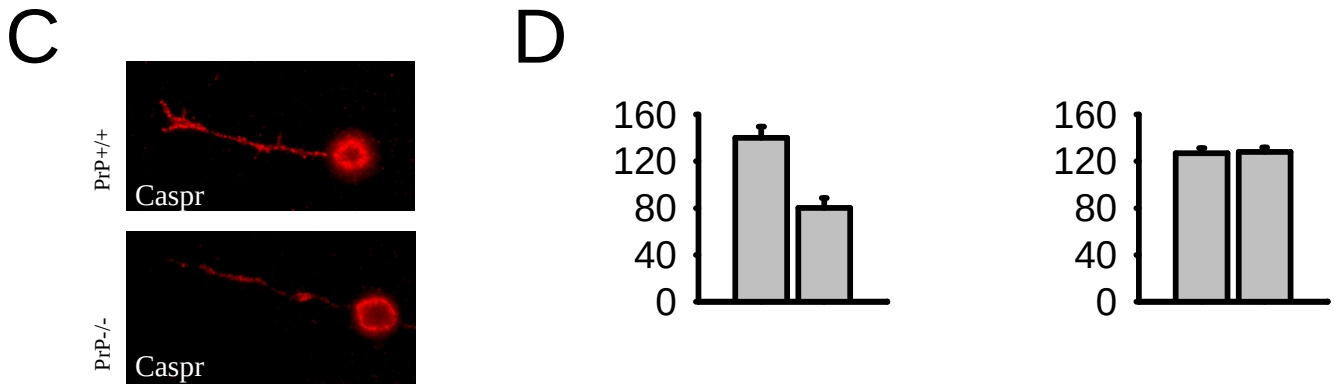

Figure 5.12 C and D: Surface labelling and quantification on PrP+/+ and PrP -/- cerebellar neurons grown invitro.

C) Cultured PrP+/+ and PrP-/- cerebellar neurons were live labeled with the antibodies against Caspr extracellular domain. Note reduced levels of Caspr at the surface of PrP-/- versus PrP+/+ neurons.

D) Graphs show mean ± SEM levels of Caspr or L1 (in arbitrary units (AU)) along neurites of PrP+/+ and PrP-/- neurons. *P<0.05, t-test.

5.3.4 Expression of Caspr on the cell surface of CHO cells is increased in the presence of PrP

To verify the role of PrP in the inhibition of Caspr shedding at the cell surface, we transfected Chinese Hamster Ovarian (CHO) cells, which are negative for Caspr and PrP, with the cDNAs encoding these proteins. For comparison, CHO cells were also transfected with F3/contactin that have been also shown to enhance Caspr levels at the cell surface (Faivre-Sarrailh et al., 2000). Cell surface proteins in transfected cells were then biotinylated and separated from the total protein pool using streptavidin-agarose. Western blot analysis of the total and biotinylated protein fractions with the antibodies against Caspr showed that levels of Caspr at the cell surface were strongly increased in cells co-transfected with Caspr and PrP versus cells transfected with Caspr alone (Fig 5.13). Interestingly, co-transfection with PrP promoted accumulation of Caspr at the cell surface to even higher extent than co-transfection with F3/contactin (Fig 5.13). Altogether, our data indicate that the cleavage of Caspr occurs at the cell surface resulting in Caspr shedding and that this process is regulated by PrP which inhibits Caspr cleavage.

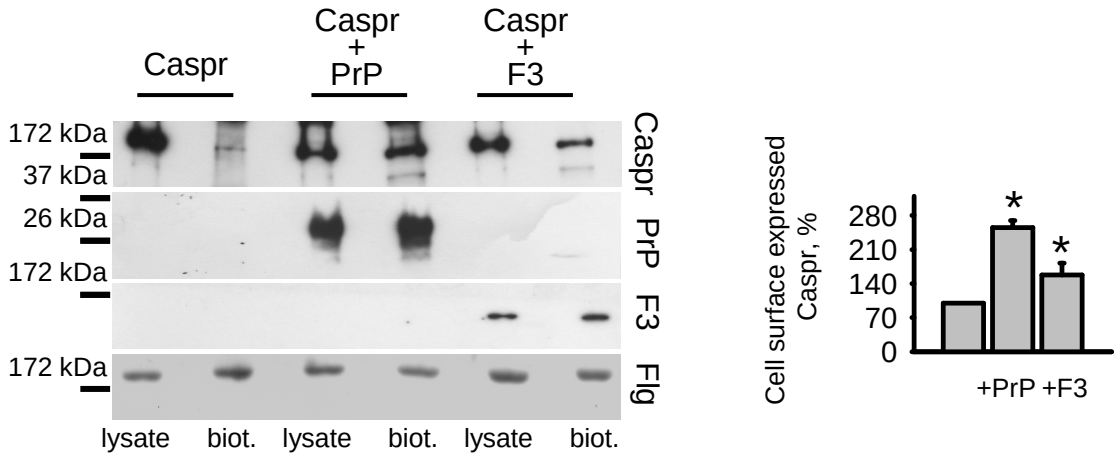

Figure 5.13 PrP increases the surface localisation of Caspr in CHO cells.

CHO cells were transfected with Caspr alone, or co-transfected with Caspr and PrP or F3. Cell surface proteins were then biotinylated and separated from the total protein pool following cell lysis. Samples containing cell surface proteins and total cell lysates were then probed by Western blot with the antibodies against Caspr, PrP, F3. Labeling for FGFR (Flg) served as a loading control. Higher levels of Caspr are observed in the cell surface protein containing samples in cells co-transfected with Caspr and PrP when compared to Caspr only transfected cells. Cell surface Caspr levels are also higher in F3 / Caspr versus Caspr only transfected cells, although this effect is less prominent than in Caspr / PrP transfected cells.

Graph shows mean ± SEM optical density of the Caspr band in the probes containing biotinylated cell surface proteins from n=3 experiments with the signal in Caspr only transfected cells set to 100%. *P<0.05, paired t-test.

5.4 PrP inhibits serine protease mediated proteolysis of Caspr at the cell surface

5.4.1 Shedding of Caspr is inhibited by aprotinin, a serine protease inhibitor

To identify proteases that are involved in Caspr shedding from the cell surface, we assayed the release of the 50 kDa fragment of the Caspr extracellular domain from cultured cerebellar neurons treated with inhibitors of different classes of proteases. Western blot analysis of culture supernatants with the antibodies against the extracellular domain of Caspr showed that inhibitors of matrix metalloproteases GM6001 and phenanthroline and an inhibitor of aspartic proteases pepstatin had no effect on Caspr shedding. In contrast, an inhibitor of serine proteases aprotinin strongly inhibited the release of the 50 kDa fragment of Caspr extracellular domain to the culture medium (Fig 5.14). Caspr shedding was inhibited by aprotinin both in PrP+/+ and PrP-/- neurons (Fig 5.15), suggesting that PrP inhibits the activity of a serine protease that cleaves Caspr at the cell surface.

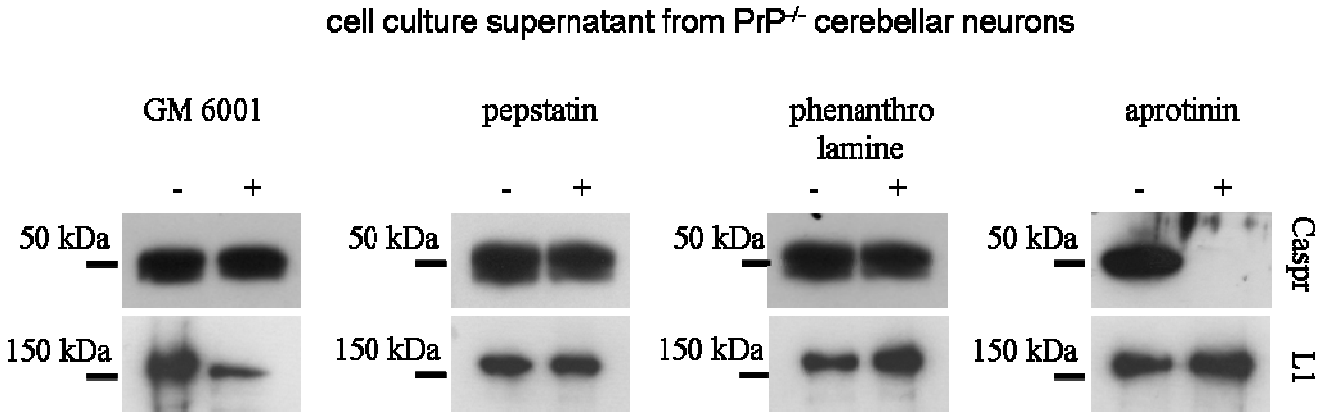

Figure 5.14: Aprotinin inhibits the formation of shedding product of Caspr.

Culture medium collected from PrP-/- cerebellar neurons that were either non-treated or treated with the inhibitors of matrix metalloproteases (GM6001 or phenanthroline), aspartic proteases (pepstatin), or serine proteases (aprotinin) was analyzed by Western blot with the antibodies against the extracellular domain of Caspr. Only aprotinin decreases levels of the 50 kDa degradation product of Caspr in the culture medium. Labeling for L1 served as a loading control.

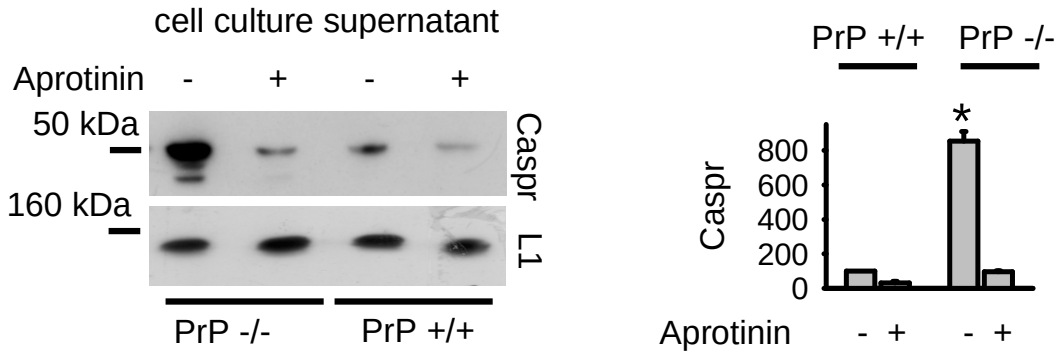

Figure 5.15: Addition of aprotinin to the cell culture medium reduces the formation of degration product.

Culture medium collected from PrP+/+ or PrP-/- cerebellar neurons that were either non-treated or treated with aprotinin was analyzed by Western blot with the antibodies against Caspr extracellular domain. Labeling for L1 served as a loading control. Note that aprotinin reduces the levels of the 50 kDa Caspr degradation product to a similar level in PrP+/+ and PrP-/- neurons. Graph shows mean ± SEM of the optical density of the 50 kDa band labeled with Caspr antibodies from n=3 experiments with the signal in non-treated PrP+/+ neurons set to 100%. *P<0.05, paired t-test.

5.4.2 PrP-Fc inhibits the formation of Caspr shedding product

In order to further verify the role of PrP in Caspr shedding, we treated PrP+/+ and PrP-/- neurons with PrP-Fc applied in the culture medium. Similarly to aprotinin, PrP-Fc strongly inhibited the release of the 50 kDa fragment of the Caspr extracellular domain to the culture medium (Fig 5.16).

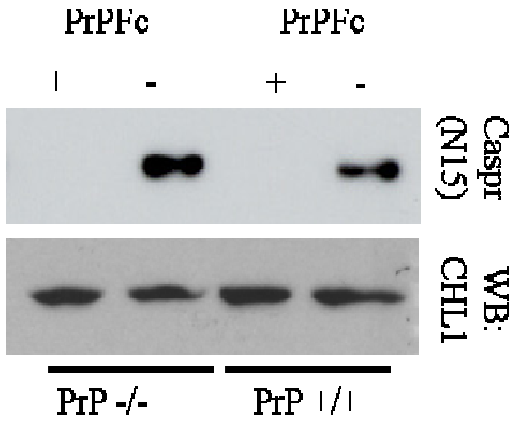

Figure 5.16: Addition of PrP-Fc to the culture medium inhibits the serine protease mediated shedding. Culture medium collected from PrP+/+ or PrP-/- cerebellar neurons that were treated with PrP-Fc was analyzed by Western blot with the antibodies against Caspr extracellular domain. Labeling for L1 served as a loading control. Note that PrP-Fc application reduces the levels ofthe 50 kDa degradation product of Caspr in the culture medium.

Results

5.5 Reelin is involved in Caspr proteolysis

5.5.1 Expression of proteolytically active reelin is increased in the absence of PrP

Reelin, an extracellular matrix serine protease is highly expressed during brain development (Tissir et al., 2003; Rice et al., 2001) and its enzymatic proteolytic activity is inhibited by aprotinin (Quattrocchi et al., 2002). Interestingly, reelin cleaves its substrates, such as fibronectin or laminin, at several sites releasing several smaller fragments (Quattrocchi et al., 2002), that makes it a plausible candidate-protease involved in Caspr cleavage. Western blot analysis with reelin antibodies showed that the levels of the full-length reelin with the molecular weight of approximately 420 kDa were decreased in PrP-/- versus PrP+/+ brain homogenates (Fig 5.17). A decrease in the levels of the full length reelin was accompanied by an increase in the levels of its self-degradation product with the molecular weight of approximately 140 kDa (Fig 5.17). Since this degradation product of reelin possesses higher proteolytic activity than that of the full length reelin (Quattrocchi et al., 2002), these observations indicate that the reelin proteolytic activity is increased in PrP-/- versus PrP+/+ brains.

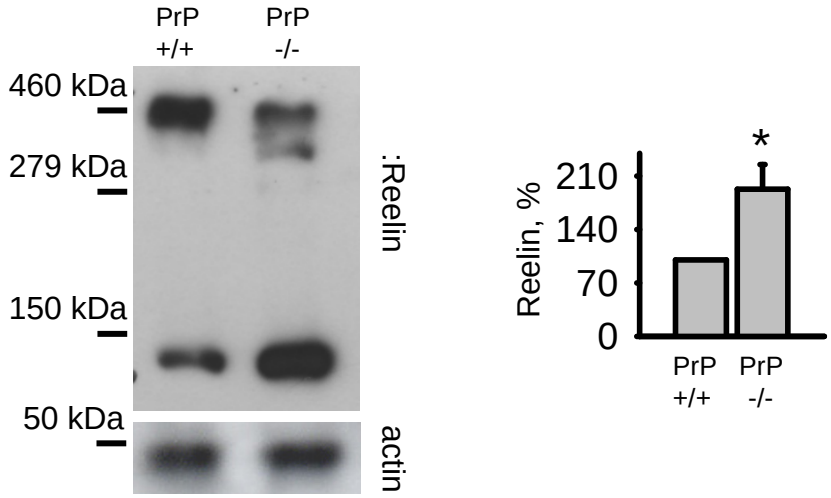

Figure 5.17: PrP inhibits reelin mediated Caspr proteolysis.

PrP+/+ and PrP-/- brain homogenates were probed by Western blot with the antibodies against reelin. Note decreased levels of the full length form of reelin with the molecular weight of approximately 460 kDa and increased levels of the 140 kDa form of reelin in PrP-/- versus PrP+/+ brain homogenates. Graph shows mean ± SEM values of the optical density of the 140 kDa band of reelin from n=3 experiments with the optical density in PrP+/+ brain homogenates set to 100%. *P<0.05, paired t-test

5.5.2 PrP protects Caspr from reelin mediated cleavage in the CHO cells

To analyze the role of reelin in Caspr cleavage, we transfected CHO cells with Caspr alone, or co-transfected them with Caspr together with reelin or PrP. Western blot analysis of CHO cell lysates showed a strong expression of the full length Caspr in CHO cells transfected with Caspr alone (Fig 5.18). The levels of the full length Caspr were further increased in cells cotransfected with Caspr and PrP (Fig 5.18) in accordance with our observation that co-transfection with PrP enhances cell surface levels of the full length Caspr (Fig 5.13). However, in cells co-transfected with Caspr and reelin, the levels of the full length Caspr were drastically reduced (Fig 5.18), that was accompanied by an increase in the 50kDa Caspr fragment levels in the culture medium, strongly suggesting that reelin is involved in Caspr proteolysis at the cell surface. In contrast, reelin-mediated Caspr proteolysis was inhibited in CHO cells cotransfected with Caspr together with reelin and PrP (Fig 5.18), indicating that PrP inhibits reelin mediated proteolysis of Caspr.

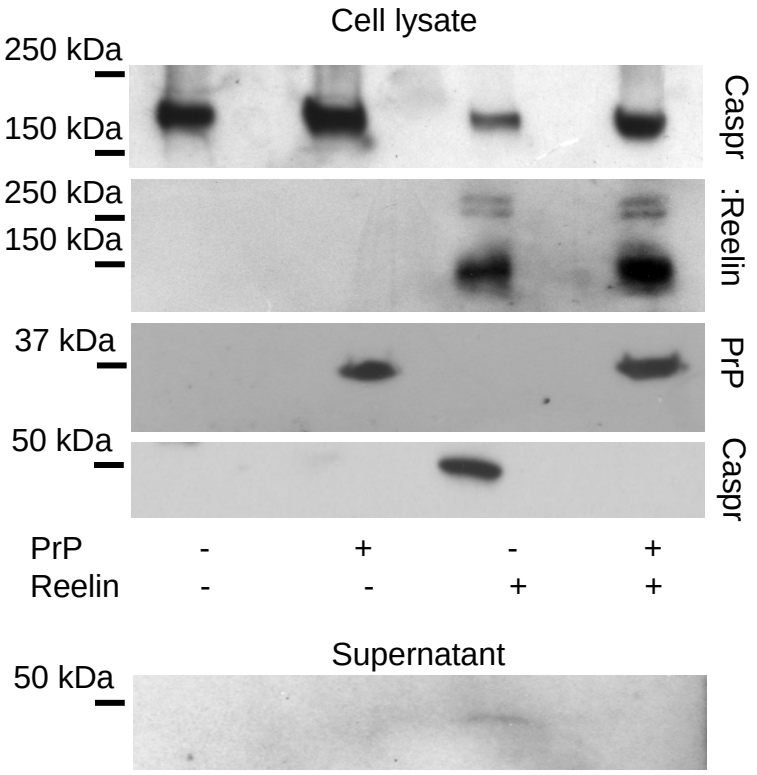

Figure 5.18: PrP inhibits reelin mediated proteolysis of Caspr. Culture medium and lysates of CHO cells that were transfected with Caspr alone, or co-transfected with Caspr and PrP, reelin or both, were analyzed with the antibodies against Caspr, reelin and PrP. Note that full length Caspr levels are reduced and 50 kDa degradation product levels increased in cells co-transfected with Caspr and reelin, and that this effect is blocked in cells co-transfected with Caspr, reelin and PrP.

5.5.3 Reelin cleaves Caspr invitro

In accordance with our data for CHO cells, preincubation of Caspr purified from the mouse brain with recombinant reelin also resulted in a pronounced reduction in the levels of the full length Caspr (Fig 5.19). Reelin, however, did not have any effect on the levels of L1 analyzed for comparison (Fig 5.19). We also observed an increase in the levels of the 50 kDa degradation product of Caspr in the probes incubated with reelin (Fig 5.19). The increase in the 50 kDa degradation product of Caspr was not proportional, however, to the decrease in the levels of full length Caspr that was probably due to faster (than in the brain) degradation of the 50 kDa degradation product of Caspr in the *in vitro* cleavage assay.

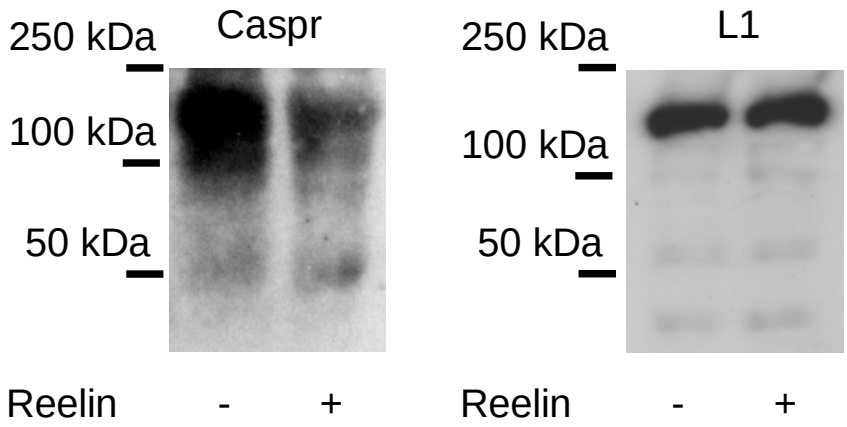

Figure 5.19: Reelin cleaves Caspr invitro.

Caspr or L1, purified from mouse brain, was either non-treated or preincubated with recombinant reelin and then probed by Western blot with the antibodies against Caspr extracellular domain. Note, that the levels of full-length 190 kDa Caspr are reduced whereas the levels of 50 kDa degradation product of Caspr increased following preincubation with reelin. Reelin does not influence L1 levels.

5.6 Caspr inhibits neurite outgrowth in cerebellar neurons

5.6.1 Neurite outgrowth is increased in Caspr -/- neurons.

While Caspr is highly expressed in cerebellar and hippocampal neurons, its role in the central nervous system remains poorly understood. High levels of Caspr in growth cones of the growing neurites suggest that Caspr may play a role in regulation of neurite outgrowth rates. To analyze this idea, we compared neurite outgrowth rates in cerebellar neurons isolated from the brains of Caspr+/+ and Caspr-/- mice and maintained on glass coverslips coated with poly-L-lysine alone or in combination with laminin or PrP-Fc. Strikingly, 24 hours after plating neurites in Caspr-/- neurons were longer than in Caspr+/+ neurons on all substrates that we have tested (Fig 5.20) suggesting that non-specific interactions of Caspr with poly-L-lysine inhibit neurite outgrowth and that this effect can not be fully compensated by outgrowth promoting agents such as laminin or PrP-Fc.

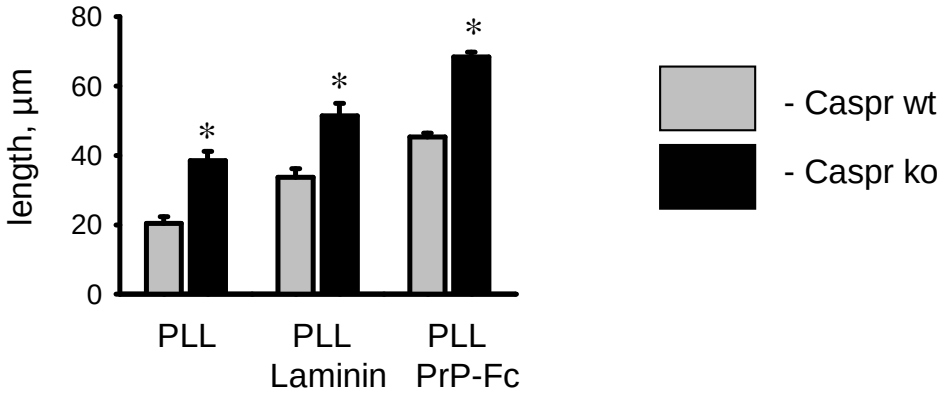

Figure 5.20: Caspr inhibits neurite outgrowth.

Graph shows mean ± SEM neurite lengths in Caspr+/+ and Caspr-/- cerebellar neurons that were maintained in culture on poly-L-lysine (PLL) that was substrate coated on glass coverslips alone or in conjunction with laminin or PrP-Fc. Neurite lengths are increased in Caspr-/- neurons.*P<0.05, t-test

To confirm this, we have grown wild type neurons on glass coverslips coated with poly-L-lysine alone or in combination with the antibodies against the extracellular domain of Caspr. All polyclonal antibodies against the epitopes within the Caspr extracellular domain that have been analyzed in our study reduced neurite outgrowth rates when compared to neurite outgrowth rates on poly-L-lysine alone (Fig 5.21) indicating that binding of Caspr to extracellular ligands, such as substrate-coated antibodies, inhibits neurite outgrowth.

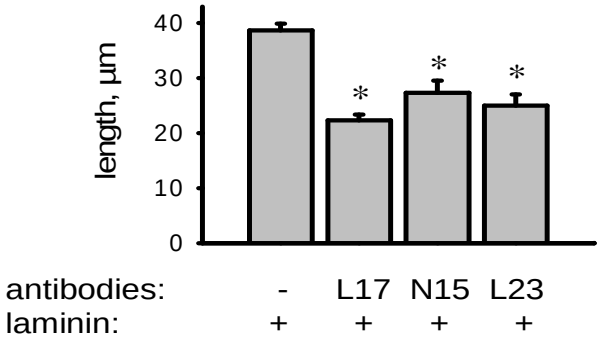

Figure 5.21: Antibodies against the extracellular domain of Caspr reduce neurite outgrowth in Caspr +/+ neurons. Graph shows mean ± SEM neurite lengths in Caspr+/+ cerebellar neurons that were maintained in culture on poly-L-lysine (PLL) that was substrate coated on glass coverslips in conjunction with laminin alone, or together with the antibodies against three different epitopes within Caspr extracellular domain (L17, N15, and L23). Neurite lengths are decreased in neurons grown in the presence of Caspr antibodies. *P<0.05, t-test

Again, antibodies against the extracellular domain of Caspr inhibited neurite outgrowth even in the presence of the outgrowth promoting agents such as laminin or PrP-Fc (Fig 5.22). Neurite outgrowth in Caspr-/- neurons was not affected by the antibodies against Caspr extracellular domain (Fig 5.22) underscoring the specificity of the effect exerted by the antibodies.

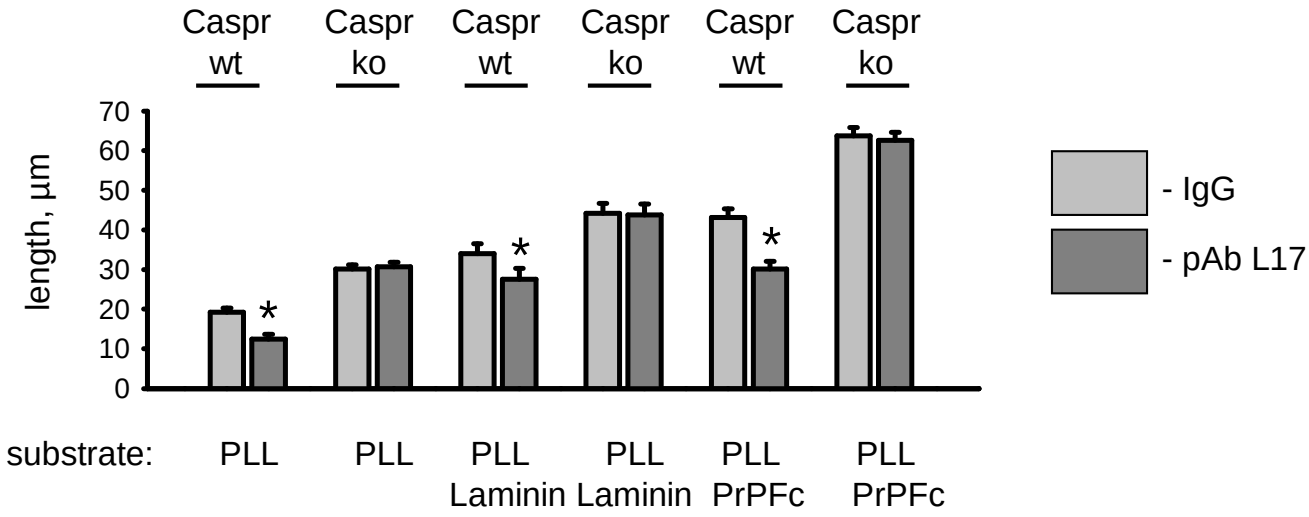

Figure 5.22: Antibodies against the extracellular domain of Caspr reduces neurite outgrowth in the Caspr+/+ neurons while they have no effect on the Caspr -/- neurons.

Graph shows mean ± SEM neurite lengths in Caspr+/+ and Caspr-/- cerebellar neurons that were maintained in culture on poly-L-lysine (PLL) that was substrate coated on glass coverslips alone or in conjunction with laminin or PrP-Fc. The coating was done with or without the antibodies against the Caspr extracellular domain (L17). Neurite lengths are decreased in Caspr+/+ but not in Caspr-/- neurons grown in the presence of Caspr antibodies. *P<0.05, t-test

5.7 PrP deficiency attenuates inhibitory effect of Caspr on neurite outgrowth

Since PrP deficiency results in reduced levels of Caspr at the neuronal surface, we also compared neurite outgrowth rates in cultured PrP+/+ and PrP-/- cerebellar neurons. Similarly to Caspr-/- neurons, PrP-/- neurons extended neurite better than PrP+/+ neurons independently of whether neurons were grown on poly-L-lysine alone or in combination with laminin or PrP-Fc (Fig 5.23). Addition of aprotinin to the culture medium, however, negated this difference by reducing neurite outgrowth rates in PrP+/+ and PrP-/- neurons to the similar levels (Fig 5.23). In a view of our results on Caspr shedding, a plausible explanation of these phenomena could be that increased Caspr shedding in PrP-/- neurons has an outgrowth promoting effect, while aprotinin mediated inhibition of Caspr shedding negates the difference between PrP+/+ and PrP-/- neurons.

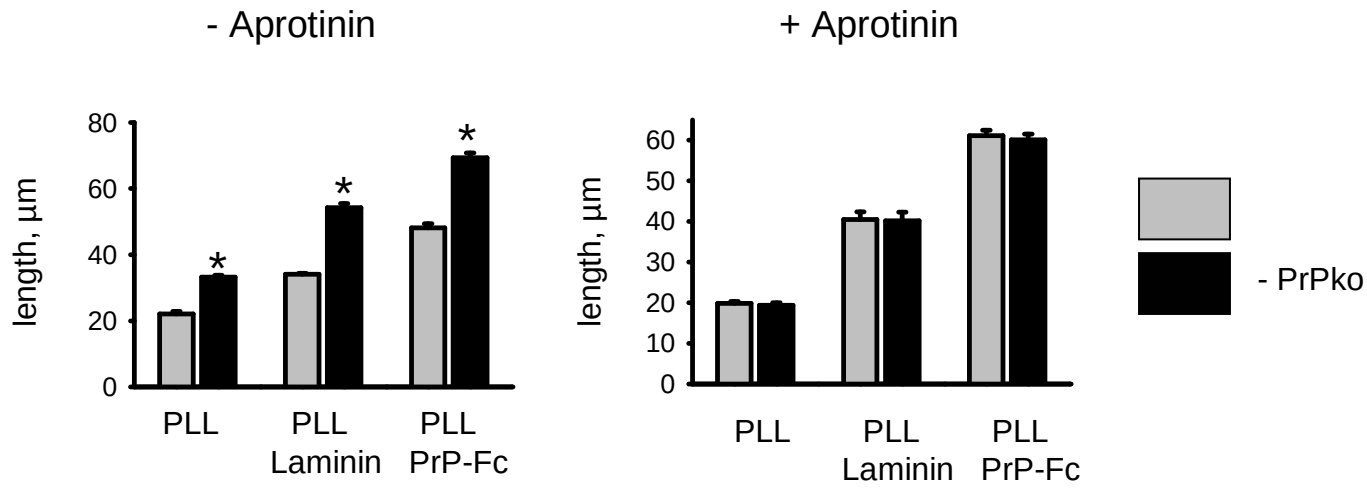

Figure 5.23. PrP deficient neurons are less responsive to Caspr mediated inhibition of neurite outgrowth.

Graph shows mean ± SEM neurite lengths in PrP+/+ and PrP-/- cerebellar neurons that were maintained in culture on poly-L-lysine (PLL) that was substrate coated on glass coverslips alone or in conjunction with laminin or PrP-Fc. Neurons were grown either in the presence or absence of aprotinin. In the absence of aprotinin, neurites in PrP-/- neurons are longer than in PrP+/+ neurons. This effect is lost in the presence of aprotinin.*P<0.05, t-test

5.7.1 Neurite outgrowth in the PrP-/- neurons is not affected by Caspr antibodies

To support this idea, we compared the sensitivity of neurite outgrowth in PrP+/+ and PrP-/- neurons to the activation of Caspr. Indeed, while neurite outgrowth was reduced in PrP+/+ neurons grown on poly-L-lysine in combination with the antibodies against Caspr extracellular domain when compared to PrP+/+ neurons grown on poly-L-lysine alone, Caspr antibodies had little effect on PrP-/- neurons (Fig 5.24). Similarly, antibodies against the Caspr extracellular domain had no effect on neurite outgrowth of PrP-/- neurons maintained on poly-L-lysine together with laminin, but inhibited neurite outgrowth in PrP+/+ neurons maintained on this substrate (Fig 5.24). Antibodies against the intracellular domain of Caspr did not influence neurite outgrowth rate in PrP+/+ neurons showing the specificity of the observed effects (Fig. 5.24). Altogether, our observations indicate that PrP deficiency results in reduced responsiveness of the neurons to Caspr activation.

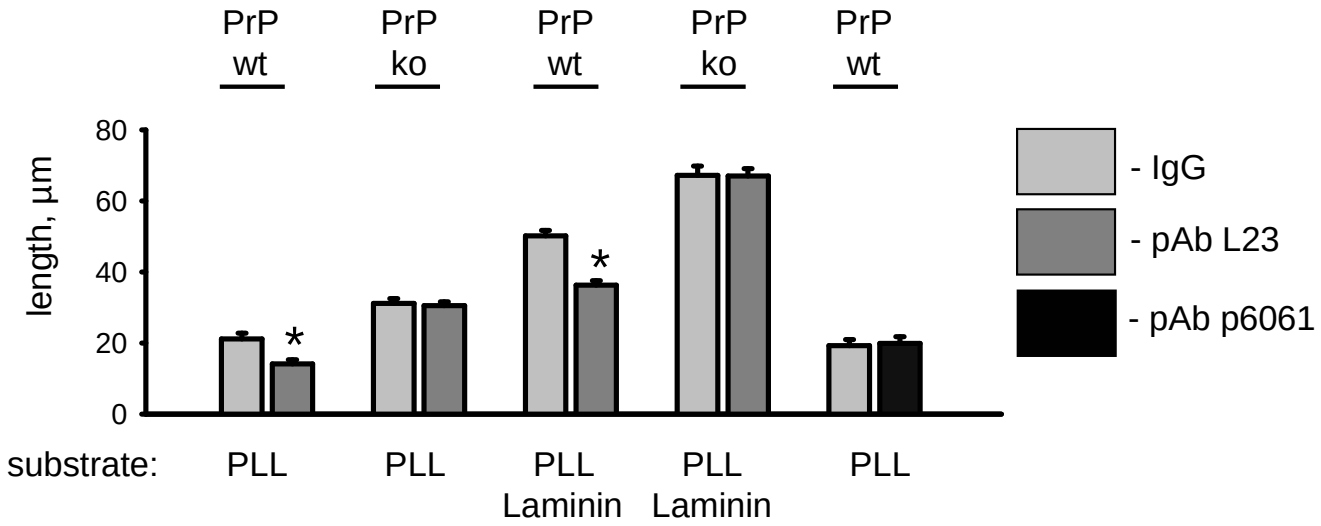

Figure 5.24: Antibodies against the extracellular domain of Caspr does not influence the neurite outgrowth in PrP+/+ and PrP-/- neurons.

Graph shows mean ± SEM neurite lengths in PrP+/+ and PrP-/- cerebellar neurons that were maintained in culture on poly-L-lysine (PLL) that was substrate coated on glass coverslips alone or in conjunction with laminin. The coating was done with or without the antibodies against the Caspr extracellular domain (L23) or Caspr intracellular domain (p6061) that served as a control. Note that PrP-/- neurons do not respond to the antibody against Caspr extracellular domain. *P<0.05, t-test.

Results

5.8 PrP deficiency promotes motor recovery after spinal cord injury

Because PrP deficiency reduces inhibitory effect of Caspr on neurite outgrowth of cerebellar neurons *in vitro*, we wanted to know if this phenomenon plays a role in CNS. Since it is difficult to evaluate cerebellar axon outgrowth *in vivo* during development, we analyzed it in regenerating nervous system by performing lower thoracic spinal cord compression lesions in PrP-/- mice and their PrP+/+ littermates and estimating recovery of the motor abilities in these mice as a function of synchronized re-growth of axons. We have previously successfully used this paradigm to assess roles of other molecules in CNS regeneration (Apostolova et al., 2006; Jakovcevski et al., 2007).

Spinal cord compression caused severe disabilities in both PrP+/+ and PrP–/– mice, as assessed by the BBB score 1 week after injury (Fig 5.25A). Between 1 and 6 weeks, the locomotor function improved significantly in both genotypes, but improvement was greater in PrP–/– mice than in their PrP+/+ littermates as revealed by analysis of BBB score values (Fig 5.25A). We also estimated plantar stepping ability with our novel parameter, the foot-stepping angle, previously shown to correlate well with the BBB score (Apostolova et al., 2006). This analysis also revealed enhanced recovery in PrP–/– mice compared with PrP+/+ mice at 3 and 6 weeks (Fig 5.25B). The rump-height index, a measure of the ability to support body weight during ground locomotion, was also significantly better at 3 and 6 weeks after injury in PrP–/– mice compared with PrP+/+ littermates (Fig 5.25C). In addition, PrP–/– mice performed voluntary movements without body weight support, estimated by the extension–flexion ratio, better than PrP+/+ mice at both 3 and 6 weeks (Fig 5.25D). However, numbers of correct steps made by the animals during inclined ladder climbing, reduced to almost 0 in both groups 1 week after injury, did not improve with time in either PrP+/+ or PrP–/– mice (data not shown). The ladder-climbing test allows estimation of the ability to perform precise movements requiring a high degree of supraspinal control, which was not re-established after being severed in either group of mice. From our analysis we conclude that after spinal cord injury there is an overall better functional outcome in PrP–/– mice compared with their PrP+/+ littermates.

Results

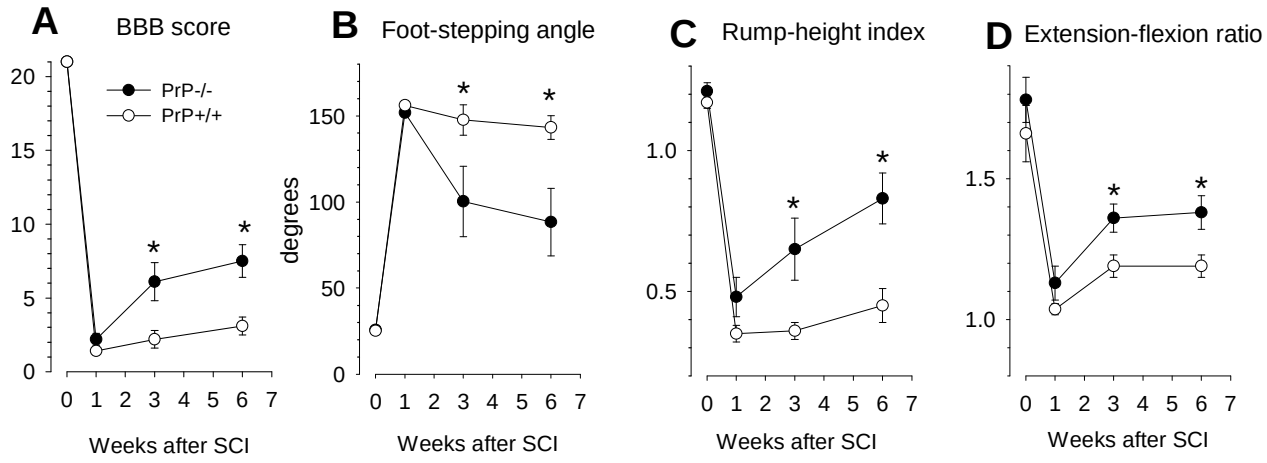

Figure 5.25: Time course and degree of motor recovery in PrP–/– and PrP+/+ mice after spinal cord injury. Shown are mean ± SEM values of open-field locomotion (BBB) scores (A), foot-stepping angles (B), rump-height indices (C), and extension–flexion ratios (D) before surgery (day 0) and at 1, 3, and 6 weeks after injury. *p < 0.05, significant differences between group mean values at a given time period (one-way ANOVA for repeated measurements with Turkey's post hoc test; n = 6 mice per genotype).

6 Discussion

Prion protein is an interesting protein from few perspectives:

Before the discovery of prions, it was thought that all pathogens used nucleic acids to mediate their replication and hence most of the diseases were nucleic acid mediated. After the discovery of prion, whose infectious agent was not nucleic acid, it was not clear how this protein can replicate without the use of nucleic acid that contradicted so-called "central dogma of molecular biology," which describes nucleic acid as the central form of replicative information. But later work led to the "protein-only hypothesis" according to which protein structure can replicate without the use of nucleic acid.

Evidence in favour of a protein-only hypothesis includes:

- Virus particles are not associated with prion diseases. Nucleic acid is not associated with infectivity; agent is resistant to degradation by nucleases.
- No immune response to infection.
- PrP^{Sc} was transmitted experimentally between species. The PrP^{Sc} in the recipient species had the amino-acid sequence of the recipient, suggesting that replication is not donor dependant, which means there is no transfer of nucleic acid.
- Infectivity level is associated with levels of PrP^{Sc}.
- PrP^{Sc} and PrP^{C} do not differ in amino-acid sequence; therefore a PrP^{Sc}-specific nucleic acid is redundant.

Familial prion disease occurs in families with a mutation in the PrP gene, and mice with PrP mutations develop prion disease despite controlled conditions where transmission is prevented.

This dual behaviour of a cellular protein to become an infectious agent makes PrP an intriguing protein. Although many studies have highlighted several functions of PrP, it is still poorly understood what are the functions of cellular PrP and how PrP is involved in cellular processes such as neurite outgrowth.

Functional interactions of PrP with its binding partner(s) have been suggested previously (Telling et al., 1995; Shmerling et al., 1998): a cis- and/or trans-interacting PrP activates an unknown binding partner or partners and disruption of this interaction competing with the dominant-negative mutant of PrP truncated at the amino terminus can lead to ataxia and cerebellar lesions (Shmerling et al., 1998). We have now identified Caspr as binding partner for

Discussion

PrP that has a role in neurite outgrowth together with PrP. In the absence of Caspr, mice exhibit tremor, paresis and ataxia. This ataxic phenotype exhibited by Caspr mutants is similar to the ataxic phenotype observed in PrP deficient mice. The defects include hypomotility, a tremor that is accentuated with movement and a wide-based gait suggestive of a cerebellar defect (Peles et al., 1997). Ataxia is a specific clinical manifestation implying dysfunction of parts of the nervous system that coordinate movement, such as the cerebellum. From the results shown above it is clear that Caspr and PrP are present in the same regions in the cerebellum. The findings in mutant mice further suggest a tight functional relationship between these two proteins.

In the absence of PrP the full length protein of Caspr is degraded and a proportional increase in the degradation product was observed. This phenomenon is not restricted to total brain homogenates but is also observed for the sub cellular fractions like axolemma and in lipid rafts. RT PCR data also supports the idea that a reduction in the levels of full length Caspr in the absence of PrP is not due to the reduction in the Caspr mRNA levels. Since Caspr mRNA levels are similar in PrP+/+ and PrP-/- mice (Fig 6.1) Since the molecular weight of full length Caspr is 155 kDa (without glycosylation) and the intracellular domain of Caspr only 10 kDa, we presumed that Caspr is cleaved at two positions which leads to the formation of 3 pieces of the same molecular weight. Therefore the degradation product observed on the western blot contains extracellular part and a part attached to the membrane. This idea is supported by the fact that the antibodies against the intracellular domain of Caspr and antibodies against the extracellular domain of Caspr (Caspr N-terminus) recognise the degradation product of Caspr.

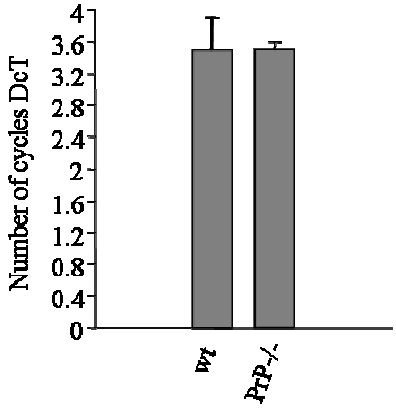

Figure 6.1: m-RNA level of Caspr is not altered in the PrP+/+ and PrP-/- mouse brains.

Total RNA was isolated from PrP+/+ and PrP-/- mouse brains and the levels of Caspr mRNA were quantified using RT PCR. Graphs show that mRNA levels of Caspr are not changed in the PrP-/- mouse brains.

Discussion

Transmembrane proteins are processed by many proteases, for instance gamma secretases in case of amyloid precursor protein, proprotein convertase PC5A and a metalloprotease are involved in the proteolytic processing of the neural adhesion molecule L1(Kalus.I et al., 2003). Interestingly, our results show that Caspr is cleaved by reelin and shedded in the extracellular space. Reelin is a known serine protease and the activity of reelin is inhibited by serine protease inhibitors (Quattrocchi et al., 2001). It is probable that the serine protease activity of reelin is important in regulating the duration of the interactions between reelin and its receptors (e.g., integrin, VLDLR, or ApoER2). Reelin is also present in the cerebellum and the absence of reelin in the reeler mutant mice leads to ataxia. The ataxic phenotype is similar to that of PrP deficient and Caspr deficient mice. Reelin cleaves certain extracellular matrix molecules such as laminin and fibronectin. An important structural feature of laminins is lam G domain. At least 1 Lam G domain is present in every laminin isoform and up to five Lam G domains are present in some isoforms of laminin. Interestingly Caspr also has equally placed Lam G domains in the extracellular part (Fig 2.5). Although the exact cleavage site for reelin is unknown; it is conceivable that reelin can cleave Caspr in LamG domain.

If Caspr is degraded by reelin in the absence of PrP, does PrP protect Caspr? Transfection and biotinylation data shows that when Caspr is cotransfected with PrP, the levels of Caspr on the surface of the CHO cell is increased as compared to when it is transfected alone. The m-RNA levels of Caspr in these transfected cells were not different, which means that the cotransfection with PrP did not alter the Caspr mRNA synthesis and that the difference in the levels of Caspr is purely attributed to the posttranslational processing of Caspr (Fig 6.2). In the absence of PrP, Caspr is also reduced at the surface of cerebellar neurons. Altogether this means that PrP stabilizes Caspr on the surface of the cell and it protects Caspr from degradation. The proper functioning of the cell requires careful control of the levels of important structural proteins, enzymes, and regulatory proteins hence protein degradation is an integral part in processing of protein.

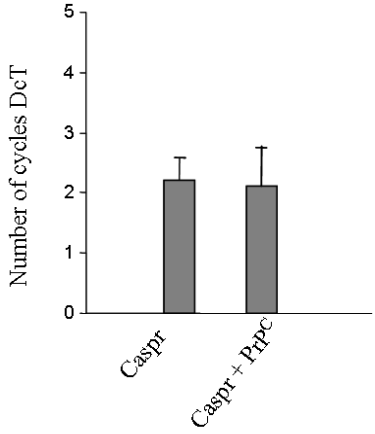

Figure 6.2: m-RNA levels of Caspr are similar in Caspr singly transfected CHO cells and Caspr-PrP cotransfected CHO cells. Total RNA was isolated from CHO cells singly transfected with Caspr and CHO cells cotransfected with Caspr and PrP. The levels of Caspr mRNA were quantified using RT PCR. Graphs show that the absence of PrP does not influence the mRNA level of Caspr

How could PrP protect Caspr from degradation? One hypothesis is that PrP physically binds to Caspr, as shown in the results from ELISA and by co immunoprecipitation thereby it may block the cleavage site within Caspr where reelin, the protease can act. Another reasonable hypothesis is that PrP influences conversion of reelin from a proenzyme form to biologically active enzyme form. This is supported by the fact that in the absence of PrP in the total brain, we see an increase in the levels of 140-180 kDa reelin, which is the biologically active form (Quattrocchi et al., 2001). How PrP hinders the conversion of reelin is yet to be resolved. However PrP and reelin are processed by a zinc dependant metalloprotease. This means that in the absence of PrP, reelin may be more exposed to metalloprotease that would activate it. Alternatively, PrP could also protect reelin from metalloproteases as it is doing for Caspr.

PrP is a known promoter of neurite outgrowth (Chen et al., 2003). Role of Caspr in neurite outgrowth is unknown. Our results show that in the absence of Caspr the neurite lengths are increased. This means that Caspr is a molecule that is sending an inhibitory signal or the stop signal to the developing neurite. Therefore while PrP is a neurite outgrowth promoting molecule (Chen et al., 2003; Santuccione et al., 2005), by protecting Caspr on the surface and increasing the levels of Caspr, PrP may also negatively regulate neurite outgrowth. PrP deficiency then reduces inhibitory effect of Caspr on neurite outgrowth.

What role does this play in the CNS? What does this inhibitory effect mean to the brain? To show that PrP regulates axonal growth in vivo, mice that underwent lower thoracic spinal cord lesions were observed for 1 to 6 weeks. These injured mice recovered between 1 to 6 weeks.

Discussion

PrP-/- mice recovered better than the PrP+/+ injured mice. In addition the PrP-/- mice performed voluntary movements without body weight support. This means that the axons regenerated in vivo more efficiently in the absence of PrP following spinal cord injury. This observation is also supported by the fact that in the axolemma fractions obtained from PrP+/+ and PrP-/- brains, we observed that the levels of full length Caspr is reduced in the absence of PrP and that the levels of the degradation product of Caspr is proportionally increased (Fig 6.3). It is also known that PrP and Caspr are both axonal proteins.

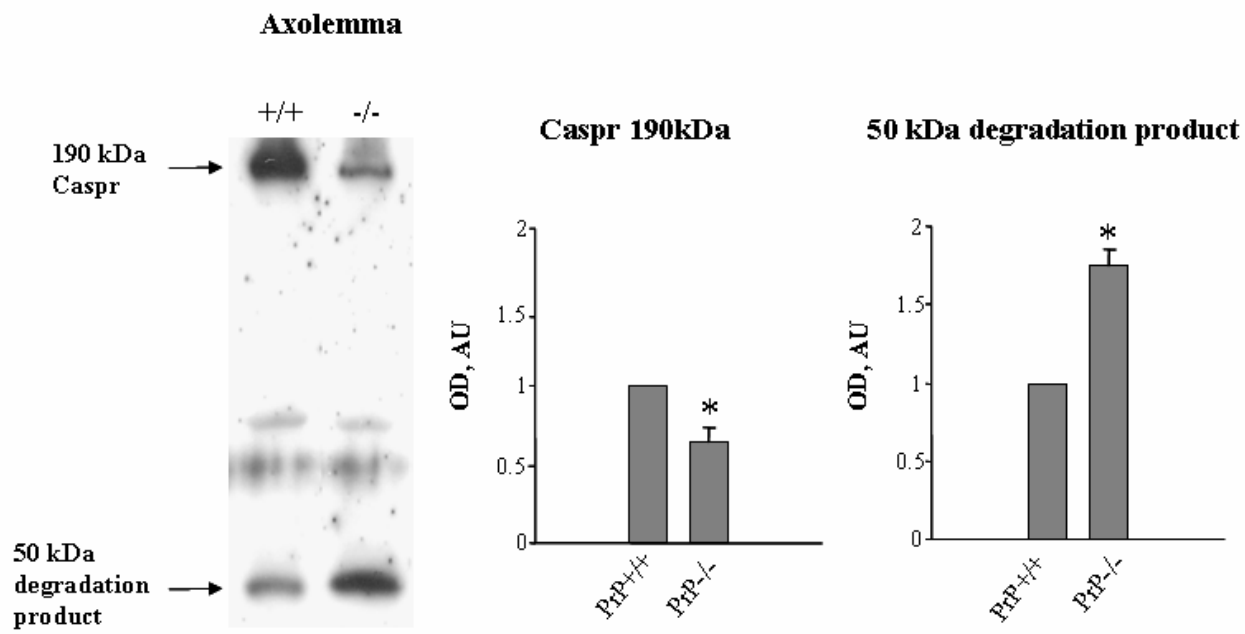

Figure 6.3 Caspr levels are reduced in axolemma from PrP-/- mouse brains.

Axolemma fractions obtained from PrP+/+ and PrP-/- brains were analysed by western blot using antibodies against the intracellular domain of Caspr. Graphs show that the levels of full length Caspr are reduced in the absence of PrP accompanied by a concomitant increase in the levels of the degradation product of Caspr. *P<0.05, t-test.

A valid explanation to this difference in the efficiency of recovery followed by spinal cord injury is that in the absence of PrP, Caspr is cleaved by reelin in excess. Therefore there is less Caspr in the brain and hence there is no inhibitory signal for the neurons to stop. This leads to more efficient and faster growth of neurons in the injured mice devoid of PrP (Fig 6.5). In the PrP+/+ situation, there is PrP to protect Caspr on the cell surface or PrP may interferes and inhibits the formation of active reelin (Fig 6.4). Hence the level of Caspr is unaltered. This allows Caspr to perform its neurite outgrowth regulation as an inhibitory molecule (Fig. 6.4). Thus we reveal a previously unrecognized role for Caspr and PrP in inhibitory modulation of neurite outgrowth in CNS neurons and a significant role in the regeneration of CNS axons.

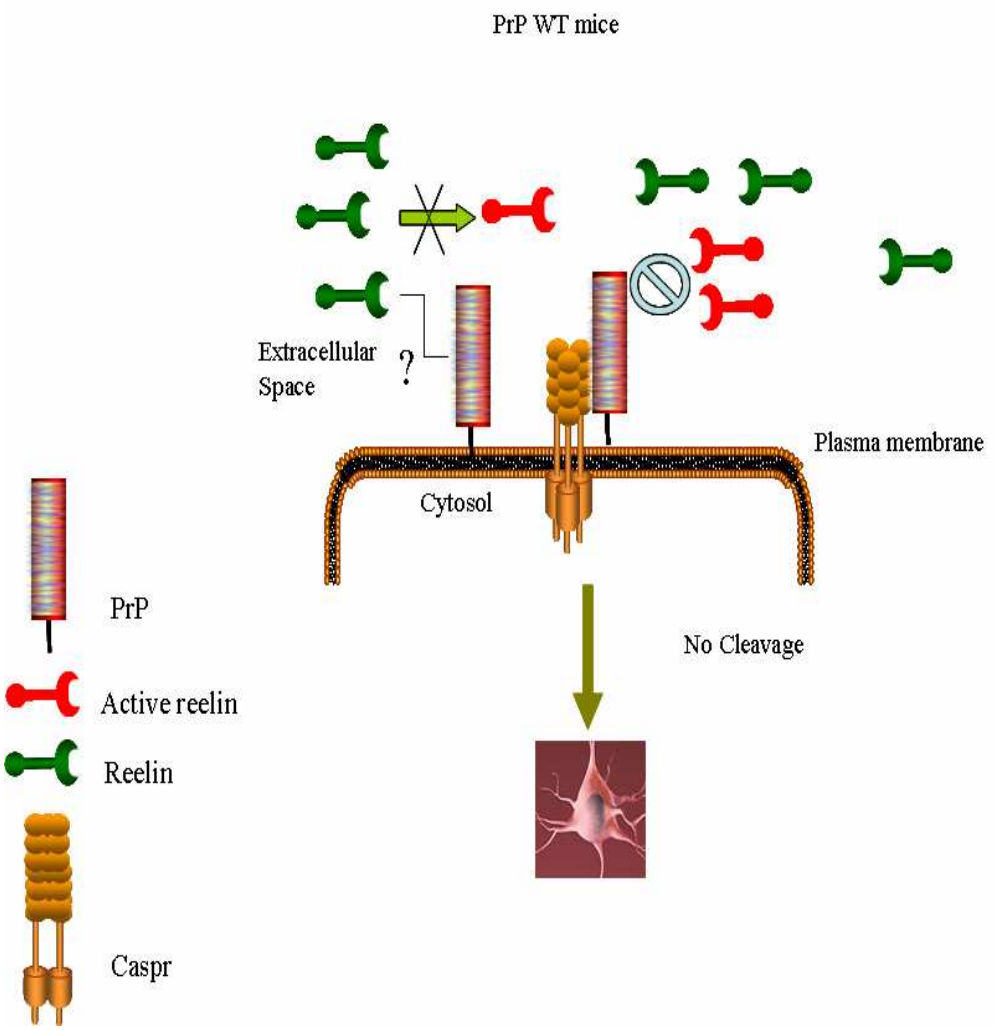

Figure 6.4: Regulation of proteolysis of Caspr and neurite outgrowth regulation in the PrP +/+ brains.

PrP is GPI anchored on the plasma membrane and reelin is in the extra cellular matrix. Caspr is a transmembrane protein. PrP physically binds to Caspr in the extracellular space and inhibits the cleavage of Caspr by reelin by competing with the binding sites. In a different scenario, presence of PrP reduces the conversion of pro form of reelin to the active enzyme form. This binding of PrP and Caspr leads to the intracellular signalling and thereby regulating the neurite outgrowth.

Discussion

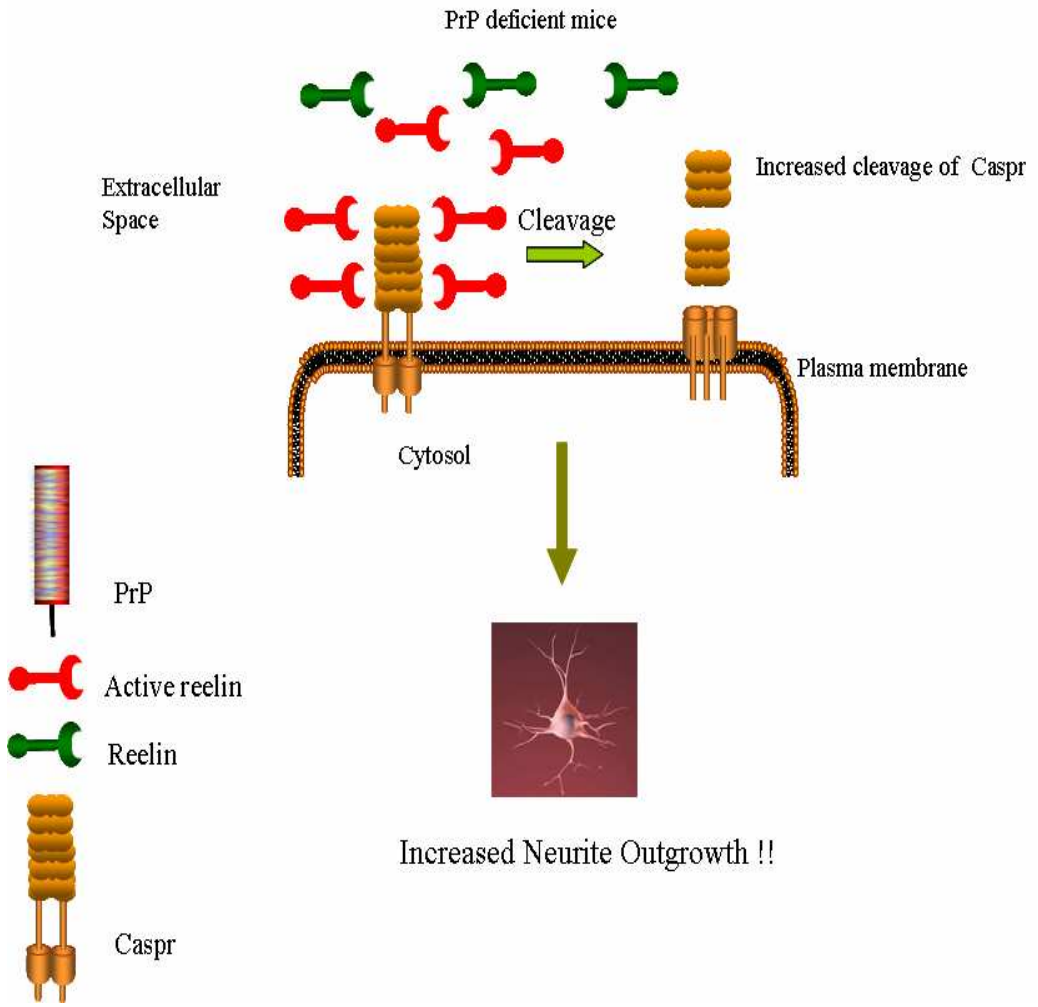

Figure 6.5: Regulation of proteolysis of Caspr and neurite outgrowth regulation in the PrP -/- brains.

There is no PrP and reelin is in the extracellular space. More active reelin is formed, perhaps because there is no PrP to inhibit the formation of active reelin. Caspr is a transmembrane protein. If PrP protects Caspr by binding physically to Caspr, then in the absence of PrP, reelin cleaves Caspr into two approximately equal half. Caspr is an inhibitory molecule, as shown above. In the event of Caspr degrading more, this is close to a Caspr -/- situation. Therefore there is an enhanced neurite outgrowth.

7 Summary

Prion protein (PrP) is an adhesion molecule anchored to the membranes of neurons by a glycosylphosphatidylinositol anchor (GPI). PrP regulates its neuronal survival, neurite outgrowth and synapse formation. Misfolding of this protein results in the formation of its scrapie form, which is the causative agent of Creutzfeldt - Jakob disease, Bovine Spongiform Encephalopathy and Transmissible Spongiform Encephalopathies. Hence, understanding of the mechanisms by which PrP regulates its cellular functions is an important issue in the field of neurobiology. We identified Contactin associated protein (Caspr) as a novel binding partner of PrP. Both proteins are associated with each other in the brain as shown by coimmunoprecipitation experiments and directly interact as shown by ELISA. We show that PrP regulates Caspr levels in neurons by inhibiting serine protease dependent shedding of Caspr from the neuronal surface membrane. Reelin was identified as a major serine protease that mediates Caspr shedding in a PrP-dependent manner. In cultured cerebellar neurons, PrP deficiency resulted in enhanced reelin-mediated shedding of Caspr from the cell surface. PrP deficiency also resulted in reduced levels of the full length Caspr in the brain accompanied by an increase in the levels of proteolytic degradation product of Caspr indicating that PrP inhibits Caspr shedding in the brain tissue also. We show that antibody-induced Caspr clustering at the surface of live neurons inhibits neurite outgrowth. In accordance with this observation, neurite outgrowth in PrP deficient cerebellar neurons maintained in vitro and locomotor recovery following spinal cord injury was enhanced correlating with reduced levels of Caspr in this mutant. Thus we reveal a previously unrecognized role for Caspr and PrP in inhibitory modulation of neurite outgrowth in central nervous system neurons.

8 Zusammenfassung

PrP (prion protein) ist ein Adhäsionsmolekül, das mittels eines Glycosylphosphatidylinositol (GPI)-Ankers mit der Membran verbunden ist. PrP reguliert Zellüberleben von Neuronen, Neuritenwachstum sowie Synapsenbildung. Eine falsche Faltung des Proteins führt zur Entstehung der Scrapie-Form, die wiederum die Creutzfeldt-Jacob-Krankheit, BSE (Bovine Spongiform Encephalopathy) und TSE (Transmissible Spongiform Encephalopathies) verursacht. Deshalb ist die Erforschung der Mechanismen, durch die PrP Zellfunktionen reguliert, ein sehr wichtiges Feld innerhalb der Neurobiologie.

Wir konnten Caspr (Contactin associated protein) als neuen Bindungspartner von PrP identifizieren. Mittels Coimmunpräzipitationen wurde eine Assoziation der beiden Proteine im Gehirn gezeigt. In ELISA-Experimenten weisen PrP und Caspr außerdem eine direkte Bindung aneinander auf. Wir zeigen, dass PrP die Expressionslevel von Caspr in Neuronen reguliert, indem es die Serinprotease-abhängige Abspaltung von Caspr von neuronalen Zelloberflächen inhibiert. Reelin wurde als hauptsächlich verantwortliche Serinprotease identifiziert, die die Abspaltung von Caspr in Abhängigkeit von PrP vermittelt. In kultivierten Kleinhirnneuronen führte die Defizienz von PrP zu erhöhter Reelin-vermittelter Abspaltung von Caspr von der Zelloberfläche. Außerdem konnte bei PrP-Defizienz eine verringerte Menge von Caspr voller Länge im Gehirn nachgewiesen werden, während gleichzeitig die Menge des proteolytischen Abbauproduktes von Caspr erhöht war. Dies weist darauf hin, dass PrP die Caspr-Abspaltung auch in Gehirngewebe inhibiert. Wir zeigen auch, dass eine durch Antikörper hervorgerufene Verdichtung von Caspr auf der Oberfläche lebender Neurone das Neuritenwachstum *in vitro* inhibiert. In Übereinstimmung mit diesen Beobachtungen waren sowohl eine Förderung des Neuritenwachstums von *in vitro* kultivierten PrP-defizienten Kleinhirnneuronen sowie eine verbesserte Erholung der Beweglichkeit nach Rückenmarksverletzungen in PrP-defizienten Mäusen zu beobachten. Beide Effekte korrelieren mit einer verringerten Menge von Caspr in dieser Mutante. Folglich konnten wir eine vorher unbekannte Rolle von Caspr und PrP in der inhibitorischen Regulierung von Neuritenwachstum im zentralen Nervensystem aufzeigen.

9 Abbreviations

α	Alpha
β	Beta
ϕ	Phi
μ	Micro (10^{-6})
°C	Degree Celsius
A	Adenosine
ADP	Adenosine biphosphate
Amp	Ampicillin
APS	Ammonium per sulphate
ATP	Adenosine triphosphate
B 4.1	Band 4.1 binding domain
B-cells	B-lymphocytes
bp	Base pairs
BSA	Bovine serum albumin
C	Cytosine
Caspr	Contactin associated protein
c-src	Cellular src kinase
C-terminus	Carboxy terminus
cDNA	Complementary deoxyribonucleic acid
CHL1	Close homologue of L1
CNS	Central nervous system
Dab-1	Disabled-1
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
EGF	Epidermal growth factor domain

Abbreviations

EGFL	Epidermal growth factor like domain
FBG	Fibrinogen related domains
FCS	Fetal calf serum
FNIII	Fibronectin III
fyn	Fyn Kinase
g	g-force
G	Guanosine
GFP	Green fluorescent protein
GPI	Glycosylphosphotidylinositol
HBSS	Hank's buffered sodium chloride solution
HEPES	2-(4-(2-Hydroxyethyl)-piperzino)-ethansulfonic acid
ICD	Intracellular domain
Ig	Immunoglobulin
IgG	Immunoglobulin subclass G
IPTG	Isopropyl-β-D-thiogalactoside
Kan	Kanamycin
kb	Kilo base pairs
l	liter
LAMG	Laminin G domain
LB	Luria Bertani
m	Milli (10^{-3})
MEM	Minimal essential medium
mRNA	Messenger ribonucleic acid
n	Nano (10^{9})
N-terminus	Amino terminus
NCAM	Neural Cell Adhesion Molecule

Abbreviations

NF 155	Neurofascin 155 kDa
NMR	Nuclear magnetic resonance
$NaHCO_3$	Sodium bicarbonate
OD_x	Optical density at x
ORF	Open reading frame
p	Pico (10^{12})
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDZ domain	post synaptic density protein (PSD95), Drosophila disc large tumour suppressor (DlgA), and zonula occuldens-1 protein (zo-1)
PMSF	Phenyl methyl sulfonyl fluoride
PNS	Peripheral nervous system
PrP/PrP^C	Cellular form of prion protein
$PrPSc/PrP^{Sc}$	Scrapie form of prion protein
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rotations per minute
RT	Room temperature
SH3 domain	Src homology 3 domain
T	Thymine
TAG-1	Transient axonal protein 1
T-cells	T-lymphocytes
TE	Tris EDTA
Tet	Tetracycline
TM	Transmembrane
Tris	tris(-hydroxymethyl)-aminomethane

Abbreviations

V	Volts
v/v	Volume per volume
w/v	Weight per volume

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12 Publications

Article

Devanathan V, Jakovcevski I, Santuccione A, Leshchyns'ka I, Sytnyk V, Schachner M (2008). PrP^{C} inhibits reelin mediated shedding of Caspr from the neuronal surface to potentiate Caspr mediated inhibiton of neurite outgrowth (Submitted).

Abstract

The following abstract is submitted in the FENS forum 2008 for a poster.

Devanathan Vasudharani, Igor Jakovcevski, Antonella Santuccione, Iryna Leshchyns'ka, Vladimir Sytnyk, Melitta Schachner (2008)Cellular form of prion protein (PrP) associates with Caspr and inhibits its reelin mediated shedding from the neuronal surface

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