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INTRACELLULAR PATHWAYS INVOLVED IN FORMATION AND DEGRADATION OF PRIONS

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Cover: Modified photomicrograph showing accumulatation of PrP^{Sc} (white) in prion-infected hypothalamic GT1-1 cells. Elin Allard and Jonas Karlsson.

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To my family

ABSTRACT

Prions cause invariably fatal neurodegenerative diseases, in which a misfolded host-encoded protein appears to be the main, if not the only, component of the infectious agent. During disease, a normal cellular protein, PrP^{C}, is converted to a disease-related isoform, PrP^{Sc}, by a post-translational process that might require auxiliary cellular cofactors. The physiological function of PrP^{C} is not completely characterized, but the protein has been implicated in synaptic function, neuroprotection and signal transduction. The studies in this thesis were undertaken with the aim to investigate whether intracellular signaling could affect the cellular accumulation of PrP^{Sc} and to examine whether a molecular cross-talk between prions and key intracellular signaling pathways occur in the infected cells.

We found that treatment of scrapie-infected hypothalamic gonadotropin-releasing GT1-1 cells with brain-derived neurotrophic factor (BDNF), or depolarization of the cells using high [KCl], activated the MEK/ERK MAP kinase cascade and unexpectedly also stimulated prion formation, resulting in increased levels of PrP^{Sc} in the cells. Conversely, inhibition of the MEK/ERK pathway using specific inhibitors of MEK cleared the cells from PrP^{Sc}, seemingly by blocking its formation. Exposure of GT1-1 cells to prions of the RML and 22L strains of scrapie caused a transient ERK activation. We, thus, demonstrated a bidirectional interaction between the MEK/ERK cascade and prions in infected cells: On the one hand, prion exposure induces ERK activation and on the other hand, ERK activation leads to increased prion formation.

Inhibition of the p38 and JNK MAP kinase pathways using specific inhibitors, led to increased accumulation of PrP^{Sc} in scrapie-infected GT1-1 cells. We demonstrate that the MEK/ERK and the p38 pathways exert opposing effects on prion formation, whereby MEK/ERK stimulates and p38 inhibits the conversion of PrP^{C} to PrP^{Sc}. This suggests that the dynamic balance between these signaling cascades may regulate the replication of prions.

Activation of not only the MEK/ERK but also the cAMP/PKA cascade stimulated cellular accumulation of PrP^{Sc} and was accompanied by phosphorylation of the cytoplasmatic S6 ribosomal protein. This protein is involved in regulation of protein translation, and is a main target for the mTOR pathway. Further studies revealed that also the mTOR signaling pathway influences the accumulation of PrP^{Sc} in GT1-1 cells, suggesting that the MEK/ERK and the mTOR pathways might converge to exert a translational regulation of prions.

By using PrP^{C}-specific siRNA we found evidence of cellular degradation of PrP^{Sc} and by using specific inhibitors as well as RNAi directed to cathepsin B and L, we identified these lysosomal proteases as important mediators of PrP^{Sc} degradation.

In conclusion, we identified five intracellular signaling pathways, including three MAP kinase cascades, to be involved in the cellular accumulation of PrP^{Sc} of different prion strains and, in addition, we identified lysosomal cathepsins as important for degradation of PrP^{Sc}. These observations might disclose novel therapeutic strategies in prion diseases, based on manipulation of intracellular signaling. Furthermore, the findings described in this thesis indicate a dual function of ERK activation in neurons during prion infections. Thus, activation of ERK, which is normally beneficial to the cell by inducing protective mechanisms, can at the same time promote formation of the pathogen.

LIST OF PUBLICATIONS

This thesis is based on the following original articles, which will be referred to in the text by their roman numerals:

I. Katarina M. Luhr, **Elin K. Nordström**, Peter Löw, Hans-Gustaf Ljunggren, Albert Taraboulos and Krister Kristensson, *Scrapie protein degradation by cysteine proteases in CD11c+ dendritic cells and GT1-1 neuronal cells*. Journal of Virology, 2004, 78(9): 4776-4782.

II. Katarina M. Luhr*, **Elin K. Nordström***, Peter Löw and Krister Kristensson*, Cathepsin B and L are involved in degradation of prions in GT1-1 neuronal cells.* Neuroreport, 2004, 15: 1663-1667.

III. **Elin K. Nordström**, Katarina M. Luhr, Carlos Ibáñez and Krister Kristensson, *Inhibitors of the Mitogen-Activated Protein Kinase Kinase 1/2 Signaling Pathway Clear Prion-infected Cells from* PrP^{Sc}. Journal of Neuroscience, 2005, 25(37): 8451-8456.

IV. **Elin K. Nordström**, Gilberto Fisone and Krister Kristensson, *Opposing effects of ERK and p38-JNK MAP kinase pathways on prion formation in GT1-1 cells.* FASEB Journal, 2009, 23(2): 613-622.

V. **Elin K. Allard**, Mirjana Grujic, Gilberto Fisone and Krister Kristensson, *Translational control of prions by MAP kinase and mTOR signaling in GT1-1 cells.* Manuscript.

LIST OF ABBREVIATIONS

4E-BP1	eukaryotic initiation factor 4E binding protein 1
BDNF	brain-derived neurotrophic factor
cAMP	cyclic adenosine monophosphate
CJD	Creutzfeldt-Jakob disease
CNS	central nervous system
eIF	eukaryotic translation initiation factor
ERK	extracellular signal-regulated kinase
GABA	gamma-aminobutyric acid
GAGs	endogenous glycosaminoglycans
GT1-1 cells	hypothalamic gonadotropin-releasing hormone neurons
GTP	guanosine triphosphate
JNK	stress-activated protein kinase 1
MAP	Mitogen-activated protein
MEK	mitogen-activated protein kinase kinase
N2a	neuroblastoma cells
NCAM	neural cell adhesion molecule
NGF	nerve growth factor
NICD	Notch-1 intracellular domain
p38	stress-activated protein kinase 2-4
PC12 cells	cell line derived from rat adrenal medulla
PKA	protein kinase A
PrP^{C}	normal cellular prion protein
PrP^{Sc}	scrapie prion protein
RML	Rocky Mountain Laboratory
RNAi	RNA interference
S6Ks	ribosomal S6 kinases
S6rp	S6 ribosomal protein
ScGT1-1	scrapie-infected GT1-1 cells
ScN2a	scrapie-infected N2a cells
siRNA	short interfering RNA
STI1	stress-inducible protein-1
Trk	tropomycin receptor kinase
TSE	transmissible spongiform encephalopathies

CONTENTS

INTRODUCTION

Prions

Prions cause transmissible spongiform encephalopathies (TSE) and constitute a novel class of infectious pathogens, in which a misfolded host-encoded protein appears to be the main component of the disease-causing agents. For many years the nature of the infectious agents remained unknown. In 1954 Björn Sigurdsson coined the concept of "slow virus infections" (Sigurdsson 1954), which included "rida" in sheep that is the Icelandic name for the prion infection scrapie. However, the pathogen was shown to be resistant to inactivation by UV and ionizing radiation (Alper et al 1967, Alper et al 1966), thus indicating an infections agent devoid of nucleic acids. A protein as the pathological agent was suggested in 1967 by Pattison and Jones (Pattison & Jones 1967) and a model for protein self-replication of the scrapie agent was proposed by the mathematician John Griffith (Griffith 1967), but these ideas were relatively neglected. Thus, Richard T. Johnson in 1982 summarized: *"The unorthodox properties of the spongiform encephalopathy agents have created a mystique about their possible nature. Exotic fantasies have evolved of an agent devoid of nucleic acids but representing self-replicating membranes, proteins or polysaccharides."* (Johnson 1982).

In 1982, a fragment of the prion protein denoted PrP27-30 was discovered and provided a molecular marker for TSE diseases (Barria et al 2009, Bolton et al 1982, Prusiner et al 1982). The term 'prions' (proteinaceous infectious particles that lack nucleic acids), was coined in 1982 by Stanley B. Prusiner (Prusiner 1982, Prusiner 1998b). Surprisingly, the infectious agent seemed to be expressed from the host's own genome. Molecular cloning studies of the PrP gene (*prnp*) and further analysis revealed that PrP27-30 was the truncated proteinase K-resistant core of a disease-related protein, designated PrP^{Sc}, which had the same amino acid sequence as a normal cellular protein, denoted PrP^{C} (Chesebro et al 1985, Hope et al 1986, Meyer et al 1986, Oesch et al 1985). PrP^{Sc} is, thus, a misfolded isoform derived from PrP^{C}, which is an abundantly expressed glycosylphosphatidyl-inositol (GPI)-anchored plasma membrane protein (for review, see (Hope & Manson 1991, Prusiner 1998b)).

The formation of PrP^{Sc} involves a change in secondary and tertiary protein structure; the α-helix and random coil structure in PrP^{C} is refolded into left-handed β-helical structures (Govaerts et al 2004, Pan et al 1993, Wille et al 2002). PrP^{Sc} has altered physiochemical properties; while PrP^{C} is readily degradable by proteinase K, PrP^{Sc} shows a remarkable resistance to proteolytic digestion. However, the occurrence of a continuum of protease-sensitive PrP^{Sc} varieties has later been recognized (Pastrana et al 2006, Safar et al 1998, Tzaban et al 2002). PrP^{C} is fully soluble in nonionic detergents, whereas PrP^{Sc} is not and forms aggregates with

characteristics often similar to amyloid fibrils, which consequently accumulates in the brain of infected individuals. PrP^{Sc} is so far the only identified component of the infectious agent and the protein promotes its own formation by acting as the template for formation of new PrP^{Sc} (fig. 1). The main arguments for a protein-only hypothesis for prion replication are presented in Table 1.

Table 1. Arguments for PrP^{Sc} being the major, if not the only, component of infectious prions

- No viral particles, bacteria, fungi or protozoan parasites have been conclusively associated with prion diseases.
- Prions are devoid of nucleic acids (Alper et al 1967, Alper et al 1966, Bellinger-Kawahara et al 1987a, Bellinger-Kawahara et al 1987b, Kellings et al 1992, Kellings et al 1994).
- PrP^{Sc} and scrapie infectivity copurify (Hope et al 1986, Prusiner et al 1982, Prusiner et al 1983).
- Procedures that hydrolyze or denature PrP^{Sc} reduce prion titers (Bolton et al 1984, McKinley et al 1983).
- PrP^{Sc} experimentally transmitted from one species to another results in PrP^{Sc} with the amino-acid sequence of the recipient species, suggesting that replication of the donor agent does not occur (Prusiner et al 1990, Scott et al 1989a, Weissmann 1991).
- PrP^{C} is essential for prion diseases and PrP^{C} knockout mice are resistant to prion infections (Bueler et al 1993, Prusiner et al 1993).
- Mutations in the human PrP gene (*PRNP*) result in formation of PrP^{Sc} and are genetically linked to inherited human prion disease (Kovacs et al 2002).
- Mice expressing MoPrP transgenes with the point mutation of the human prion disease Gerstmann-Sträussler-Scheinker syndrome spontaneously develop spongiform encephalopathy, and the disease can be transmitted to transgenic mice that express the same mutation at low levels (Hsiao et al 1991, Hsiao et al 1994, Telling et al 1996).
- Overexpression of PrP^{C} increases the rate of PrP^{Sc} formation and leads to shorter incubation times (Scott et al 1989b).
- Synthetic prions produced in *Escherichia coli* can infect transgenic mice overexpressing truncated PrP (Legname et al 2004).

PrP^C – putative functions

The normal prion protein, PrP^C, is evolutionally conserved and is predominantly expressed in neurons, but also in other cell types, with a primary localization to cholesterol-rich lipid rafts or "caveolae-like" domains of the plasma membranes. Lipid rafts serve as organizing centers for the assembly of signaling molecules, and are involved in regulation of neurotransmission as well as receptor trafficking (Paratcha & Ibanez 2002, Taylor & Hooper 2006).

Synaptic function

PrP^C is particularly abundant in brain regions with a high degree of synaptic plasticity, e.g. hippocampus (Sales et al 1998), and because of its localization to synapses (Fournier et al 1995, Godsave et al 2008, Haeberle et al 2000, Herms et al 1999, Kovacs et al 2005, Laine et al 2001, Sales et al 1998) PrP^C has been attributed a role in synaptic function. In line with this, there are several studies describing alterations in neuronal function in PrP^C-deficient mice, including impaired $GABA_A$ receptor-mediated signaling with reduced synaptic inhibition (Collinge et al 1994), decreased EPSPs (Carleton et al 2001) and reduced slow afterhyperpolarization (Colling et al 1996, Fuhrmann et al 2006, Mallucci et al 2002) recorded in hippocampal slices from mice lacking PrP^C. Reduced long-term potentiation in hippocampal slices from PrP knock-out mice was observed in some studies (Collinge et al 1994, Manson et al 1995), but was not seen in another study (Lledo et al 1996).

Signal transduction and neuroprotection

The localization to lipid rafts implies a potential role for PrP^C in signal transduction. PrP^C have been suggested to play a role in neuroprotection, for instance by acting as a sensor of oxidative stress and/or by counterbalancing Bax-mediated apoptotic signals (Milhavet & Lehmann 2002, Roucou et al 2005, Roucou & LeBlanc 2005). Moreover, the interaction between PrP^C and stress-inducible protein-1 (STI1) induce endocytosis of PrP^C and this is a necessary step in the activation of intracellular signaling that can protect neurons from anisomycin-induced apoptotic cell death (Caetano et al 2008, Zanata et al 2002). Incubation of cultured hippocampal neurons with recombinant STI1 stimulates PrP^C-dependent neurite outgrowth via activation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) and contributes to neuroprotection via activation of protein kinase A (PKA) (Lopes et al 2005). Furthermore, PrP^C-binding peptides activate the cAMP/PKA and mitogen-activated protein kinase kinase (MEK)/ERK pathways (described below) and can partially prevent anisomycin-induced cell death in retinal explants from wild-type rodents, but not in explants from PrP^C knock-out mice (Chiarini et al 2002). PrP^C specific antibodies also exert neuroprotective effects by stimulating the PKA pathway (Chiarini et al 2002). In addition, PrP^C-deficient neuronal primary cultures are more sensitive than wild-type cells to apoptotic stimuli, such as serum deprivation, further supporting the idea of a neuroprotective role for PrP^C (Kuwahara et al 1999).

Antibody-mediated cross-linking of PrP^C, which induce clustering of PrP^C at the cell surface in the neuroectodermal 1C11 cell line, has been used as a model of PrP^C activation. PrP^C has thereby been suggested to stimulate a caveolin-1-dependent activation of the non-receptor tyrosine kinase Fyn (Mouillet-Richard et al 2000). Downstream activations include phosphoinositide 3-Kinase (PI3K), protein kinase C, NAPDH and ERK1/2 (Mouillet-Richard et al 2007, Schneider et al 2003). These results could be reproduced in hypothalamic gonadotropin-releasing hormone neurons (GT1 cells), but not in primary embryonic neuronal cultures or rat neuroblastoma cells (Monnet et al 2004). It should be noted that cross-linking of membrane proteins using antibodies might induce signaling that are not specific to PrP^C, but instead reflects an antibody-antigen interaction that by itself could elicit intracellular signaling.

Other suggested functions

There are indications that PrP^C may be involved in the regulation of circadian rhythms, since PrP knockout mice show a disruption of both circadian rhythms and sleep patterns (Huber et al 1999b, Huber et al 2002, Tobler et al 1997, Tobler et al 1996).

PrP^C has, in addition, been proposed to play a role in cell adhesion processes, based on the findings that it interacts with the laminin receptor and its precursor (LRP/LR) as well as the neural cell adhesion molecule (NCAM) (Gauczynski et al 2001b, Rieger et al 1997, Schmitt-Ulms et al 2001). Several lines of evidence indicate that PrP^C promotes neurite outgrowth (Chen et al 2003, Graner et al 2000, Kanaani et al 2005, Lopes et al 2005, Santuccione et al 2005). Moreover, PrP^C has recently been implicated in processing of sensory information by the olfactory system (Le Pichon et al 2009).

Conversion of PrP^C to PrP^{Sc}

Normal PrP^C is converted to its abnormal isoform, PrP^{Sc}, by a post-translational process (fig. 1), which might require auxiliary cellular cofactors. The $PrP^C \rightarrow PrP^{Sc}$ conversion involves formation of a partially unfolded monomer, PrP*, which is considered to be a crucial intermediate in the formation of PrP^{Sc} (Cohen et al 1994). The conversion process may occur either at the cell surface in association with lipid rafts or in early endosomal compartments (Borchelt et al 1992, Caughey & Raymond 1991, Caughey et al 1991, Godsave et al 2008, Jeffrey et al 1994, Marijanovic et al 2009, Taraboulos et al 1995). However, the exact subcellular site for PrP^{Sc} formation has not yet been determined. Irrespective of its site of formation, PrP^{Sc} has been shown to localize to lipid rafts in the plasma membrane (Naslavsky et al 1997, Taraboulos et al 1995, Vey et al 1996). In neurons, PrP^{Sc} has been suggested to localize to both electrical (Kovacs et al 2005) and chemical synapses (Fournier et al 2000, Kitamoto et al 1992, Kovacs et al 2005). However, in a recent cryo-EM study no such synaptic localization of PrP^{Sc} was observed (Godsave et al 2008). In addition, PrP^{Sc} accumulation in both early and late endocytic, as well as lysosomal, compartments has been described (Arnold et al 1995, Fournier et al 2000, Godsave et al 2008, Laszlo et al 1992, McKinley et al 1991).

Studies of the species barriers employing transgenic mice indicate that auxiliary molecules ("protein/cofactor X") of the host might be involved in the $PrP^{C} \rightarrow PrP^{Sc}$ reaction (Kaneko et al 1997, Telling et al 1994, Telling et al 1995). Although the mechanism for the conversion process is not yet determined in detail, several factors that facilitate the conversion process have been discussed, and are described briefly in the following section:

Endogenous glycosaminoglycans, heparan sulfat

Heparan sulfates (HS), which belong to a family of endogenous glycosaminoglycans (GAGs), can operate as cellular receptors for endocytosis of prions as well as cofactors for the $PrP^{C} \rightarrow PrP^{Sc}$ conversion process (Ben-Zaken et al 2003, Díaz-Nido et al 2002, Horonchik et al 2005, Schonberger et al 2003). Cellular uptake of purified prion "rods" and *de novo* infection of cell cultures are blocked by treatment with soluble sulfated glycans, GAG degrading bacterial enzymes (heparinase III) or the sulfation inhibitor chlorate (Horonchik et al 2005). In addition, HS has been shown to accumulate in prion amyloid plaques (Snow et al 1989) and associate with diffuse PrP^{Sc} deposits at early stages of prion diseases (McBride et al 1998). The interactions between prions and HS are reviewed in (Díaz-Nido et al 2002).

A variety of sulfated polyanions, such as heparin (another endogenous GAG) (Gabizon et al 1993) or semi-synthetic GAG analogues, e.g. HS mimetics (Adjou et al 2003, Schonberger et al 2003), pentosan polysulfate (PPS) (Caughey et al 1994, Caughey & Raymond 1993) and dextran sulfate (Ehlers & Diringer 1984) can block the conversion of PrP^{C} to PrP^{Sc} presumably by a competitive inhibition of the interaction between endogenous GAG molecules and PrP^{C} and/or PrP^{Sc}. In addition, treatment of cells with estradiol β-D-xyloside, which specifically inhibits glycosylation of endogenous proteoglycans, leads to reduced levels of PrP^{Sc} (Ben-Zaken et al 2003, Díaz-Nido et al 2002, Horonchik et al 2005).

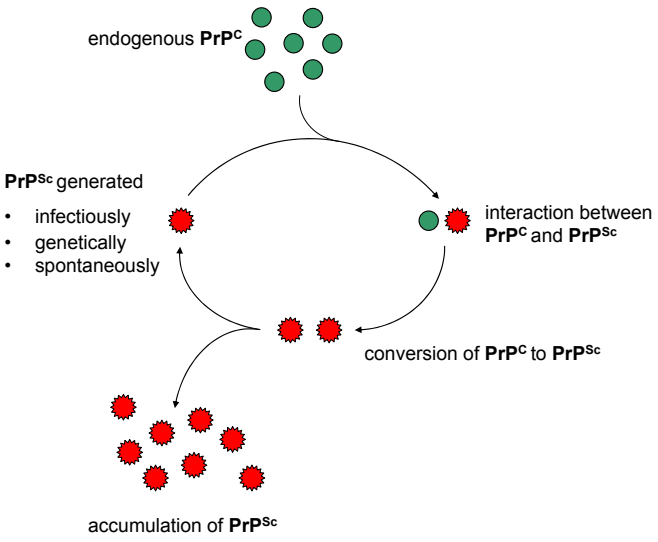

Figure 1. Propagation of PrP^{Sc}

Normal endogenous PrP^{C} can be converted to a misfolded isoform, PrP^{Sc}, and this process can be initiated in 3 different ways:

A. By the exposure of PrP^{C} to exogenous PrP^{Sc} (e.g. variant CJD).

B. By inherited mutations in PrP^{C} rendering the protein prone to misfold (e.g. familial CJD).

C. By somatic mutations in PrP^{C} or spontaneous conversion of PrP^{C} to PrP^{Sc} (e.g. sporadic CJD).

Laminin receptors

PrP^{C} can interact with the laminin receptors (LRP/LR) directly as well as in a HS-dependent manner (Gauczynski et al 2001a, Gauczynski et al 2001b, Hundt et al 2001, Rieger et al 1997), and LRP/LR is required for PrP^{Sc} propagation (Leucht et al 2003, Vana & Weiss 2006). Anti-LRP/LR antibodies, siRNA directed against LRP, polysulfated glycanes and LRP decoy mutants can be used to block or down-regulate the laminin receptor and reduce cellular levels of PrP^{Sc} (Leucht et al 2003, Vana et al 2009).

Blockage of PrP^{Sc} formation

Insight to the PrP conversion process has further been gained by studying drugs that block the formation of PrP^{Sc} both *in vivo*, *in vitro* and in cell free conversion assays (Kirby et al 2003).

Attention has been paid to the anti-malarial drug quinacrine (and its derivates), which is effective in clearing cultured cells from PrP^{Sc} as well as infectivity (Korth et al 2001), but has had no or little effect in clinical trials so far (Collinge et al 2009). Quinacrine is proposed to inhibit PrP^{Sc} formation by redistributing cholesterol from the plasma membrane to intracellular compartments, thereby destabilizing membrane domains and induce ultrastructural and morphological changes in endosomal compartments (Klingenstein et al 2006).

Other compounds, e.g. lovastatin and squalestatin, may interfere with PrP^{Sc} formation by affecting cholesterol synthesis and the lipid composition of rafts (Bate et al 2004a, Bate et al 2008b, Hagiwara et al 2007, Taraboulos et al 1995). Although several cholesterol inhibitors reduce PrP^{Sc} accumulation, others like docosahexaenoic acid and eicosapentaenoic acids can instead stimulate the formation of PrP^{Sc} (Bate et al 2008a). Klingenstein and colleagues suggest a combination of quinacrine, desipramine (a tricyclic antidepressant) and simvastatin (an inhibitor of cholesterol biosynthesis) as a novel pharmacological treatment for Creutzfeldt-Jakob disease, based on the positive synergistic effects observed in cell cultures (Klingenstein et al 2006).

The transport of PrP^{C} (and also of other proteins) to the plasma membrane can be blocked by the sulfated polyanion suramin. Thereby the amount PrP^{C} of available for conversion to PrP^{Sc} is decreased and subsequently also the cellular accumulation of PrP^{Sc} (Doh-Ura et al 2000, Gabizon et al 1993, Gilch et al 2001, Ladogana et al 1992). Compounds related to sulfated polyanions, such as Congo red and its analogues, may block prion formation by stabilizing the template, PrP^{Sc}, so that the partial denaturation of PrP^{Sc} needed for the $PrP^{C} \rightarrow PrP^{Sc}$ reaction is prevented (Caspi et al 1998, Caughey & Race 1992, Rudyk et al 2003, Rudyk et al 2000).

Moreover, there are drugs that directly interfere with the interaction between PrP^{C} and PrP^{Sc} and thereby block the formation of new prions, i.e. PrP antibodies (Enari et al 2001, Peretz et al 2001), anti-PrP aptamers (Proske et al 2002, Rhie et al 2003) PrP-derived peptides (Chabry et al 1999) and β-sheet breaker peptides (Soto et al 2000).

Other compounds, like Cp60 and its derivates, may inhibit the interaction between PrP^C and a proposed conversion cofactor X (Perrier et al 2000). In addition, cell-penetrating peptides derived from the prion protein N-terminus reduce PrP^{Sc} levels without affecting PrP^C levels in the cell (Löfgren et al 2008). This reduction might be caused by a mechanism by which the signal sequence guides the prion protein-derived peptide into a cellular compartment, where it specifically binds to PrP^{Sc} and inhibits formation of prions (Löfgren et al 2008).

Furthermore, a number of endogenous factors can affect the levels of PrP^C and thereby influence the formation of PrP^{Sc}. In that way, the expression of PrP^C is increased when intracellular signaling is induced via nerve growth factor (NGF) (Kuwahara et al 2000, Mobley et al 1988, Sauer et al 2003, Wion et al 1988), insulin (Kuwahara et al 2000), epidermal growth factor (EGF), tumor necrosis factor alpha (TNF –α) (Sauer et al 2003), phospholipase A_2 (PLA_2) and platelet-activating factor (Bate et al 2004b, Bate et al 2004c) as well as retinoic acid (Bate et al 2004a). However, there are, to my knowledge, no studies investigating whether intracellular signaling pathways could affect the efficiency of the conversion of PrP^C to PrP^{Sc}.

Proteolytic processing of PrP^C and PrP^{Sc}

For many years prions were considered "non-degradable" and once formed almost impossible to break down completely. This notion was based on the resistance of prions to conventional procedures for sterilization, e.g. heat and formalin-treatment. There was so far no convincing evidence for a cellular turnover of PrP^{Sc} in neuronal cells. Degradation of prions *in vivo* by immune cells, i.e. macrophages (Beringue et al 2000, Carp & Callahan 1981) and migrating CD11c+ dendritic cells (Luhr et al 2002) has, however, been described.

The two PrP isoforms, PrP^C and PrP^{Sc}, undergo different patterns of proteolytic cleavage depending on their secondary and tertiary protein structure and, in addition occur in different glycosylation forms and therefore display different band patterns in Western blots (Lawson et al 2005). In uninfected cell or tissue samples, three PrP^C-specific bands corresponding to un- (~25 kDa), mono- (~30 kDa) and di- (~35 kDa) glycosylated forms of the protein are seen in Western blots. PrP^{Sc} is partially resistant to proteinase K (McKinley et al 1983), while PrP^C is readily digested (Oesch et al 1985, Rubenstein et al 1986). These distinct properties of the prion proteins are used not only in research, but also in diagnostic tests to distinguish between the two isoforms. In Western blots of infected cell or tissue samples, three PrP^{Sc}-specific bands can be seen after proteinase K digestion, corresponding to un- (~19-21 kDa), mono- (~24 kDa) and di- (~27-30 kDa) glycosylated forms. Proteolytic processing of PrP^C by ADAM/TACE matrix metalloproteases, which is called α-cleavage, occurs at amino acid 111 and yields a 17 kDa fragment denoted C1 (Vincent et al 2001). The N-terminal cleavage product from PrP^C is released extracellularly and the C1-fragment is further degraded in the cell. PrP^{Sc} cleavage is instead carried out by calpains at amino

acid 88 and is known as β-cleavage. This processing yields a 19-21 kDa fragment called C2, which corresponds to unglycosylated PrP27-30 (Chen et al 1995, Yadavalli et al 2004). The PrP^{Sc}-derived C2-fragment can accumulate both intracellularly and extracellularly (Borchelt et al 1992, Caughey et al 1989, Hope & Manson 1991), indicating that the rate of PrP^{Sc} formation is higher than its rate of degradation.

The possibility that degradation of PrP^{Sc} might also occur in neurons was suggested in studies where prion-infected neuroblastoma (N2a) cells were treated with antibodies against PrP^{C}, which led to decreased levels of PrP^{Sc} in the cells (Enari et al 2001, Peretz et al 2001). This effect was thought to reflect an inhibition of the conversion of PrP^{C} to PrP^{Sc} by the blocking antibody, paralleled by a cellular degradation of the preexisting PrP^{Sc}. The synthesis of PrP^{C} is rapid (minutes) and once in the plasma membrane this protein has a half-life of approximately 3-6 hours. In contrast, the formation of PrP^{Sc} has been estimated to take about 15 hours and its degradation has been predicted to exceed 24 hours (Borchelt et al 1990, Caughey et al 1989, Peretz et al 2001). Studies of branched polyamine-induced clearance of PrP^{Sc} (described above) indicate that lysosomal proteases might be involved in the slow degradation of prions in infected cells (Supattapone et al 2001), but the specific proteases responsible for this remain to be determined.

Stimulation of PrP^{Sc} degradation

A few anti-prion compounds that presumably act by stimulating endogenous degradation of PrP^{Sc} have been described. For instance, branched polyamines, such as polypropyleneimine, are lysosomotropic compounds that induce cellular clearance of prions by directly targeting PrP^{Sc} and increasing its sensitivity to proteolytic digestion, possibly by dissociating the PrP^{Sc} aggregates (Supattapone et al 1999, Supattapone et al 2001, Winklhofer & Tatzelt 2000). Moreover, there are substances such as imatinib mesylate, trehalose and lithium that act by increasing the efficiency of autophago-lysosomal breakdown of proteins and they have recently been described to increase the degradation of PrP^{Sc} in prion infected cell lines (Aguib et al 2009, Ertmer et al 2004, Heiseke et al 2009). However, neither imatinib mesylate nor trehalose was effective in curing scrapie infected mice from disease (Aguib et al 2009, Yun et al 2007).

Prion diseases and pathology

Prions cause invariably fatal neurodegenerative disorders that can be both infectious, genetic and sporadic, i.e. Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome, fatal familial insomnia and kuru in humans, bovine spongiform encephalopathy ("mad cow disease") in cattle, scrapie in sheep and goats, chronic wasting disease in deer and elk, as well as feline and mink spongiform encephalopathy in cats and minks (Prusiner 1998a). CJD occur in all three forms; the disease can be transmitted (variant CJD), inherited or sporadic.

Prion diseases are characterized by accumulation of PrP^{Sc} in the central nervous system (CNS), and to a minor extent also in peripheral organs or tissues, e.g. spleen, skeletal muscles and peripheral nerves (Glatzel et al 2003, Hainfellner & Budka 1999). The histopathological changes are, however, confined to the CNS and can include vacuolization of nerve cell processes resulting in the characteristic spongiform neurodegeneration, dendritic atrophy and synaptic loss, astrogliosis and activation of microglia cells. So far, the cellular signals that induce neurodegeneration in prion diseases are not known, but apoptotic mechanisms appear to be involved (Dorandeu et al 1998, Giese et al 1995, Gray et al 1999, Lucassen et al 1995, Williams et al 1997). Small non-fibrillar particles with masses equivalent to 14−28 PrP molecules, have been suggested to be more efficient initiators of prion disease than larger amyloid fibrils or plaques (Silveira et al 2005).

Neurotoxicity

Neuronal damage has been suggested to result from a loss of the normal function of PrP^{C} or a gain of toxic properties of PrP^{Sc} (reviewed in (Chiesa & Harris 2001).

The potential role of PrP^{C} loss in prion diseases has been studied in PrP^{C} knock-out mice. These mice are described as overtly normal in their development and behavior (Bueler et al 1992, Manson et al 1994). However, there are, as described above, several reports of subtle alterations in neuronal function, such as synaptic dysfunctions, in PrP^{C}-deficient mice (Carleton et al 2001, Colling et al 1996, Colling et al 1997, Collinge et al 1994, Fuhrmann et al 2006, Herms et al 2001, Manson et al 1995), but no synaptic loss, myoclonic seizures or neuronal degeneration are seen. To avoid compensatory mechanisms during development that may mask the effect of PrP^{C} loss, post-natal neuronal PrP^{C} knock-outs were generated by Mallucci and coworkers using the Cre-*lox*P system for conditional gene knock-outs (Mallucci et al 2002). These mice showed no signs of neurodegeneration and were not overtly different from wild-type mice. However, reduced slow afterhyperpolarization in hippocampal slices was observed (Mallucci et al 2002), in line with previous findings in hippocampal slices from regular PrP^{C} knock-out mice (Colling et al 1996, Fuhrmann et al 2006). Overall, these studies show that loss of PrP^{C} function does not contribute to any major extent to the pathological changes in prion diseases.

Neurotoxic properties of PrP^{Sc}, has been suggested based on studies showing that PrP^{Sc} colocalizes with the histopathological changes observed in prion diseases (DeArmond et al 1987, Jeffrey et al 1994, Jendroska et al 1991). Moreover, synthetic PrP fragments, corresponding to amino acids 106-126 in the human prion protein, show neurotoxic properties and a tendency to aggregate into fibrils *in vitro* (Forloni et al 1993). It is, however, not clear whether or not the pathogenicity of the aggregation of the PrP106-126 peptides in cultured cells mimics the properties of PrP^{Sc} accumulation in the CNS, and not all studies confirm the reported neurotoxicity of the peptides (Kunz et al 1999).

There are several inherited prion diseases in which only low levels of PrP^{Sc} are detected despite fatal clinical disease (Collinge et al 1995, Medori et al 1992, Tateishi et al 1992). Further arguments against neurotoxicity of extraneuronal PrP^{Sc} have been obtained in experiments by Brandner and colleagues, in which neuronal tissue from PrP^{C} overexpressing mice was transplanted to PrP^{C} knock-out mice that were later inoculated with scrapie prions. High levels of PrP^{Sc} accumulation and scrapie-characteristic histopathological changes were observed in the transplanted grafts, but were completely absent in PrP^{C}-deficient tissue even though graft-derived PrP^{Sc} was spread extracellularly to the host brain (Brandner et al 1996). In addition, extracellular accumulation of PrP^{Sc}, derived from glial cells, continued after conditional knock-out of PrP^{C} in neurons in the brains of scrapie-infected mice, but early spongiform changes were reverted, and neuronal loss and progression to clinical disease were prevented (Mallucci et al 2003). Moreover, scrapie-inoculated mice expressing mutant PrP^{C} lacking the GPI-anchor developed minimal brain pathology and neurological dysfunction despite the accumulation of numerous PrP^{Sc}-containing amyloid plaques (Chesebro et al 2005).

Early impairments associated with prion diseases are most likely linked to neuronal dysfunctions that probably precede neuronal loss. Damage to several neurotransmitter systems, have been described. Diez and coworkers showed scrapie-strain related alterations in neuropeptide expression, with increased neuropeptide Y, enkephalin and dynorphin-like immunoreactivity paralleled by decreased expression of cholecystokinin in infected mice (Diez et al 2007, Diez et al 1996, Diez et al 1997). Although loss of GABAergic neurons was one of the first detectable neuropathological changes in experimental models of scrapie (RML, 22L and Me7 strains) and CJD in mice (Guentchev et al 1998), no selective vulnerability of GABAergic neurons was observed in scrapie-infected hamsters (Bouzamondo-Bernstein et al 2004). However, loss of presynaptic boutons was associated with significantly reduced [KCl]-depolarization evoked [^{3}H]-GABA release from synaptosomes, as well as with PrP^{Sc} accumulation in the plasma membrane and synaptosomes (Bouzamondo-Bernstein et al 2004). The authors suggested that these changes might result in overactivity of neuronal networks by disrupting the balance between inhibitory and excitatory connections (Bouzamondo-Bernstein et al 2004).

Moreover, when scrapie-infected GT1-1 cells, which are GABA-ergic (Ahnert-Hilger et al 1998, Mellon et al 1990), are depolarized using high [KCl] they show reduced responses in the N-type, but not the L-type, voltage-gated Ca^{2+} channels (Sandberg et al 2004). Whether this change reflects a pathogenetic prion activity or a host cell response to the infection is not known. However, this observation indicates that the prion infection can cause disturbances in synaptic vesicle release in these cells, since the N-type voltage-gated Ca^{2+} channels are localized to synapses and are involved in regulation of neurotransmitter release (Evans & Zamponi 2006).

Increased levels of Notch-1 transcripts as well as elevated levels of the Notch-1 intracellular domain (NICD) in prion-infected mice have been observed in association with dendritic atrophy and PrP^{Sc} accumulation (Ishikura et al 2005). In addition, increased levels of NICD were seen in scrapie-infected N2a (ScN2a) cells, which also exhibited shorter neurites than uninfected cells. Activation of Notch-1 signaling, by treating ScN2a cells with Notch-1 specific short interfering RNA (siRNA), reinduced long neurites in the cells (Ishikura et al 2005).

The observations described above indicate that neither loss of PrP^{C} function nor gain of toxic PrP^{Sc} properties can fully explain prion-induced neurodegeneration. The possibility, therefore, arises that the $PrP^{C} \rightarrow PrP^{Sc}$ conversion process, which might include a toxic intermediate PrP-species (Collinge & Clarke 2007, Hill & Collinge 2003), may elicit signaling that could alter cell functions and/or induce toxic cascades contributing to neurodegeneration in prion diseases.

Intracellular signaling –mitogen-activated protein kinase pathways

Mitogen-activated protein (MAP) kinase cascades are important signaling pathways that convey extracellular cues to intracellular targets via sequential activation (i.e. phosphorylation) of kinases. There are four MAP kinase pathways: the MEK/ERK1/2, stress-activated protein kinase 2-4 (p38), stress-activated protein kinase 1 (c-Jun N-terminal kinase; JNK) (fig. 2) and the yet not well characterized Big MAP kinase ERK5 cascade (Rubinfeld & Seger 2005).

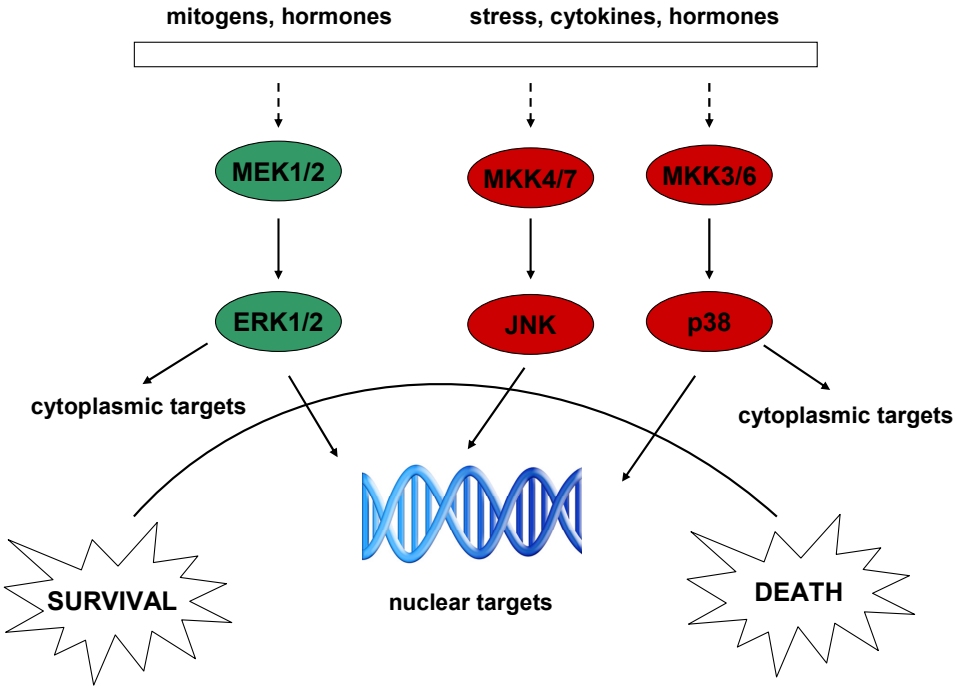

Figure 2. MAP kinase signaling

There are three major MAP kinase pathways: The protective MEK/ERK cascade involved in cell survival, and the stress-induced p38 and JNK pathways promoting apoptosis.

The MEK/ERK cascade

The MEK/ERK cascade is a central signaling pathway in neurons and other cell types. In neurons ERKs are localized primarily in neuronal cell bodies and dendrites, consistent with a postsynaptic function in neuronal signaling (Fiore et al 1993). This cascade regulates proliferation and differentiation and is typically activated by growth factors (e.g. neurotrophins), hormones and neurotransmitters (Kaplan & Miller 2000). In addition, depolarization of neuronal cells leads to the opening of L-type voltage-dependent Ca^{2+}-channels and the increase in intracellular [Ca^{2+}] causes phosphorylation of ERK1/2 (Rosen et al 1994). Stimulation of this MAP kinase cascade by growth factors first results in activation of small guanosine triphosphate (GTP)-binding proteins (e.g. Ras and Rap) that activate Raf kinases, which convey the signal via phosphorylation of MEK1/2 on two serine residues. Thereafter, the signal is transmitted down the cascade via MEK-dependent phosphorylation of threonine and tyrosine residues on ERK1/2, which then phosphorylate several downstream targets located in both the nucleus and the cytoplasm (Chuderland & Seger 2005, Kolch 2005). ERKs phosphorylate, among other proteins, the mitogen- and stress-activated kinase 1 that is responsible for phosphorylation of histone H3, a nuclear protein participating in chromatin remodeling and transcriptional regulation (Dunn et al 2005, Thomson et al 1999). The ribosomal S6 kinase 1 (RSK1) is a cytoplasmatic target of ERK that upon activation phosphorylates the S6 ribosomal protein (S6rp), which regulates protein translation (Roux et al 2007).

ERK activity is negatively regulated by phosphatases. All three groups of cellular protein phosphatases can participate in this important regulation and are crucial in determining the magnitude, duration and compartmentalization of the cellular responses controlled by this cascade (Torres et al 2004). ERKs also interact with various scaffolding proteins that are important in recruiting proteins belonging to the signaling cascade and bring them within close proximity. The scaffold proteins, thus, induce faster kinetics, higher specificity, compartmentalization and modified cross-talk with other signaling cascades (Kolch 2005, Levchenko et al 2000). Molecular cross-talk has been described between the MEK/ERK cascade and other intracellular signaling pathways, e.g. the cAMP-PKA pathway and the mammalian target of rapamycin (mTOR).

Malfunction of the MEK/ERK pathway may cause several diseases, including cancer (Dunn et al 2005, Wong 2009). Selective activation of MEK/ERK can promote neuronal survival, but MEK-inhibitors have no, or minimal, effects on cell survival (Klesse et al 1999, Mazzoni et al 1999). This indicates that the MEK/ERK cascade, although biologically capable of supporting neurotrophin mediated cell survival, is not biologically required for this process (Klesse et al 1999, Mazzoni et al 1999).

Neurotrophins – brain-derived neurotrophic factor

Neurotrophins, which consist of NGF, brain-derived neurotrophic factor (BDNF) as well as neurotrophin 3 and 4/5, are common activators of the MEK/ERK cascade. BDNF is abundantly expressed in the CNS, unlike NGF which is typically expressed in the peripheral nervous system. Synthesis and release of neurotrophins by neurons are dependent on neuronal activity, and they regulate neuronal development, differentiation, survival, repair and death, as well as synaptic morphogenesis and function (for review, see (Bibel & Barde 2000)). All neurotrophins can bind to the low-affinity p75 neurotrophin receptor ($p75^{NTR}$; "death receptor") and to another high-affinity tropomycin receptor kinase (Trk); the TrkB receptor in the case of BDNF (fig. 3). There is an important cross-talk between these receptors. The neurotrophin-mediated cellular response in terms of death or survival appears to be directed by the expression and signaling activities of the $p75^{NTR}$ and Trk tyrosine kinase receptors and their downstream effector molecules. Trk-mediated survival is most often conveyed by the small GTP-binding protein Ras, which transmits the neurotrophin signal primarily through the PI3K pathway, inducing suppression of apoptotic proteins. In addition, the MEK/ERK cascade can be partially involved in Trk-mediated survival by inducing activation of anti-apoptotic proteins. Moreover, BDNF can activate the phospholipase C-gamma (PLC-γ) pathway, involved in activity-dependent synaptic plasticity (Bibel & Barde 2000).

Reduced expression of BDNF in the brain is seen with aging and in association with neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. In other brain diseases, e.g. epilepsy, the expression of BDNF is instead increased (Murer et al 2001, Pezet & Malcangio 2004).

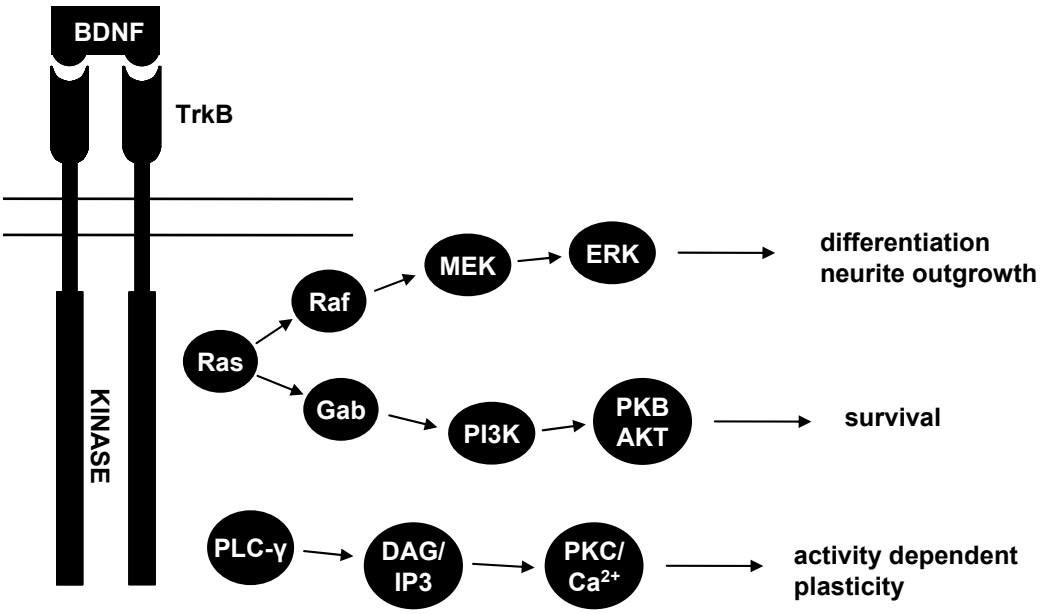

Figure 3. BDNF signaling

BDNF binds extracellularly to the TrkB receptor dimer, which have an intracellular kinase domain that can activate the MEK/ERK, the PI3K/Akt and the PLC-γ pathways.

The p38 and JNK cascades

The p38 and JNK MAP kinase pathways are predominantly activated by environmental stresses (e.g. radiation, oxidative stress, DNA damaging agents) as well as proinflammatory stimuli, and they regulate cellular stress responses and apoptosis (Kyriakis et al 2004). Both p38 and JNK require dual phosphorylation on threonine and tyrosine residues to become activated and may relocalize from the cytoplasm to the nucleus upon stimulation, similar to ERK activation (Rubinfeld & Seger 2005).

Opposing effects of MAP kinase pathways

Neurotrophic factors, such as NGF, have been shown not only to activate survival pathways like the MEK/ERK cascade, but also to inhibit signaling through the stress-related p38 and JNK pathways. Xia and coworkers showed that apoptosis in NGF-differentiated PC12 cells is regulated by the opposing actions of the ERK and p38/JNK cascades; activation of p38 or JNK is necessary, but not sufficient for apoptosis, which also requires inactivation of ERK. Hence, a stronger activity of ERK relative to that of p38/JNK may promote neuronal survival, whereas a more pronounced p38/JNK activity relative to that of ERK might instead trigger apoptosis (Xia et al 1995). Opposing actions of these MAP kinases pathways have also been implicated in regulation of other biological functions. Thus, the ERK and p38 cascades have been suggested to play opposing roles in the regulation of cardiac contractility (Szokodi et al 2008). In airway smooth muscle cells, LPS treatment leads to proasthmatic-like changes in the muscle function. This is accompanied by coactivation of MAP kinase pathways; activation of p38 serves to homeostaticly down-regulate the proasthmatic effects of ERK activation (Shan et al 2006). Several other studies demonstrate cross-talk between these pathways as a central event in MAP kinase signaling; p38 has been shown to regulate dephosphorylation of ERK and thereby down-regulation of this cascade (Grethe & Porn-Ares 2006, Lee et al 2002, Li et al 2003, Liu & Hofmann 2004). Accordingly, there is a dynamic balance between these MAP kinase pathways, which may be influenced by extracellular stimuli and intracellular signaling events, shifting the balance towards either ERK or p38, depending on the specific situation.

Pathogens and intracellular MAP kinase signaling

Microbial pathogens have developed a wide range of strategies for manipulation of host cell functions to the benefit of their own survival and spread. The MAP kinase pathways, the MEK/ERK cascade in particular, has proven to be suitable targets for a variety of pathogens.

Bacterial toxins, for instance, can activate MAP kinase signaling in order to deregulate the progression of the host cell cycle to promote bacterial replication. In that way, ERK-activation is induced by *Helicobacter pylori* (Higashi et al 2004) and ERK- and JNK-activation by *Pasteurella multocida* (Seo et al 2000). An even more sophisticated mechanism for bacterial control of the host cell is depicted

by Tapinos and Rambukkana in a detailed study of how *Mycobacterium leprae* "hijacks" the ERK signaling pathway in a MEK-independent way, to drive the proliferation of un-myelinated Schwann cells, thereby increasing the number of potential host cells to the advantage of further bacterial spread (Tapinos & Rambukkana 2005). They observed that *M. leprae* infections in Schwann cells lead to an increase in the phosphorylation of ERK1/2 in a PKC-dependent manner, followed by increased proliferation rate of the host cells.

Also protozoan parasites have recently been described to manipulate the host cell metabolic pathways and thereby interfere with host signaling cascades (for review see (Plattner & Soldati-Favre 2008)). As an example, *Toxoplasma gondii* has developed a variety of strategies to manipulate signal transduction pathways of the host in order to survive and this includes activation of the MEK/ERK pathway in an oscillatory manner to suit the parasite's needs (Molestina et al 2008).

Viruses have developed advanced techniques in order to manipulate the host metabolism, since they fully depend on the cellular molecular machinery for their replication. In that way, many DNA viruses use the host cell cycle machinery through activation of the MEK/ERK signaling pathway, in order to drive the host cell into a proliferative state, in which the virus can promote its own replication. Certain RNA viruses, e.g. some strains of influenza virus (Ludwig et al 2004, Pleschka et al 2001), hepatitis C virus (Erhardt et al 2002, Fukuda et al 2001, Hayashi et al 2000) and coxsackievirus B3 (Huber et al 1999a, Lim et al 2005, Luo et al 2002, Opavsky et al 2002) also depend on the cellular MEK/ERK pathway for their replication.

An interesting example is infection with the neurotropic Borna disease virus (BDV), during which the MEK/ERK pathway is activated. Inhibition of MEK using U0126 blocks spread, but not replication, of the virus in cultured cells (Planz et al 2001). Moreover, persistent infection of PC12 cells with BDV completely blocks NGF-induced neurite outgrowth, by down-regulating TrkA NGF receptors and impairing translocation of ERK1/2 to the nucleus (Hans et al 2001). In primary cultures of rat hippocampal neurons, infection with BDV blocks BDNF-mediated activation of ERK1/2, despite normal expression of TrkB BDNF receptors. Furthermore, BDNF-induced expression of synaptic proteins was impaired, leading to abrogated synaptogenesis. Interestingly, the observed alterations in BDV-infected cells in response to BDNF were accompanied by increased levels of viral proteins, indicating that the virus is capable of "hijacking" the neurotrophin signaling pathway in order to increase its own replication (Hans et al 2004). These studies inspired us to investigate whether an interaction between the pathogen and intracellular MAP kinase signaling pathways also occur during prion infections.

AIMS

The overall aim of this thesis was to investigate the potential occurrence of a molecular cross-talk between prions and key cellular signaling networks of the host. We were specifically interested in examining whether prions could take advantage of intracellular signaling pathways, such as the MAP kinase cascades, in order to support their own replication, as depicted for other pathogens. Moreover, we aimed at studying the balance between formation and degradation of prions in cultured cells, and to examine if this delicate balance can be modulated by intracellular signaling.

RESULTS AND DISCUSSION

Involvement of the MEK/ERK pathway in prion formation

As described in the Introduction, these studies were prompted by the observations that Borna disease virus impairs NGF- and BDNF-signaling by "hijacking" the intracellular MEK/ERK pathway in order to support its own replication (Hans et al 2004, Hans et al 2001).

NGF was shown to regulate the expression of PrP^C at the transcriptional level in developing hamster brains as well as in PC12 cells and neuronal mouse cells lines (Kuwahara et al 2000, Mobley et al 1988, Wion et al 1988), and at the translational level in glioma spheroids (Sauer et al 2003). NGF was also shown to be essential for scrapie replication in PC12 cells (Rubenstein et al 1990).

For our studies we choose the neurotrophin BDNF, based on its importance as a growth factor and signaling molecule in the adult brain, which is the primary target for prions. In **paper III**, we investigated whether accumulation of PrP^{Sc} in scrapie-infected cells, could be affected by BDNF-induced intracellular signaling. Hypothalamic GT1-1 cells, infected with the RML-strain of scrapie (ScGT1-1 cells), were treated with BDNF in medium containing low concentration of serum (1%) for 3-4 days. Western blot analysis and subsequent quantification, revealed a two-fold increase in PrP^{Sc} levels in BDNF-treated, compared to untreated, samples (**paper III**). The levels of PrP^C did not change after BDNF-treatment of uninfected GT1-1 cells, showing that BDNF affected the metabolism of PrP^{Sc}.

Similar to our results, Bate and coworkers reported in a recent publication that BDNF induces an increase in PrP^{Sc} levels in ScGT1-1 cells and that this is accompanied by an increase in free cholesterol, as well as phosphorylation of PLA_2 (Bate et al 2008b). PLA_2 inhibitors had previously been shown to decrease PrP^{Sc} formation by reducing the levels of PrP^C available for conversion (Bate et al 2004b). In our studies BDNF-induced PrP^{Sc} accumulation was not associated with elevated PrP^C levels. We, therefore, next investigated whether it was due to an increase in PrP^{Sc} formation or a decrease in PrP^{Sc} degradation.

Degradation of PrP^{Sc}

As mentioned in the Introduction, little was known about cellular degradation of PrP^{Sc}. In **paper I**, we used the, at that time novel, RNA interference (RNAi) technique to study whether GT1-1 cells possess an intrinsic capacity to degrade these misfolded proteins. We designed short interfering RNA (siRNA) directed to the mRNA for PrP^C and by using *in vitro* transcription we subsequently synthesized the siRNAs, which were then used for transfection of ScGT1-1 cells. A 50% reduction of PrP^C levels led to a clearance of PrP^{Sc}. This finding

indicates that GT1-1 cells are able to degrade the pre-existing PrP^{Sc}, if the efficiency of conversion of PrP^{C} to PrP^{Sc} is hampered by reducing, but not completely eliminating, the substrate for conversion, i.e. PrP^{C}. These results are in line with another study, in which chemically synthesized siRNA manufactured from a company were employed (Daude et al 2003).

To further analyze the mechanisms for the observed clearance of PrP^{Sc}, ScGT1-1 cells were treated with chemical inhibitors of cellular proteases. Inhibition of cysteine proteases, using E-64c, E-64d and leupeptin, led to increased accumulation of PrP^{Sc} in the cells, in contrast to inhibition of aspartic, serine or metalloproteases, which did not affect PrP^{Sc} levels. PrP^{Sc} has been shown to accumulate in lysosomes (Arnold et al 1995, McKinley et al 1991) and studies of branched polyamine-induced clearance of PrP^{Sc} indicate that lysosomal proteases might be involved in the slow degradation of prions in infected cells (Supattapone et al 2001). We, thus, focused on lysosomal proteases and, in **paper II,** we identified cathepsin B and L as two proteases involved in PrP^{Sc} degradation, by using specific inhibitors as well as RNAi directed to these cathepsins.

From these observations it was clear that PrP^{Sc} could be degraded by lysosomal cathepsins and that this degradation could be blocked by inhibitors of cysteine proteases. Thus, we were able to perform experiments aiming to determine if the increase in PrP^{Sc} accumulation following BDNF treatment was due to increased formation or decreased degradation of the protein.

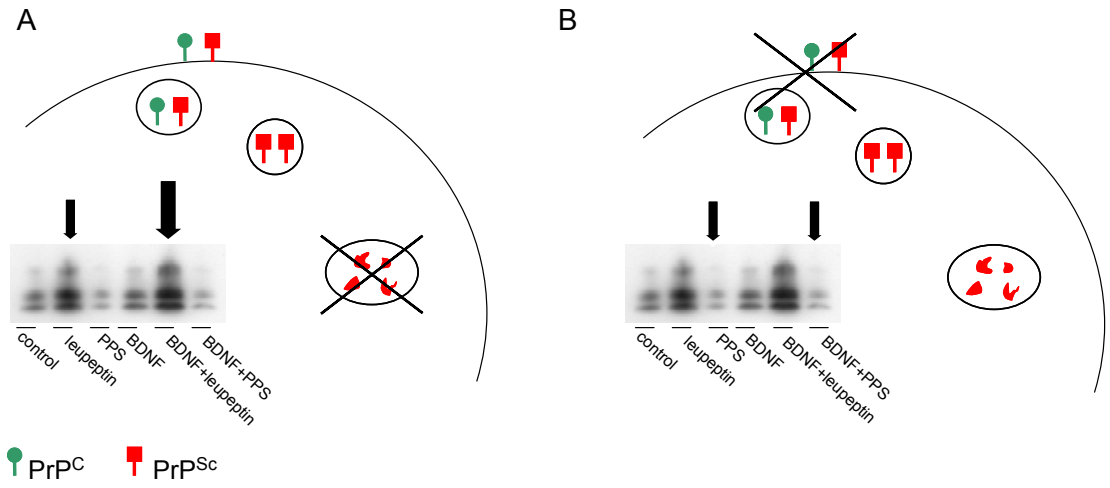

Figure 4. BDNF increases the formation of PrP^{Sc}.

A. When lysosomal *degradation* of PrP^{Sc} was blocked by leupeptin, BDNF still caused an increase in PrP^{Sc} levels. **B**. When the *formation* of PrP^{Sc} was blocked by PPS, BDNF-treatment did not cause an increase in PrP^{Sc} levels. Taken together, the experiments depicted in **A** and **B** demonstrate that BDNF acts by increasing the formation rather than impeding the degradation of PrP^{Sc}.

In order to study the effects of BDNF on PrP^{Sc} formation, we in **paper III** blocked its degradation using the cysteine protease inhibitor leupeptin and found that BDNF still caused a marked increase in PrP^{Sc} levels when degradation of PrP^{Sc} was blocked (fig. 4 A). Conversely, to study the effects of BDNF on PrP^{Sc} degradation, we blocked its formation using the polyanion PPS and then found no effect of BDNF treatment on PrP^{Sc} accumulation (fig. 4 B). Altogether, these results indicate that BDNF is involved in *formation* rather than degradation of prions.

Inhibition of the MEK/ERK cascade

As described in the Introduction, BDNF can activate the MEK/ERK cascade and therefore we next treated the scrapie-infected cells with specific inhibitors of this pathway (Shaul & Seger 2004). Inhibition of MEK1/2 using U0126 cleared the cells from PrP^{Sc}, but had no effect on the levels of PrP^{C}. In addition, the MEK-inhibitors PD098059 and SL327 also caused a marked decrease in PrP^{Sc} accumulation in the cells. The MEK inhibitor SL327 is of particular interest since the brain is the primary target for prions and this drug can pass the blood-brain barrier (BBB). New and less toxic MEK inhibitors have been developed and are now undergoing clinical trials for certain forms of tumors (Sebolt-Leopold 2008, Wong 2009). These inhibitors may be of interest for evaluation of their therapeutic value also in prion diseases.

To determine if the effect of U0126 was on formation or degradation of PrP^{Sc}, we performed similar experiments as described above. The MEK-inhibitor U0126 was still effective in reducing the levels of PrP^{Sc} when degradation of PrP^{Sc} was blocked with the cysteine protease inhibitor leupeptin. On the other hand, when PPS was used in combination with U0126, the level of PrP^{Sc} was reduced as compared to treatment with PPS alone. Thus, inhibition of the MEK/ERK pathway seems to hinder the formation, but not accelerate the degradation of prions, which is in line with our previous findings on the effects of BDNF.

In addition to the MEK/ERK cascade, BDNF can activate the PI3K pathway. In our next set of experiments we therefore treated ScGT1-1 cells with an inhibitor of PI3K. Inhibition of PI3K, using Ly294002, did not affect cellular levels of PrP^{C} or PrP^{Sc}, suggesting that this pathway is not involved in prion formation.

As an approach to inhibit ERK activation by other means than chemical inhibitors, we used RNAi directed to ERK1 and 2 and achieved an approximately 50% reduction of total ERK1/2 levels in Western blots. The levels of phosphorylated ERK1/2 were concomitantly reduced. No significant reduction in cellular PrP^{Sc} levels could be seen (unpublished results, data not shown), indicating that the levels of activated ERK1/2, although somewhat reduced, was still sufficient to maintain prion replication. These observations show that the modest RNAi-mediated reduction in ERK activity was not enough

to affect the formation of PrP^{Sc}, in contrast to the more efficient PrP^{Sc} clearance seen when applying chemical MEK-inhibitors. In these experiments I have not yet examined transfection efficiency and I have not looked at ERK or PrP^{Sc} using immunofluorescence. Using immunofluorescence, I might be able to see differences at the level of individual cells, and identify cells that have been efficiently transfected with siRNA.

Activation of ERK via depolarization

In **paper IV**, we examined whether activation of the MEK/ERK signaling cascade by other stimuli than BDNF could also accelerate prion formation. We, therefore, cultured GT1-1 infected with the RML-strain of scrapie in cell culture medium containing high [KCl] that is known to depolarize cells (Rosen et al 1994). We observed an increase in the cellular accumulation of PrP^{Sc} after high [KCl] treatment. This accumulation was accompanied by ERK1/2 activation, as seen by increased phosphorylation, and could be blocked by MEK inhibitors. This again indicates a crucial role for the MEK/ERK cascade in PrP^{Sc} formation. To further study whether activation of the MEK/ERK signaling pathway is a more general event in the formation of prions, we examined the effects of MAP kinase signaling on another prion strain, the 22L strain of scrapie, described in Methodological considerations. In line with our observations using the RML-strain of scrapie, we in **paper V** show that also the formation of 22L-strain prions is under the influence of MEK/ERK signaling. Treatment of 22L strain-infected ScGT1-1 cells with high [KCl] led to increased accumulation of PrP^{Sc}, while inhibition of MEK using U0126 had the opposite effect. In contrast to cells infected with the RML strain, the 22L strain-infected cells could not be completely cleared from PrP^{Sc}, not even when higher concentrations of the MEK-inhibitor were applied; only a 40% decrease in PrP^{Sc} was seen. This indicates that, although MEK/ERK signaling affects formation of prion of different strains, the relative influence of this signaling pathway may vary between strains.

GT1-1 cells have membrane characteristics of neurons and release GABA and gonadotropin releasing hormone in response to depolarization with high [KCl] (Ahnert-Hilger et al 1998, Mellon et al 1990). Scrapie-infected GT1-1 cells that are depolarized using high [KCl] show reduced responses of the N-type, but not the L-type, voltage-gated Ca^{2+} channels, indicating that the infection can cause disturbances in synaptic vesicle release in these cells (Sandberg et al 2004). As described in the Introduction, prion infections in hamsters are associated with decreased [KCl]-depolarization evoked [^{3}H]-GABA release from synaptosomes (Bouzamondo-Bernstein et al 2004). These findings suggest that a reduced release of inhibitory GABA transmitters may contribute to the overactivity of neuronal networks and seizure-like activities observed in both humans suffering from prion diseases (Brown et al 1986) and in animal models of scrapie infections (Bassant et al 1984, Bassant et al 1987, Collinge et al 1994, Jefferys et al 1994, Strain et al 1986). The observed increase in PrP^{Sc} formation following [KCl]-depolarization of scrapie-

infected GT1-1 cells in my studies might therefore be of pathobiological relevance, suggesting that vicious circles may operate in the brains of infected individuals. Thereby, the prion infection causes an increased excitability in nervous tissues that in turn may promote the formation of more prions, via MEK/ERK activation (fig. 5).

Prions influence intracellular signaling

The above described studies show that the MEK/ERK cascade can significantly influence prion formation. We next examined if this relationship was bidirectional, i.e. if a prion infection could influence MAP kinase signaling. In **paper V**, the levels of activated (i.e. phosphorylated) ERK1/2 were analyzed at three different passages post exposure of GT1-1 cells to three different mouse-adapted scrapie strains, namely the RML, 22L and Me7 strains (described in Methodological considerations). The levels of phosphorylated ERK1/2 were significantly elevated in RML- and 22L strain-infected cells at 2 passages post exposure. At 6 passages post exposure only 22L strain-infected cells showed increased activation of ERK1/2, and after 10 passages the levels of phosphorylated ERK1/2 returned to baseline levels in all groups. In contrast to the infections with the RML and 22L strains of scrapie, the levels of phosphorylated ERK1/2 remained unchanged at all passages analyzed in cells exposed to the Me7 strain, and no accumulation of PrP^{Sc} was seen by Western blotting. The activation of ERK was more pronounced and more prolonged in 22L- than RML strain-infected cells. This correlates well with the higher levels of PrP^{Sc} observed in cells infected with the 22L prion strain. In addition to the RML, 22L and Me7 scrapie strains we also exposed GT1-1 cells to brain homogenates from mice infected with the Nor98 atypical scrapie strain, but these cells did not accumulate PrP^{Sc} and did not show any increase in ERK-phosphorylation (unpublished results, data not shown).

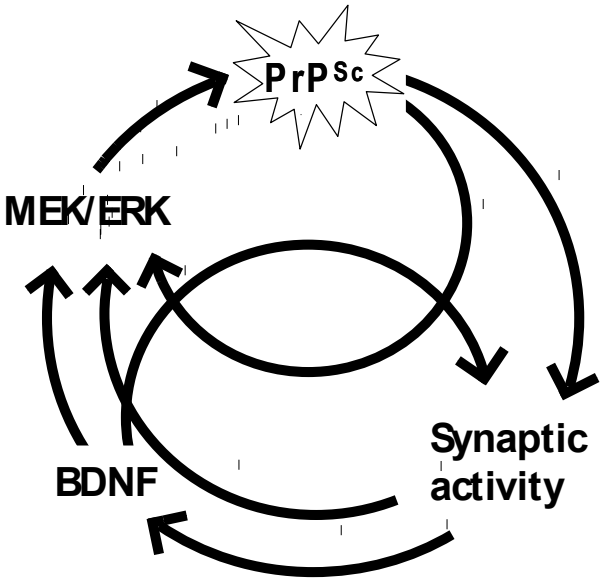

Figure 5. Vicious circles in the prion-infected brain?

Prion infections are associated with increased synaptic activity, i.e. myoclonic seizures (Wadsworth et al 2003), and activation of ERK (paper V). Synaptic activity, here modeled by depolarization of GT1-1 cells, can induce increased PrP^{Sc} levels via activation of ERK (paper IV and V), as well as increased BDNF expression and secretion, which can cause a further increase in synaptic activity (Murer et al 2001) and an increase in PrP^{Sc} levels via ERK (paper III, IV, V). Taken together, these observations suggest that vicious circles may operate in the brains of prion-infected individuals.

A basal level of ERK activation was seen in both uninfected and scrapie-infected GT1-1 cells, but no sustained increase in ERK activation was observed in persistently scrapie-infected GT1-1 cells compared to uninfected controls. In hippocampal neurons and astrocytes in hamsters infected with the 263K strain of scrapie (Lee et al 2005) increased ERK activation was seen. LaCasse and coworkers also describe ERK activation in astrocytes, as well as a transient ERK activation in cortical large neuron-like cells in mice infected with the RML strain of scrapie (Lacasse et al 2008). The latter observations on neurons are in line with our data on ScGT1-1 cells.

In summary, by using PrP^{C}-specific siRNA, evidence of cellular degradation of PrP^{Sc} was found and by using specific inhibitors as well as RNAi directed to cathepsin B and L, we identified these lysosomal proteases as important mediators of PrP^{Sc} degradation. Furthermore, I showed that BDNF and high [KCl] treatments led to activation of the MEK/ERK signaling cascade and stimulated prion formation in scrapie-infected GT1-1 cells. Inhibition of MEK/ERK using specific inhibitors cleared the cells from PrP^{Sc}, seemingly by blocking its formation. Moreover, a transient activation of ERK was observed upon exposure of GT1-1 cells to prions. In my studies, I have, thus, identified the MEK/ERK cascade as a pathway involved in the formation of PrP^{Sc} of different prion strains and a potential therapeutic target for antiprion drug design.

Involvement of the p38 and JNK pathways in prion formation

Inhibition of the p38 and JNK cascades

In our previous experiments, we characterized the involvement of the MEK/ERK MAP kinase pathway in prion formation. To further study the influence of intracellular signaling on the formation of PrP^{Sc}, we examined whether other MAP kinase pathways, i.e. p38 and JNK, could also be involved. In **paper IV**, ScGT1-1 cells infected with the RML strain of scrapie were treated with specific inhibitors of the stress-induced MAP kinases p38 (SB202190) and JNK (SP600125). Increased levels of PrP^{Sc} were observed in cells treated with either of the inhibitors. We then combined the p38 inhibitor SB202190 with PPS and leupeptin, to study if the effects on PrP^{Sc} were due to increased formation or decreased degradation of the protein. When formation of PrP^{Sc} was blocked with PPS, SB202190 did not cause increased levels of PrP^{Sc}, but when degradation of PrP^{Sc} was impeded by leupeptin the p38 inhibitor still caused an increase in PrP^{Sc} levels in the cells. This indicates that the observed increase in PrP^{Sc} reflects an enhanced formation. In **paper V**, we obtained analogous results in cells infected with the 22L strain of scrapie, pointing to a more general role for the p38/JNK MAP kinase pathways in formation of PrP^{Sc}.

Stimulation of p38 and JNK

Knowing that inhibition of the p38/JNK pathways leads to increased accumulation of PrP^{Sc}, we wanted to examine if stimulation of the same pathways would instead impede prion formation. Stimulation of these molecules promotes cell death and most studies describing activation of p38/JNK are short-term studies, in which the cells are harvested after 30 seconds to 90 minutes of stimulation (for review and protocols, see (Kyriakis et al 2004)). Such short-term treatments do not enable detection of any changes in PrP^{Sc} levels since, as described in the Introduction, both formation and degradation of prions are slow processes (Borchelt et al 1990). Attempts were made to activate p38 and JNK by H_2O_2-treatment and short pulses of UV-C radiation (Kyriakis et al 2004). However, no concentration or radiation that was low enough to prevent cell death, but high enough to activate p38/JNK (without activation of ERK) and affect PrP^{Sc} was found (unpublished results, data not shown).

Opposing effects of MAP kinase pathways on prion formation

Collectively, our data regarding the MEK/ERK and the p38 pathways demonstrate that these MAP kinase cascades exert opposing effects on prion formation in GT1-1 cells, i.e. ERK stimulates whereas p38 inhibits the conversion of PrP^{C} to PrP^{Sc} (fig. 6). Thus, in ScGT1-1 cells the ERK-mediated prion formation might be counterbalanced by p38-mediated inhibition. Opposing actions of the MEK/ERK and the p38/JNK signaling pathways was first described by Xia and coworkers regarding cellular control of apoptosis (Xia et al 1995) and has, as described in the Introduction, also been implicated in the regulation of other biological machineries. We now found that the dynamic balance between these pathways is also relevant for the formation of PrP^{Sc} in GT1-1 cells.

In **paper V**, we examined the activity of p38 in GT1-1 cells exposed to prions of various strains. The levels of phosphorylated p38 were analyzed at different passages after prion infection, as described for phosphorylated ERK in the previous section. No induction of the p38 pathway was observed after exposure of GT1-1 cells to RML, 22L or Me7 prions, in line with previous findings by Uppington and Brown comparing SMB cells (a cell line derived from the brain of a mouse infected with the Chandler strain of scrapie (Clarke & Haig 1970)) with PPS-cured SMB cells (Uppington & Brown 2008).

In certain cell types, the balance between the MAP kinase pathways may be shifted towards p38 activation, which leads to cell death. This is described for human SH-SY5Y neuroblastoma cells which undergo apoptosis via activation of p38 upon exposure to the prion protein peptides PrP106-126 or hPrP90-231 (Corsaro et al 2009, Thellung et al 2002). Corsaro and coworkers demonstrated that the pro-apoptotic activity of hPrP90-231 is determined by alterations in the homeostatic balance between the ERK and p38 MAP kinase pathways, favoring the latter (Corsaro et al 2009). In line with this, our observations indicate that during

establishment of a prion-infection in GT1-1 cells, the dynamic balance between the MEK/ERK pathway and the p38 pathway is shifted in favor of the former, thereby stimulating cell survival and prion formation, enabling persistence of the infection in the cells. Later, when the cells are stably infected, ERK-activation returns to base-line and it could be speculated that a new equilibrium between these opposing signaling pathways is formed in order to maintain the infection.

Taken together, I have shown that inhibition of the p38 and JNK MAP kinase pathways lead to increased accumulation of PrP^{Sc} in GT1-1 cells infected with the RML and 22L strains of scrapie. Our data demonstrate that the MEK/ERK and the p38 pathways exert opposing effects on prion formation, whereby MEK/ERK stimulates and p38 inhibits the conversion of PrP^{C} to PrP^{Sc} (fig. 6). The balance between these MAP kinase pathways may therefore influence the susceptibility of cells to prion infections.

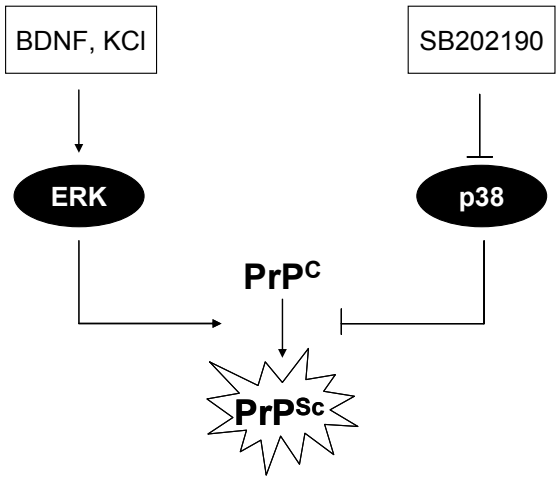

Fig 6. Opposing effects of MAP kinase pathways on conversion of PrP^{C} to PrP^{Sc}
The MEK/ERK pathway stimulates, while the p38 pathway suppresses the conversion process. Thereby, conversion of PrP^{C} to PrP^{Sc} can be increased by *stimulation* of the former pathway with high [KCl] or BDNF, or by *inhibition* of the latter pathway by the specific inhibitor SB202190.

Translational or transcriptional regulation of prions?

Prion infections and expression of MAP kinase related transcripts
In **paper V**, we examined whether a prion infection would alter the expression of MAP kinase related transcripts in GT1-1 cells, by using a PCR array designed for this purpose. Alterations in gene expression in mice infected with different strains of scrapie have been demonstrated by others; Skinner and coworkers found alterations in the expression of more than 400 genes in RML, 22L and Me7 strain-infected mouse brains, using a cDNA microarray (Skinner et al 2006). Differential expression in that study included transcripts encoding proteins that function in protein folding or degradation, localize to endosomal/lysosomal systems or involved in cell signaling. I compared expression profiles of uninfected GT1-1 cells and cells exposed to the RML, 22L and Me7 strains of prions. Nine deviant transcripts were found and further analyzed. However, no differential expression of any of these transcripts could be confirmed by quantitative real-time RT-PCR. Thus, we found no evidence of alterations in transcripts coupled to MAP kinase signaling during prion infections.

Translation or transcription?

In **paper IV**, I observed that treatment of ScGT1-1 cells with the diterpene forskolin caused increased levels of PrP^{Sc}. This increase was not mediated by ERK-activation and could not be impeded by MEK inhibition. Instead the effects of forskolin were blocked by inhibition of PKA, using the inhibitor H89. In contrast, high [KCl]-induced PrP^{Sc} accumulation was PKA-independent. Cross-talk between the MEK-ERK and the cAMP-PKA pathways both upstream and downstream ERK1/2 has been described, but these pathways can also operate separately (for review, see (Gerits et al 2008)). We next aimed at finding a common down-stream target for high [KCl]-mediated ERK activation and forskolin-induced PKA activation, since both pathways could stimulate PrP^{Sc} accumulation. The MEK/ERK, as well as the PKA pathway, is known to activate both nuclear and cytoplasmic targets, and in this study we chose to study activation of histone H3, which regulates transcriptional activity of the cell and of S6 ribosomal protein (S6rp), which is involved in translational regulation.

We found that high [KCl]-mediated prion formation is accompanied by increased phosphorylation of S6rp at Ser-235/236. In contrast, we did not observe any change in the phosphorylation of the acetylated form of histone H3. On the other hand, forskolin-induced PrP^{Sc} accumulation was paralleled by phosphorylation of both S6rp and histone H3. Treating the cells with high [KCl] led to a larger increase in phosphorylated S6rp accompanied by higher levels of PrP^{Sc} as compared to forskolin treatment. We, thus, identify the cytoplasmatic S6rp as a common target for MEK/ERK and PKA signaling in GT1-1 cells and speculate that this protein might be linked to the conversion of PrP^{C} to PrP^{Sc}. These findings indicate that the accumulation of prions requires translational rather than transcriptional activity, and are in line with the above data describing a lack of evidence for alterations in MAP kinase related transcripts during the establishment of prion infections in cell culture.

The mTOR pathway and prion accumulation

In **paper V**, we found that the high [KCl]-induced phosphorylation of S6rp at Ser-235/236 could be partially blocked by inhibition of MEK1/2 using U0126. This is in line with recent observations that serum-induced S6rp phosphorylation at Ser-235/236 is partly blocked by MEK-inhibition (Roux et al 2007). The authors demonstrate that phosphorylation of S6rp is modulated by both ERK- and mTOR-dependent mechanisms (Roux et al 2007). In our next set of experiments, we therefore further characterized the involvement of mTOR signaling on the accumulation of prions of the RML and 22L strains. In **paper V**, we treated high [KCl]-stimulated ScGT1-1 cells infected with these scrapie strains with rapamycin, a specific inhibitor of mTOR. Western blot analysis showed increased accumulation of PrP^{Sc} in the rapamycin treated 22L and RML strain infected cells and subsequent quantification revealed a more pronounced effect of rapamycin in cells infected with the 22L strain. No change in PrP^{C} levels was detected in uninfected GT1-1 cells.

In contrast to our findings, a decrease in PrP^{Sc} levels was observed when ScN2a cells infected with the RML strain of scrapie were treated with rapamycin (Aguib et al 2009, Heiseke et al 2009). N2a cells maintain a higher rate of cell division than GT1-1 cells and this might increase the kinetics of PrP^{Sc} clearance in these cells (Ghaemmaghami et al 2007). Furthermore, the different outcomes between the studies, might be explained by the opposing effects of acute and chronic rapamycin treatment (Choo et al 2008) (fig. 7). In our studies, we treated the GT1-1 cells with rapamycin for 4 days compared to 2 days in the case of the ScN2a cells (Aguib et al 2009, Heiseke et al 2009). Long-term treatment may reverse the effects of rapamycin in certain cell lines (Choo et al 2008). In that way, protein translation is decreased upon acute rapamycin treatment, which inhibits the function of ribosomal S6 kinases (S6Ks) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1). However, in cell lines, like HeLa and HEK 293, prolonged rapamycin treatments can cause hyperphosphorylation of 4E-BP1, which instead leads to increased translation (Choo et al 2008). In these cells, rapamycin inhibits S6Ks activity through the whole duration of the treatment, but 4E-BP-1 is only inhibited initially and recovers in phosphorylation after a few hours and can even overcompensate for the loss of S6K activity and increase translation.

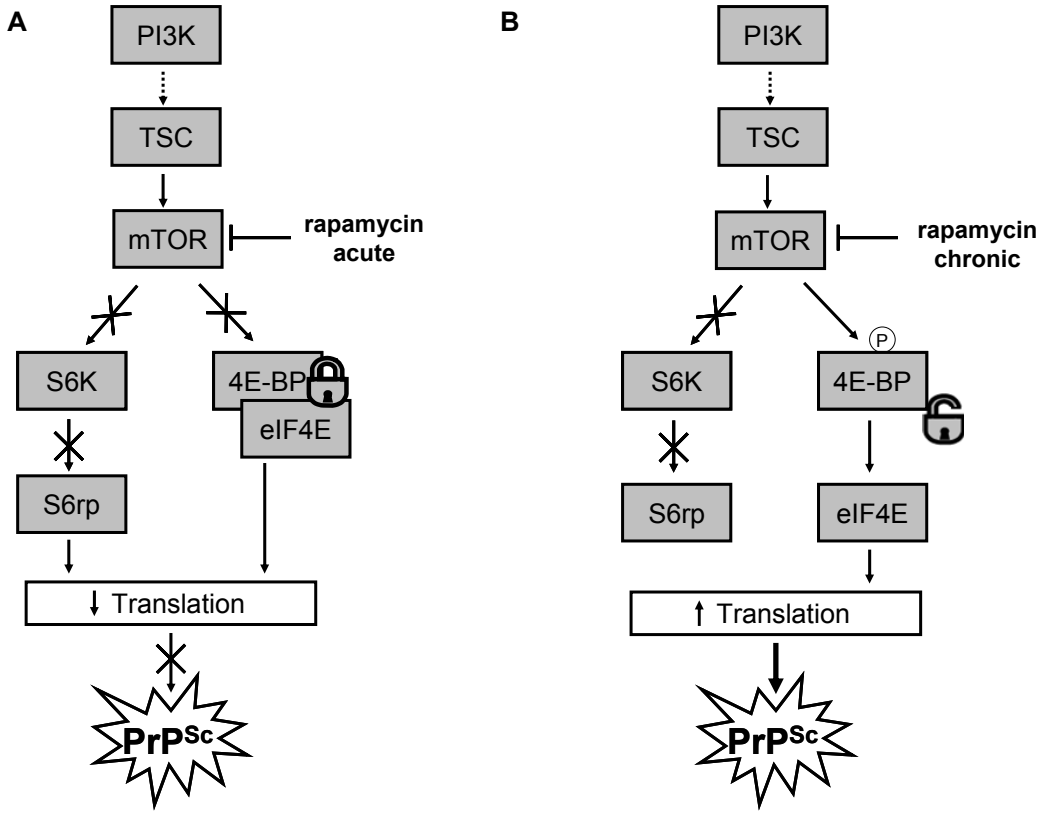

Figure 7. Acute (short) versus chronic (long) treatment of ScGT1-1 cells with rapamycin

A. Short-term treatment with rapamycin commonly leads to inhibition of both S6K and 4E-BP, preventing activation of S6rp and keeping eIF4E in a locked position, causing a decrease in protein translation. B. Long-term treatment with rapamycin can in certain cell lines leads to increased translation, caused by hyperphosporylation of 4E-BP and freed eIF4E. This was accompanied by increased levels of PrP^{Sc} in ScGT1-1 cells, as seen in our studies in paper V.

4E-BP1 binds to the eukaryotic translation initiation factor (eIF) 4E and upon phosphorylation at multiple sites it dissociates from eIF4E, which then can interact with eIF4G and other molecules to start cap-dependent mRNA translation (reviewed in (Klann & Dever 2004)). Therefore, in **paper V**, we also treated high [KCl]-stimulated ScGT1-1 cells with 4EGI-1, a specific inhibitor of the eIF4E/eIF4G interaction (Moerke et al 2007). Treatment with 4EGI-1 had no effect on the levels of PrP^{C} in uninfected GT1-1 cells, in line with our observations on the effects of rapamycin. In contrast, 4EGI-1 treatment caused a reduction in the levels of PrP^{Sc} in ScGT1-1 cells infected with the 22L strain and a not statistically significant reduction in the RML strain-infected cells. Our observations indicate that the mTOR downstream targets eIF4E and eIF4G can play a role in regulation of PrP^{Sc} accumulation in prion-infected cells (fig. 8).

In contrast to our previous studies showing that the MAP-kinase pathways can affect prion formation, we have not yet determined whether the mTOR pathway affects formation or degradation of PrP^{Sc}. However, reduced and not increased levels of PrP^{Sc} would have been anticipated in our study if the observed effects would relate to degradation of PrP^{Sc}, since rapamycin can stimulate autophagy (Sarkar & Rubinsztein 2008) and concomitant PrP degradation (Aguib et al 2009, Heiseke et al 2009).

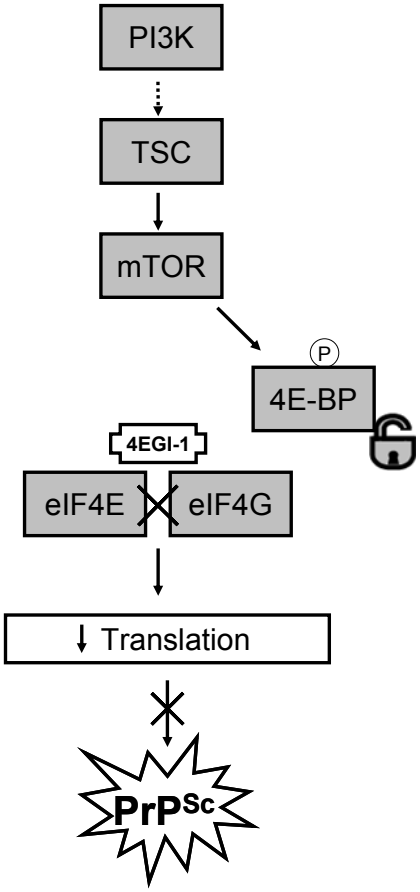

Figure 8. eIF4E-eIF4G inhibition
Inhibition of the eIF4E-eIF4G interaction cause reduced protein translation and decreased levels of PrP^{Sc}.

In summary, in my studies the cytoplasmatic S6 ribosomal protein was identified as a common down-stream target for the MEK/ERK and the PKA pathways and this protein may play a role in regulation of the $PrP^{C} \rightarrow PrP^{Sc}$ conversion process. The S6 ribosomal protein is involved in protein translation and can also be activated by mTOR-dependent mechanisms. Accumulation of PrP^{Sc} in prion infected cells was, in addition to the MAP kinase and cAMP-PKA cascades, found to be under the influence of the mTOR signaling pathway. Our findings suggest that the MEK/ERK and the mTOR signaling pathways might converge to exert a translational regulation of prions (fig. 9). However, the relative influence of these signaling pathways on PrP^{Sc} accumulation seem to vary between prion strains.

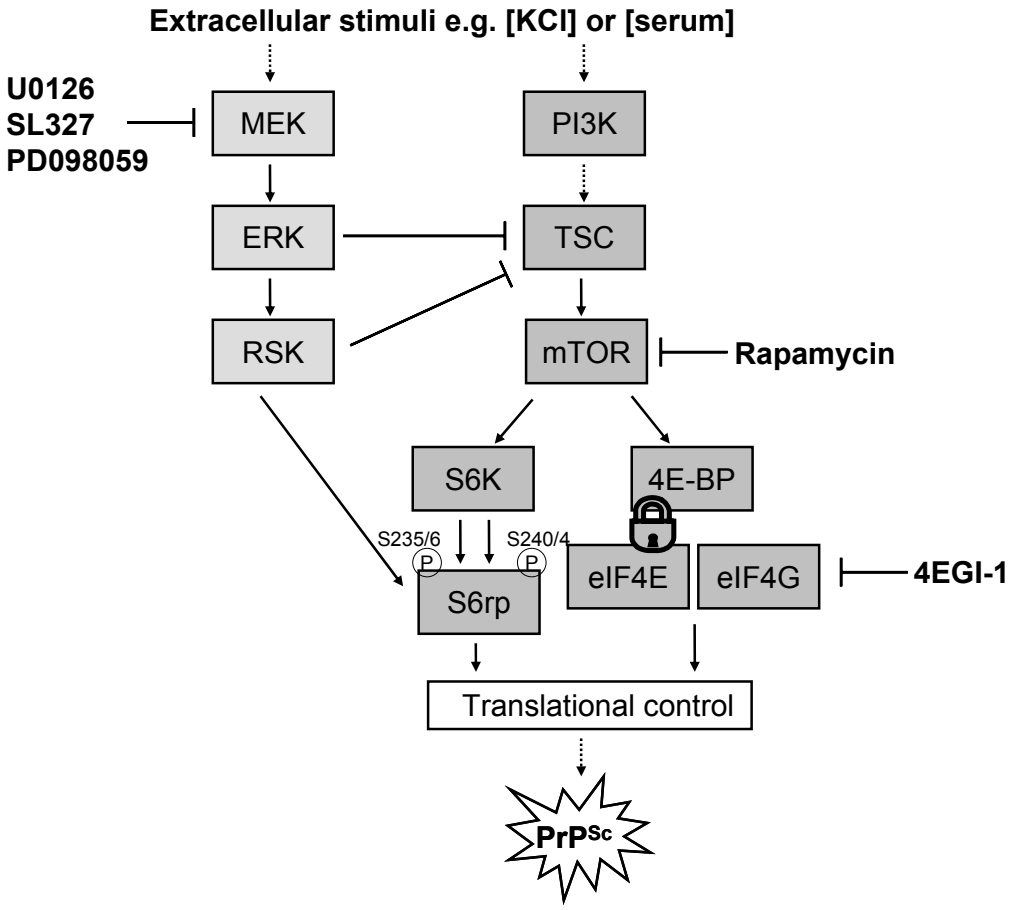

Figure 9. Convergence of the ERK and mTOR pathways might exert translational control of PrP^{Sc}
The MEK/ERK and the mTOR signaling pathways are both involved in the phosphorylation of S6rp. S6rp is an important regulator of the cellular translational machinery. We show that inhibition of MEK/ERK blocks the formation of PrP^{Sc}. Inhibition of mTOR, using rapamycin, leads to increased accumulation of PrP^{Sc}, while inhibition of the eIF4E-eIF4G interaction instead causes a decrease in PrP^{Sc} levels. Taken together, these results indicate that both the MEK/ERK and the mTOR signaling pathways are involved in accumulation of PrP^{Sc} in ScGT1-1 cells, and that these pathways may converge to exert a translational control of PrP^{Sc}.

METHODOLOGICAL CONSIDERATIONS

The model system – GT1 cells

GT1 is a hypothalamic cell line, generated by targeted tumorigenesis in transgenic mice, using immortalization with SV40Tag under the control of the gonadotropin-releasing hormone (GnRH) promoter (Mellon et al 1990). These GABA-ergic cells extend multiple lengthy neurites and express several neuronal characteristics, such as voltage-gated ion channels and proteins involved in synaptic function (Ahnert-Hilger et al 1998, Costantin & Charles 1999, Favit et al 1993, Hales et al 1994, Hiruma et al 1997, Mellon et al 1990, Watanabe et al 2004). The cells exhibit many of the known characteristics of hypothalamic GnRH neurons *in situ* (Wetsel 1995) and can be persistently infected with some prions strains (Schätzl et al 1997). We have, by using immunofluorescence, shown that cultures of RML strain-infected GT1-1 cells, which is the GT1 cell clone we have used in our studies, show a high proportion of infected cells, with more than 80% of the cells immunopositive for PrP^{Sc}. Based on their neuron-like properties and their susceptibility to scrapie prions, the GT1-1 cells comprise a suitable model system for *in vitro* studies of prion infections.

Prion strains

Distinct prion strains are described, and they constitute prion populations that differ in physiochemical characteristics, e.g. cleavage patterns and relative proportions of the three PrP glycoforms, and also in the induced pathological changes and the clinical course of disease (for review, see (Collinge & Clarke 2007)).

For our studies, we have chosen three experimental mouse adapted scrapie strains of various origins, namely the RML, 22L and Me7 strains of scrapie. The RML strain is derived from brain homogenates of scrapie-infected goats with the clinical manifestation described as drowsy (Chandler 1961). The 22L strain is derived from brain homogenates of a scrapie infected sheep (Dickinson 1976), while the Me7 strain is derived from the spleen of a scrapie-infected sheep (Dickinson & Meikle 1969, Zlotnik & Rennie 1963). *In vivo,* brains from 22L strain-infected mice show the highest levels of PrP^{Sc} compared to brains from mice infected with the Me7 and 139A strain, another strain derived from the same Chandler isolate as RML (Pan et al 2005). Cultured cells have been shown to be susceptible to infections with both the RML and the 22L strain (Nishida et al 2000, Schätzl et al 1997), while the Me7 strain is less infectious (Bosque & Prusiner 2000, Rubenstein et al 1992).

Media composition

GT1-1 cells are normally cultivated in Dulbecco's modified Eagle's medium 4.5 g/l glucose with GLUTAMAX I (DMEM) supplemented with 5% fetal bovine serum (FBS), 5% horse serum (HS) and 50 U/ml penicillin-streptomycin. In **paper III**, we observed that the level of PrP^{Sc} in the cells is dependent on the media composition. GT1-1 cells grown in DMEM supplemented with 1% serum displayed significantly lower levels of PrP^{Sc} than cells grown in medium containing 10% serum.

For our studies on intracellular MAP kinase signaling, we wanted for several reasons to keep the concentration of serum in the medium low. First, serum is a well known activator of ERK (Ferrell & Martin 1990) and we wanted to keep the levels of activated ERK as low as possible in cells that were not treated with BDNF or other factors/compounds. Second, the content of serum is not characterized or controlled, and it might contain growth factors, antibodies or other molecules that could affect cellular signaling. Third, in CNS tissue, which is the microenvironment we wanted to model, there is no serum present.

A controlled synthetic medium would be ideal and we therefore tested the serum-free neuron-specific Neurobasal medium (supplemented with B27 and L-glutamine). We observed that even higher levels of PrP^{Sc} were obtained in culture of cells grown in the Neurobasal medium compared to regular cell culture medium with 10% serum. We have up till now not analyzed the mechanism by which the Neurobasal medium affect PrP^{Sc} levels. However, our studies using MEK-inhibitors in this medium indicate that the observed increase in PrP^{Sc} accumulation is MEK/ERK-dependent, since longer incubation times and higher doses of the MEK-inhibitors were needed in order to clear the cells from PrP^{Sc} as compared to DMEM supplemented with 1% serum. In addition, the cells could only be kept in this synthetic medium for 5 days; longer incubations led to extensive cell death. This medium was, therefore, not suitable for our purposes. For our studies on intracellular MAP kinase signaling, we instead used DMEM supplemented with 1% serum (HS:FBS, ratio 1:1).

However, in our studies on the mTOR signaling pathway, DMEM with low concentrations of serum could not be used, since mTOR-inhibitors in DMEM with only 1% serum caused extensive cell death. Inhibitors of mTOR, e.g. rapamycin, induce cellular autophagocytosis (Sarkar & Rubinsztein 2008), and very low serum concentrations can also lead to increased autophagocytosis in the cells, partly mediated by the mTOR pathway. Combining medium containing 1% serum with mTOR inhibitors might, thus, be toxic to the cells due to an enhanced degree of cellular autophagocytosis. In the experiments employing mTOR-inhibitors we therefore supplemented the medium with 10% serum.

Cell counts and cell death markers

Cell division modulates prion accumulation in cells in culture, i.e. a higher rate of cell division might benefit prion clearance since it acts as a constant diluting factor (Ghaemmaghami et al 2007). The MAP kinase cascades are involved in proliferation and regulation of cell survival and death and we, therefore, carefully examined the effects of the applied treatments on cell death and total cell number.

First, concentrations of signaling pathway inhibitors/stimulators that caused no overt cell death were chosen for the experiments. To further control for this, the number of cells both floating in the medium (which was not replaced during the 4 day incubation periods) and attached to the culture dish (after their detachment with trypsin-EDTA) were counted in a Bürker chamber.

In addition, cultures were incubated with Hoechst 33342 and propidium iodide, and thereafter fixed in 10% formalin, to determine the proportion of apoptotic and necrotic cells, respectively, in cultures exposed to the various treatments. The proportion dead to living cells was estimated by counting cells in defined areas of the dishes 4 dishes/treatment.

The above described treatments did not affect cell death and total cell number.

RNA interference

In order to find out whether GT1-1 cells encompass an intrinsic capacity to degrade PrP^{Sc}, a method to eliminate the substrate for PrP^{Sc} production, i.e. PrP^{C}, from the cells was necessary. We used the RNA interference (RNAi) technique, which was a relatively new method that could be used to perform a selective knock-down of a protein of interest. The knock-down of the target protein, by RNAi, is mediated by the introduction of short sequence specific, double stranded RNAs (siRNAs) to the cells. The steady-state level of the target mRNA is reduced by degradation of the transcribed mRNA, without an alteration in the rate of transcription of the target gene itself (Hannon 2002). The method was originally in discovered and developed in the worm *Caenorhabditis elegans* (Fire et al 1998), and was subsequently modified in order to work in cultured mammalian cells (Elbashir et al 2001a, Elbashir et al 2001b). In mammalian cells siRNAs by the size of 21-23 bp must be used, since longer fragments elicit a strong anti-viral response (Elbashir et al 2001a, Elbashir et al 2001b). The knock-down of a specific protein can be maintained for about 6-10 days depending on the generation time of the cells.

In 2002, when I started this project, chemically synthesized siRNA could be purchased from certain companies, but they were expensive and there was no guarantee that the purchased siRNA would be effective in silencing the protein

in question (Donze & Picard 2002). Thus, in paper I, I designed and synthesized the siRNAs myself, using T7 RNA polymerase for *in vitro* transcription (Donze & Picard 2002). The design of the siRNA is of great importance, since targeting different regions of the mRNA yields different efficiency of target knock-down. Previous work correlated low G/C content within target mRNA regions with efficient siRNA silencing (Elbashir et al 2002) and today much is known about the pitfalls involved in siRNA design (Reynolds et al 2004).

In paper II, I instead purchased chemically synthesized siRNA, which by then hade become affordable and effective, directed to the mRNA for cathepsin B and L, and by doing so saved time. Today there are libraries of pre-designed siRNAs directed to all known mRNAs in the common model systems.

PCR array

The expression of 84 transcripts involved in MAP kinase signaling was analyzed in a 96-well PCR Array (PAMM -061, RT^{2}Profiler^{TM} PCR Array System; SuperArray Bioscience Corporation). The expression profiles of uninfected cells and cells exposed to the 22L, RML and Me7 scrapie strains were compared and analyzed using RT^{2}Profiler PCR Array Data Analysis (SuperArray Bioscience Corporation). Deviant transcripts were picked out; however, none of these transcripts could be confirmed to be differentially expressed when further analyzed by quantitative real-time RT-PCR. This indicates that the PCR Array was not a reliable tool in identifying differentially expressed genes, and other transcripts that were not picked up by the array might turn out to be differentially expressed when analyzed by another method. All together, we did not find any evidence of altered expression of MAP kinase related transcription, using these methods.

CONCLUDING REMARKS

The studies described in this thesis were undertaken with the aim to examine a potential cross-talk between prions and intracellular signaling pathways. This was done based on the notion that hijacking of host signaling networks during infections has favored replication and spread of other pathogens, such as certain viruses, bacteria and protozoan parasites. In my studies, I demonstrate the occurrence of a molecular cross-talk between prions and key signaling cascades of the host cells, i.e. the MEK/ERK and p38/JNK MAP kinase pathways, the cAMP/PKA cascade as well as the mTOR pathway. I show that the formation of PrP^{Sc} in GT1-1 neuronal cells infected with the RML or 22L strain of scrapie can be stimulated by activation of the MEK/ERK pathway using two different stimuli, i.e. the growth factor BDNF and high [KCl]-mediated depolarization. Conversely, inhibition of the same pathway efficiently clears prion-infected cells from PrP^{Sc} by blocking its formation. I, thus, identify the MEK/ERK signaling cascade as a crucial pathway involved in the formation of PrP^{Sc} of different prion strains. Moreover, our results indicate that the MEK/ERK cascade and the mTOR pathway might converge to activate the cytoplasmatic S6 ribosomal protein to exert a translational regulation of PrP^{Sc} formation.

The MEK/ERK and p38 MAP kinase pathways were shown to exert opposing effects on prion formation. Thus, in prion-infected GT1-1 cells ERK-mediated formation of PrP^{Sc} might be counterbalanced by p38-mediated inhibition of PrP^{Sc}. In humans and animals the homeostatic balance between these pathways may vary between different cell types and tissues. This might influence the susceptibility of the cells to prion replication and possibly contribute to the selective targeting of prions to certain cell populations, such as neurons.

The cross-talk between prions and the MEK/ERK cascade was found to be bidirectional. In other words, the exposure of GT1-1 cells to prions causes a transient ERK activation, and the formation of PrP^{Sc} is increased by activation of ERK. It should be noted that only exposure of cells to the scrapie strains that eventually led to persistent infections, i.e. the RML and 22L strains, caused an activation of ERK. This could possibly reflect that the activation of ERK is coupled to cellular PrP^{Sc} replication, since exposure to the Me7 and Nor98 scrapie strain, which did not result in an infection, did not elicit ERK activation.

My studies may have certain therapeutic implications: The MEK/ERK pathway might be a potential target for antiprion drug design. The MEK inhibitor SL327 that we used in our studies is of particular interest since it can pass the BBB and have been used for systemic administration *in vivo* in mice (Atkins et al 1998, Wang et al 2003). Notably, there are newly developed MEK-inhibitors that are undergoing clinical trials for certain forms of tumors and these are well tolerated by the

patients (Sebolt-Leopold 2008, Wong 2009). The efficiency of such MEK-inhibitors in animal models of prion-disease would therefore be interesting to study.

In our initial studies, I used RNA interference, which by then was a newly developed technique (Elbashir et al 2001a, Elbashir et al 2001b, Fire et al 1998), to knock-down the expression of PrP^{C}, and by doing so enabled cellular clearance of PrP^{Sc}. The same method has recently been used *in vivo* and is in fact the first therapeutic intervention that results in neuronal rescue, prevents clinical signs of disease and increases survival in prion-infected mice (Pfeifer et al 2006, White et al 2008, White & Mallucci 2009).

The question whether the observed transient activation of the MEK/ERK cascade in our cell system is a direct effect of pathogenetic prion activity or a secondary protective host cell response to the infection, remains to be answered. However, the findings in this thesis indicate a dual function of ERK activation in neurons during prion infections, whereby activation of ERK, which is normally beneficial to the cells, can at the same time promote formation of the pathogen.

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"Prion people"

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