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Pharmacological and genetic manipulation of autophagy and its impact on diverse prion infection scenarios

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Doubt grows with knowledge.
Johann Wolfgang von Goethe

Knowledge of the self is the mother of all knowledge. So it is incumbent on me to know my self, to know it completely, to know its minutiae, its characteristics, its subtleties, and its very atoms.
Khalil Gibran

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Summary

Prion diseases are infectious and fatal neurodegenerative disorders of humans and animals which are characterized by spongiform degeneration in the central nervous system. The accumulation of an abnormal „scrapie“ isoform (PrP^{Sc}) of the cellular prion protein (PrP^{c}) is the main feature of prion diseases. PrP^{Sc} is closely associated with infectivity and has been proposed as the protein-only agent responsible for prion diseases. Prion propagation involves the endocytic pathway; endosomal and lysosomal compartments are implicated in trafficking and re-cycling as well as final degradation of prions.

Until now prophylactic and therapeutic regimens against prion diseases are highly limited. A profound understanding of cellular metabolic pathways involved in prion infections might lead to key knowledge for novel strategies to activate cellular clearance mechanisms for prion aggregates or to prevent infection and cell death. The autophagic pathway is suggested to play a key role in the CNS and in neurodegenerative diseases. Autophagy is a fundamental cellular bulk degradation process for e.g. organelles or cytoplasmic proteins, which can be protective or detrimental to neuronal cell survival and degeneration. As it is crucial to perform a thorough investigation of this cellular mechanism, which might have the potential to interfere with accumulation of intracellular PrP^{Sc}, the role of autophagy in transmissible spongiform encephalopathies (TSEs) and its potential as a target for prion therapeutics is investigated in this work.

The distinctiveness of this study is that it was conceptually designed to address two complex and dynamic processes, namely the autophagic pathway and the neurodegenerative prion diseases, by manipulating the first and studying the impact of this manipulation on the latter. Autophagy monitoring and modulation, induction and inhibition, were used as research tools to analyze the effect of autophagic alteration on prion infection.

In the first part of this study it was shown that trehalose, an alpha-alpha-1,1-linked glucose disaccharid, is able to significantly reduce PrP^{Sc} in a dose- and time-dependent manner while at the same time it induced autophagy in persistently prion-infected neuronal cells, one of the most prominent models for studying prion infection. Inhibition of autophagy, either pharmacologically by known autophagy inhibitors like 3-methyladenine, or genetically by siRNA targeting Atg5, counteracts the anti-prion effect

of trehalose. This study provides the first direct experimental evidence for the impact of autophagy in prion disease.

In order to identify a candidate with high potential, in respect to its ability to induce autophagy as well to its suitability for future therapies, the widely used anti-cancer drug tamoxifen was investigated in the second part of this work. In this study tamoxifen had a double role: i) It was examined for its potential as an anti-prion drug and ii) was a tool that helped dissecting further aspects in the interconnection between the autophagic pathway and cellular prion infections. Tamoxifen had an anti-prion potential in primary, persistent and in vivo prion infection. The data display that the efficient mode of action of the drug is directly connected to the autophagy machinery; as soon as tamoxifen-mediated autophagy was constrained genetically by siRNA knock down of autophagy essential genes, Beclin 1 or Atg5, the effect on PrP^{Sc} was diminished. These results uncover a novel function for the selective estrogen receptor modulator (SERM), Tamoxifen and suggest the anti-cancer drug and derived compounds as a candidate for further in vivo studies with respect to therapeutics for prion infections and other related diseases, in which disease is triggered by aggregate-prone or aggregated proteins, such as Huntington`s, Parkinson`s and Alzheimer’s disease. In addition, it confirms a rather general connection between drug-induced autophagy and prion infections.

In the final part of this study the role of basal constitutive autophagy and the consequence of its deceleration were addressed in the scenario of persistent prion infections. Attenuation of basal autophagy enhanced persistent prion infection and Beclin 1 was shown to be an important player in the intracellular degradation of PrP^{Sc} providing another interesting therapeutic target in the context of prion diseases.

Overall, this work demonstrates a fascinating interplay between autophagy and prion infection that seems to manifest itself in various ways throughout different cellular conditions and scenarios and introduces for the first time the drug tamoxifen and beclin 1 as two involved and interesting players.

Zusammenfassung

Prionerkrankungen sind fatale, infektiöse neurodegenerative Erkrankungen, die bei Mensch und Tier vorkommen und sich in einer fortschreitenden schwammartigen (spongiformen) Degeneration des zentralen Nervensystems (ZNS) manifestieren. Die Ablagerung der pathologischen „scrapie“ Isoform (PrP^{Sc}) des normalen zellulären Prionproteins (PrP^{c}) ist das Hauptmerkmal von Prionerkrankungen. PrP^{Sc} stellt nach heutigen Erkenntnissen den ausschließlich aus Protein bestehenden Erreger der Prionerkrankungen dar. Bei der Vermehrung von Prionen ist der endozytotische Weg beteiligt; endosomale und lysosomale Kompartimente sind sowohl beim Trafficking und Recycling als auch bei der finalen Degradation von Prionen involviert.

Die prophylaktischen und therapeutischen Möglichkeiten zur Behandlung dieser Krankheiten sind bisher sehr begrenzt. Ein gründliches molekulares Verständnis der für Prioninfektionen relevanten zellulären Stoffwechselwege kann zu neuartigen Strategien zur Aktivierung von zellulären Abbau- und Beseitigungsmechanismen führen und somit zur Prävention von Infektion und Zelltod beitragen. Der Prozess der Makroautophagie (hier auch als Autophagie bezeichnet) scheint generell eine Schlüsselrolle im ZNS und in neurodegenerativen Erkrankungen zu spielen. Autophagie ist ein fundamentaler zellulärer Abbauweg, und er ist in erster Linie für größere Strukturen, etwa Zellorganellen oder cytoplasmatische Proteine oder einzelne Proteine, verantwortlich. Der autophagische Prozess kann protektiv oder auch schädlich für neuronale Zellen sein. Es ist von hoher Relevanz, solch einen zellulären Prozess, der möglicherweise in die Akkumulation von intrazellulärem PrP^{Sc} eingreift, sorgfältig zu analysieren und zu verstehen. Deshalb wurde die Rolle der Autophagie in Prionerkrankungen und ihr Potential, neue Ziele für therapeutische anti-Prion Ansätze zu liefern, in dieser Arbeit untersucht.

Die Besonderheit dieser Studie besteht in der Konzeption, welche einerseits auf der gezielten Beeinflussung des Autophagischer Prozess und anderer Seite auf der Analyse des zellulären Krankheitsgeschehen beruht. Die Herausforderung liegt darin, dass zwei komplexe und dynamische Prozesse molekular adressiert werden: die Autophagie und die neurodegenerativen Prionerkrankungen. Der Nachweis der Autophagie und ihre Modulation, nämlich durch Aktivierung und Inhibition, wurden als Forschungswerkzeuge eingesetzt, um den Effekt von Modifikationen der autophagischen Aktivität im Kontext der zellulären Prioninfektion zu analysieren.

Im ersten Teil dieser Arbeit wurde gezeigt, dass Trehalose, ein alpha-alpha-1,1-glyosidisch verknüpftes Disaccharid, welches bekannter Maßen die Autophagie induziert, in der Lage ist, in persistent prion-infizierten neuronalen Zellen, einem der prominentesten Untersuchungsmodelle für Prioninfektionen, PrP^{Sc} dosis- und zeitabhängig signifikant zu reduzieren. Die Inhibition der Autophagie, sei es mittels bekannter pharmakologischer Inhibitoren wie 3-Methyladenin, oder genetisch mittels siRNA gegen Atg5, führt zu einer deutlichen Abnahme des anti-Prion Effekts von Trehalose. Diese Studie verschafft somit den ersten direkten experimentellen Beweis zur Bedeutung der Autophagie in Prionerkrankungen.

Mit dem Ziel, einen Kandidaten zu identifizieren, der sowohl über ein hohes Potential in seiner Fähigkeit Autophagie zu induzieren als auch eine vielversprechende Eignung für zukünftige Therapien verfügt, wurde der Wirkstoff Tamoxifen im zweiten Teil dieser Arbeit untersucht. In dieser Studie hatte Tamoxifen eine Doppelrolle: i) Tamoxifen wurde auf sein Potential als anti-Prion Therapeutikum untersucht, und ii) es diente als experimentelles Werkzeug zur Untersuchung des Zusammenhangs zwischen Autophagie und zellulären Prioninfektionen. Tamoxifen führte zu einer signifikanten Reduktion von PrP^{Sc} sowohl in primären, persistenten als auch in *in vivo* (Mausmodell) Prioninfektionen. Die Daten zeigen, dass der Effekt des Wirkstoffes direkt mit der autophagischen Maschinerie zusammenhängt; das heißt, sobald Tamoxifen-induzierte Autophagie durch genetischen Knockdown von Beclin 1 oder Atg5 mittels siRNA eingeschränkt ist, wird der Effekt der PrP^{Sc} Reduktion deutlich abgeschwächt. Diese Ergebnisse enthüllen eine neuartige Funktion des selektiven Östrogenrezeptormodulators (SERM) Tamoxifen und indizieren dieses Krebsmedikament und seine Derivate als Kandidat für weitere *in vivo* Studien für Therapeutika gegen Prioninfektionen und verwandte Erkrankungen, bei denen aggregierte oder zur Aggregatbildung neigende Proteine die Krankheit auslösen können, wie z. B. bei Huntington-, Parkinson- und Alzheimer- Erkrankungen. Im Weiteren bestätigen die Ergebnisse einen generellen Zusammenhang zwischen Wirkstoff-induzierter Autophagie und Prioninfektionen.

Im dritten und letzten Teil der Arbeit wurde die Rolle der unmodullierten, basalen Autophagie und die Auswirkung ihrer Attenuierung im Szenarium der persistenten Prioninfektionen untersucht. Die Schwächung der basalen Autophagie führt zur Verstärkung der persistenten Prioninfektion. Knock-down Experimente mittels siRNA zeigten, dass Beclin 1 ein wichtiger Faktor bei der intrazellulären Degradation von PrP^{Sc} ist und somit ein interessantes weiteres Target im Kontext der Prionerkrankungen liefert.

Zusammengenommen ist in dieser Arbeit ein bisher kaum bekanntes Zusammenspiel zwischen der Autophagie und der Prioninfektion, und die Fähigkeit der Autophagie intrazelluläres, akkumuliertes PrP^{Sc} zu beseitigen, demonstriert. Tamoxifen und Beclin sind hier zum ersten Mal als Ausgangspunkte für eine Therapeutische Intervention aufgezeigt.

1 Introduction

1.1 Prions and prion diseases

Transmissible spongiform encephalopathies (TSEs), also known as “prion diseases”, are inevitably fatal and belong to a family of neurodegenerative disorders that occur in humans and animals and can, under certain circumstances, be transmitted within or even between species (Weissmann, 1996; Prusiner, 1998). Long incubation times, a short symptomatic phase, the always fatal progression of the disease and, usually, the lack of preclinical diagnostic are main features of prion diseases. The epidemic occurrence of BSE (bovine spongiform encephalopathy) in Great Britain, later on in Europe, made TSEs win broad public attention and prion diseases became a significant scientific subject: TSEs display an unusual group of disease that apparently are not caused by viruses or other nucleic acid-encoding agents. They are caused by prions. The following chapters present facts and theories about prion diseases.

1.1.1 The prion gene (*Prnp/PRNP*)

The gene family of the prion protein consists of three members: *Prnp*/*PRNP* encoding the prion protein (Basler *et al.*, 1986), *PRND* for doppel (Moore *et al.*, 1999), and recently *SPRN* (Premzl *et al.*, 2003) standing for shadoo (shadow) of the prion protein was described.

As for doppel, it is usually not expressed in the brain and it is suggested that it is involved in male fertility (Premzl *et al.*, 2003). In certain $PrP^{0/0}$ mouse lines, in which expression of doppel was accidentally triggered (Moore *et al.*, 1999), doppel was responsible for the neurodegenerative phenotype observed. Shadoo is expressed within the brain mainly complementary to PrP and its function in physiology and pathology is so far unknown (Watts *et al.*, 2007).

PRNP (the PrP gene) is the gene of which the expression is absolutely required for the development of prion disease (Bueler et al., 1993). *Prnp* was first described 1986 (Basler *et al.*, 1986), the year that BSE first emerged. Up to now, PrP-genes of over 70 species have been analysed and found to be highly conserved in evolution of mammals, marsupials, birds and amphibians (Schätzl *et al.***,** 1995; Windl *et al.*, 1995; Wopfner *et al.*, 1999; Strumbo *et al.*, 2001; Suzuki *et al.*, 2002; Rivera-Milla *et al.*, 2003).

The PrP gene is mapped to the short arm of human chromosome 20 and in the same region in the mouse chromosome 2 (Robakis *et al.*, 1986b; Sparkes *et al.*, 1986).

The number of exons, the exon in which the ORF is located and the location of untranslated regions may alter from species to the other. PrP genes consist of one (e.g. in hamster and humans) or two (e.g. in rat, mouse, bovine, sheep) short exons at the 5'-end and one bigger exon at the 3'-end, the latter coding for the entire open reading frame (ORF) of the prion protein (Hsiao *et al.*, 1989; Gabriel *et al.*, 1992; Schatzl *et al.*, 1995). In all known PrP genes, the entire open reading frame resides within a single exon at the 3`-end (Hsiao *et al.*, 1989; Gabriel *et al.*, 1992; Schätzl *et al.*, 1995). Thus, the possibility for alternative splicing producing two protein isoform does not exist (Basler, 1986, Westaway *et al.*, 1994) (→Figure 1).

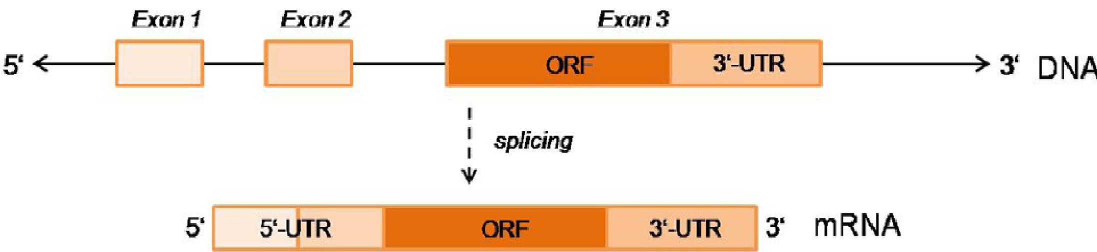


Figure 1. Structure of murine Prnp. Murine Prnp consists of the short exons 1 and 2 and the long exon 3. The entire open reading frame (ORF) is encoded in exon 3, followed by a long 3' untranslated region (3'UTR).

The size of transcribed PrP-mRNA is between 2,1 and 4 kb and encodes for a protein of approx. 250 amino acids. The promoter of PrP gene lacks a typical TATA-box but contains multiple copies of G+C rich repeats that bind transcription factors Sp-1 and AP-1 (McKnight and Tijan, 1986), a typical feature for "house-keeping" genes (Basler *et al.* 1986). Transcription factors SP1 and MTF-1 indeed appear to be involved in regulation of PrP^c expression (Bellingham *et al.*, 2009). Interestingly, some insulinoma and pheochromocytoma cell lines down-regulate PrP^c expression on the mRNA level in response to challenge with prion infected brain homogenates which confers some resistance to prion infection on those cells (Aguib *et al.*, 2008). The high conservation of the PrP gene indicates its important biological function (Schätzl *et al.*, 1995; Shmerling *et al.*, 1998; Wopfner *et al.*, 1999).

1.1.2 Cell biology, life-cycle and putative function of the prion protein

The cellular isoform of the prion protein, namely PrP^c, is not specifically expressed throughout the CNS. It is particularly expressed in its highest concentration in neurons (Kretzschmar *et al.*, 1986; Moser *et al.*, 1995) and localizes mainly at synaptic (Fournier *et al.*, 1995) or presynaptic ends (Herms *et al.*, 1999), but also – though to a lesser extent – in extraneural tissues (Bendheim *et al.*, 1992; Ford et al., 2002). PrP^c is also widely expressed in cells of the immune system and seems to excert important functions there (Dodelet and Cashman, 1998).

PrP^c can occur in different topologies. Its main form is as a glycoprotein anchored to the outside of the cell by a glycophosphoinositol (GPI) anchor (Stahl *et al.*, 1990). Researchers also report transmembrane forms of the protein (Hegde *et al.*, 1998; Stewart *et al.*, 2000; Holme *et al.*, 2003). Reports about dimeric forms of PrP^c are unconfirmed (Kaneko *et al.*, 1997a; Meyer *et al.*, 2000). There are studies describing a somatic expression of PrP^c in neurons with no signal or only a minor signal in the neuropil (DeArmond *et al.*, 1987; Piccardo *et al.*, 1990; Ford *et al.*, 2002). Some data indicated PrP^c localization in the neuropil with synaptic membrane prevalence (Sales *et al.*, 1998; Haeberle *et al.*, 2000; Moya *et al.*, 2000; Fournier *et al.*, 1995, 2000). Other data have favoured a predominantly plasma membrane location of PrP^c with no expression on synaptic vesicles or in the cytoplasm (Herms *et al.*, 1999, Laine *et al.*, 2001). However, a subset of neurons was discovered in which PrP is located predominantly in the cytosol: Cytosolic PrP (PrP_{cyto}) (Mironov *et al.*, 2003). These cells may have implications in the pathogenesis of prion diseases. Studies on the effects of proteosome inhibitors on PrP^c degradation and expression of cytosolic PrP suggested that cytosolic localization of PrP is sufficient to induce neurodegeneration (Ma and Lindquist, 2002; Ma *et al.*, 2002; Rambold *et al.*, 2006).

The primary translation product of the human PrP consists of 253 amino acids and of rodents consists of 254. The first N-terminal 22 amino acids function as a signal peptide for of the nascent polypeptide chain into the endoplasmic reticulum (ER) via the translocon and is co-translationally cleaved off by a signal peptide peptidase (Oesch *et al.*, 1985) (→Figure 2). The C-terminal peptide promotes the posttranslational addition of a glycosyl-phosphatidyl-inositol (GPI) anchor, which mediates membrane linkage of PrP^c, and is cleaved afterwards (Stahl *et al.*, 1992). After cleavage of the N- and C-terminal signal peptides, the mature prion protein comprises 208-209 aa. Then the

polypeptide undergoes several posttranslational modifications. Two N-linked carbohydrate chains can be added at N180 and N196 (Endo *et al.*, 1989), which results in the non-, mono- and di-glycosylated protein forms. Misfolding can occur to at least a small portion of PrP^c, which might be retro-translocated to the cytosol and is there subjected to ER-associated degradation (ERAD) (Ma & Lindquist, 2001).

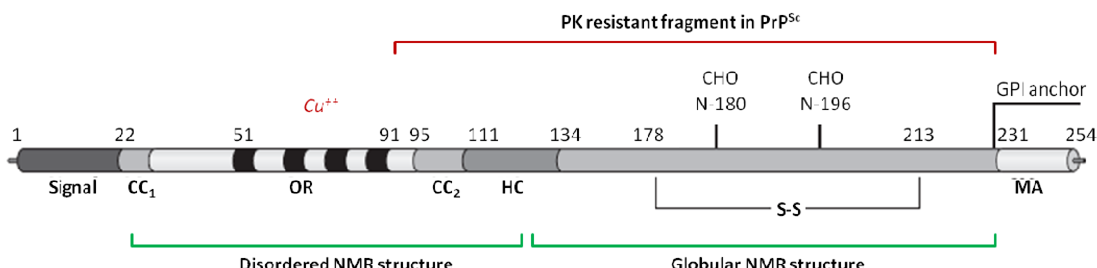


Figure 2. Primary structure of the cellular prion protein including posttranslational modifications contains a secretory signal peptide that resides at the extreme NH2 terminus (aa 1 – 22) . CC_1 and CC_2 define the charged clusters. The N-terminal half of PrP^c comprises the octarepeat region (OR; aa ~ 51 - 90) consisting of the G/P-rich octapeptide repeats. HC defines the hydrophobic core. MA denotes the C-terminal membrane anchor region (aa 232 – 254). In mature PrP^c both signal peptides are cleaved off and at the C-terminus, a glycosylphosphatidyl-inositol (GPI-) anchor is covalently linked to serine 231. The protein can be modified by two N-linked carbohydrate moieties at N180 and N196 (CHO), and an intramolecular disulfide bond is formed between C178 and C213. The C-terminal portion (aa 91 – 231) encompasses the PK resistant fragment in the PrP^{Sc} isoform. The numbers describe the position of the respective amino acids.

An intramolecular di-sulfid bridge is built between the two cysteine residues (Cys178 and Cys 213) within the ER. PrP^c follows the ER secretory pathway, through the Golgi compartment and transport vesicles and is then attached to the outer leaflet of the plasma membrane by the GPI-anchor (Borchelt *et al.*, 1990; Tarabolous *et al.*, 1990b; Caughey, 1991). Here the protein is localized in so called “lipid rafts” (Madore *et al.*, 1999) which are specialized and organised domains in the membrane rich in cholesterol and sphingolipids (Simons and Ikonen, 1997). PrP^c – shedding was also described (Parkin *et al.*, 2004; Heiseke *et al.*, 2008) and was associated with exosomes (Fevrier *et al.*, 2004; Vella *et al.*, 2007). From here internalization seems to occur through clathrin-mediated endocytosis (Sunyach *et al.*, 2003). It might also be occurring through caveolin-related endocytosis and transport (Prado *et al.*, 2004) or in rafts (Tarabolous *et al.*, 1995) (→Figure 3). The octapeptide repeat domain build complexes with copper ions within the PrP^c aminoterminus and promotes its clathrin-mediated internalisation (Shyng *et al.*, 1995). The low-density lipoprotein receptor-related protein 1 (LRP1) seems to play a role in supporting transport of PrP^c through the secretory pathway by interaction with the N-terminal domain of PrP^c and its knock-down led to PrP^c retention in biosynthetic compartments (Parkyn *et al.*, 2008). It was also observed that deletion of N-terminal PrP

(aa 23 – 90) decelerated the transport of PrP^c through the secretory pathway to the cell surface (Nunziante *et al.*, 2003a). Glycosaminoglycans (GAGs) and the 37 kDa/67-kDa laminin receptor (LRP/LR) are further transmembrane receptors that seem to promote PrP^c internalization (Hundt *et al.*, 2001; Pan *et al.*, 2002). PrP^c cell surface localisation is thought to be essential for its conversion into PrP^{Sc} (Borchelt *et al.*, 1990; Tarabolous *et al.*, 1990b; Caughey, 1991) as the conversion seems to occur close to the plasma membrane along the endocytic pathway in caveolae-like domains (CLDs) or lipid rafts (Borchelt, 1992; Tarabolous *et al.*, 1995; Vey *et al.*, 1996; Kaneko *et al.*, 1997a). After internalization PrP^c is transported to endosomes. From the endosomes it can be transported back to the cell surface (Vey *et al.*, 1996) or to acidic compartments, such as lysosomes, for its final degradation and has a half-life in N2a neuroblastoma cells of ~ 3 - 4 hours (Borchelt *et al.*, 1990; Caughey & Raymond, 1991).

High expression levels of the PrP^c in the CNS and in the immune system (Dodelet and Cashman, 1998; Kretzschmar *et al.*, 1986) and its high conservation in evolution (Schätzl *et al.*, 1995; Wopfner *et al*, 1999) as described →1.1.1 indicate an important biological role, which still has not been revealed.

One fact is confirmed about PrP knock-out mice ($PrP^{-/-}$): They are completely resistant to inoculated prions and fail to replicate infectivity (Bueler *et al.*, 1993). Thus, PrP^c expression is necessary for onset of prion disease (Bueler *et al.*, 1992) and for development of the neurotoxic phenotype that parallels TSEs (Mallucci *et al.*, 2003). Mice lacking PrP^c show normal development, behaviour and reproductivity (Bueler *et al.*, 1992, Manson *et al.*, 1994). There are some reports of impairments of PrP knockout mice on the neuronal level, such as abnormalities in synaptic physiology (Colling *et al*, 1996, Whittington *et al.*, 1995), in circadian rhythms and sleep (Tobler *et al.*, 1996) and of a suggested function in neurotransmission (Manson *et al.*, 1994; Colling *et al.*, 1996). In contrast, other studies stated normal neuronal excitability and synaptic transmission in the hippocampus of PrP null mice (Lledo *et al.*, 1996). Several neuroprotective effects of PrP^c are described. A possible protective function of PrP^c against oxidative stress is suggested (Brown & Besinger, 1998; Haigh & Brown, 2006; Dupiereux *et al.*, 2007). It can bind Cu (II) ions (Hornshaw *et al*, 1995; Brown *et al.*, 1997; Stockel, 1998) and it may have some superoxide dismutase activity (Brown *et al.*, 1997). This binding also stimulates its internalisation, suggesting a role in copper homeostasis (Hornshaw *et al.*, 1995; Brown *et al.*, 1997; Pauly & Harris, 1998).

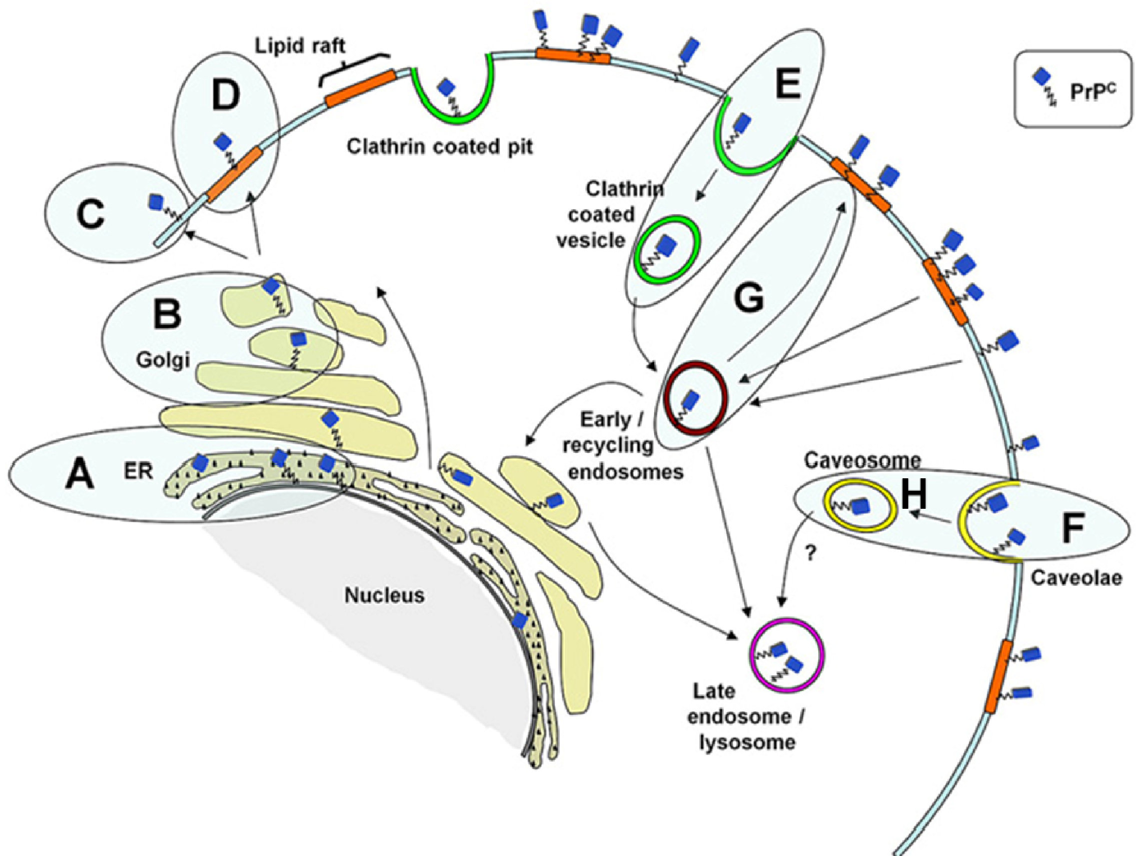


Figure 3. Trafficking and localization of the cellular PrP^c. PrP^c (blue square) is synthesized in the ER (A) and is trafficked through the Golgi (B) to the cell surface where it resides in the plasma membrane (C) predominantly in lipid raft domains (orange) (D). PrP^c endocytosis can be mediated via clathrin coated pits(green) (E) or caveolae (yellow) (F), and PrP^C can be transported back to the cell surface via recycling endosomes (brown) (G) and reaches late endosomes and lysosomes (violet) (H) for degradation or is recycled back to the plasma membrane. (adapted from Lewis and Hooper, 2011)

Furthermore, the antiapoptic function of PrP^c is in debate (Bounhar *et al.*, 2001; Kuwahara *et al.*, 1999; Roucou *et al.*, 2003; 2005, Diarra-Mehrpour, 2004, Li & Harris, 2005; Nishimura *et al.*, 2007; Rangel *et al.*, 2007; Rambold *et al.*, 2008). Interestingly, the stress-protective function of PrP^c seems to be impaired by the presence of PrP^{Sc} (Rambold *et al.*, 2008).

Attempts to gain further insight into the putative function of PrP^c were made by searching for interacting proteins using various biophysical assays. Candidate proteins found were Bcl-2 (Kurschner and Morgan, 1996), Hsp60 (Edenhofer *et al.*, 1996), and the 37-kDa transmembrane laminin receptor precursor (LRP) (Rieger *et al.*, 1997). Recently, direct interactions between LRP/LR and PrP^{Sc} in mediating binding of exogenous PrP^{Sc} to enterocytes (Morel *et al*. 2005) and baby hamster kidney (BHK) cells (Vana and Weiss, 2006) were reported, implying that LRP/LR might have a role in the initial infection process. Its localization on the cell surface and its binding to neural cell adhesion

molecules (NCAMs) points at a possible role in cell adhesion (Stahl *et al.*, 1987; Schmitt-Ulms *et al.*, 2001; Mange *et al.,* 2002). Lipid rafts enhance signal transduction events resulting from protein interactions due to the spatial proximity of proteins in them. PrP^c, a lipid raft protein, might play a role in signalling, supported by observations in neuronal cells. Here, cross-linking of PrP^c led to the activation of the tyrosine kinase Fyn (Mouillet-Richard *et al.*, 2000).

Moreover, interaction between PrP and proteins involved in neuronal signalling, such as synapsin Ib and Grb2 (Spielhaupter and Schätzl, 2001), strongly indicate its role in signal transduction processes.

Furthermore, involvment of PrP^c in haematopoietic-stem-cell renewal (Zhang *et al.* 2006), neural-stem-cell differentiation (Steele *et al.* 2006) and in mediating amyloid β (Aβ)-oligomer-induced synaptic dysfunction (Lauren *et al.*, 2009) were described.

1.1.3 Mechanisms of prion conversion

In 1982, S. Prusiner and coworkers were able to isolate a protease-resistant glycoprotein from brains of diseased hamsters that obviously represented the infectious fraction (Prusiner, 1982). To clearly restrain this new group of infectious agents from classical pathogens like viruses, he coined the word 'prion' by combining '*pro*teinaceous *in*fectious particle' (Prusiner *et al.*, 1984). This designation indicates the presumption that the infectious agent solely consists of protein and is devoid of any genetic information encoded by nucleic acid (Prusiner, 1998; Legname *et al.*, 2004). For proposing and elucidating the protein-only hypothesis, Stanley Prusiner was awarded the Nobel Prize for Medicine in 1997.

The composition of the infectious unit exclusively of protein provoked many discussions during the following decades. Although the theory remains controversial, evidence in support of the protein-only model (elaborated in →1.1.4) is substantial (Bolton *et al.*, 1982, 1987; McKinley *et al.*, 1983; Prusiner *et al.*, 1984; Hsiao *et al.*, 1989; Scott *et al.*, 1989, 1993; Büler *et al.*, 1992, Legname *et al.*, 2004; Soto *et al.*, 2002). As described in →1.1.2, PrP^c expression is absolutely necessary for the conversion event. The conversion of the host encoded protein, PrP^c, into its abnormally folded aggregated isoform, PrP^{Sc}, which then, predominantly in the central nervous system of the infected host, is the main event during prion infection for its establishment.

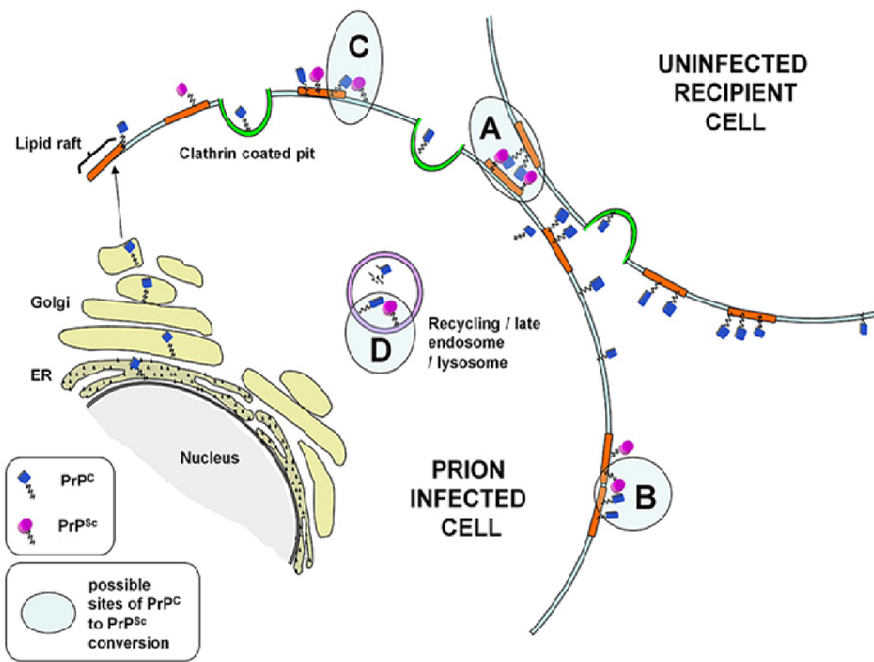


Figure 4. Possible sites of PrP^c to PrP^{Sc} conversion and propagation. (A) Initiation of conversion at the cell surface after direct contact between uninfected and infected cells. (B) Lipid raft clustering, enabling interaction of otherwise separated PrP^c and PrP^{Sc}. (C) Non-raft associated PrP^{Sc} promoting conversion of contiguous raft associated PrP^c. (D) Conversion of PrP^C to PrP^{Sc} in endocytic vesicles. Mode (A) is represented by lipid raft associated PrP, but could involve non-raft associated PrP. Mode (B) highlights conversion at the cell surface, but could occur intracellularly in vesicles known to contain raft-associated proteins (from Lewis and Hooper, 2011).

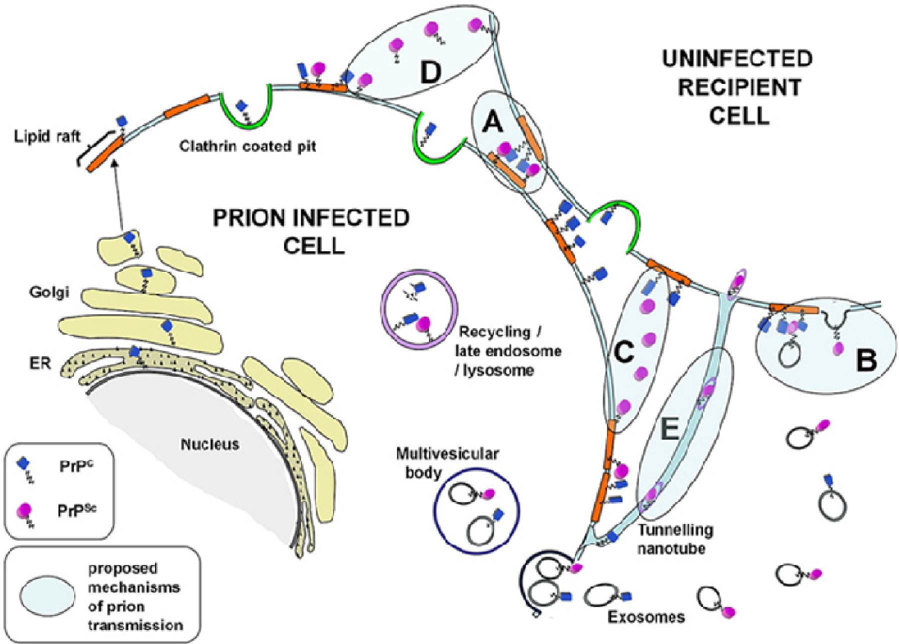


Figure 5. Proposed mechanisms of cell-to-cell spread of prion infectivity. (A) Prion transmission through direct cell-to-cell contact (conversion of recipient PrP^c without internalization of donor PrP^{Sc}). (B) Transmission of prions through exosomal PrP^{Sc} association; both a direct interaction of exosome-associated PrP^{Sc} with cell-associated PrP^c and incorporation of exosomal membrane with recipient cell membrane are represented. (C) C-terminal truncation of PrP^{Sc} allowing release from an infected cell and movement to an uninfected recipient cell. (D) "GPI-painting" mode of prion transfer. (E) PrP^{Sc} spread through tunnelling nanotubes, in association with small vesicles of lysosomal origin. Mode (A) is represented by lipid raft associated PrP, but could involve non-raft associated PrP. Mode (D) is depicted by transfer of cell surface PrP^{Sc}, but could potentially occur with exosomal PrP^{Sc} (from Lewis and Hooper, 2011).

According to the protein-only hypothesis a direct interaction between host PrP^c and exogenous PrP^{Sc} catalyses the conversion of the normal host protein into the pathogenic form. Recently, profound support for this hypothesis was provided by studies on the in vitro generation of prion infectivity out of brain-derived PrP^c (Castilla *et al.*, 2005) or prion protein expressed recombinantly in E. coli (Legname *et al.*, 2004). This, increasing evidence states that PrP^{Sc} or a folding intermediate PrP^{Sc} present the infectious agent responsible for prion disease (Legname *et al.*, 2004; Castilla *et al.*, 2005).

On the cellbiological level, the conformational change of PrP^c into PrP^{Sc} is suggested to occur at the cell surface, either in lipid rafts or caveolae-like domains (CLDs), and/or along the early endocytotic pathway (Caughey *et al.* 1991; Borchelt *et al.* 1992), involving a direct contact between PrP^c and PrP^{Sc} isoforms (→Figure 4). Possible co-factors for prion generation are the laminin receptor or its precursor (Leucht *et al.* 2003) or glycosaminoglycans (Priola & Caughey 1994). PrP^{Sc} mainly localizes in secondary endosomes and lysosomes (McKinley *et al.* 1991; Arnold *et al.* 1995; Mironov, Jr. *et al.* 2003) and a small fraction was observed at or near the plasma membrane (Borchelt *et al.* 1990; Caughey & Raymond 1991; Vey *et al.* 1996). Additionally, a small portion of PrP^{Sc} appears to accumulate in cytosolic aggresomes under certain experimental conditions (Kristiansen *et al.* 2005) where it seems to impair proteasomal function (Kristiansen *et al.* 2007). The cell-to-cell spread of prion infectivity can then take place via exosomes (Vella *et al.* 2007), via cell-to-cell contact (Kanu *et al.* 2002) or other proposed meachnisms (→Figure 5). On the molecular level, conversion of the two short β–sheet structures and the first α–helix into a large β–sheet formation is the major event which is responsible for conversion of PrP^c into the pathogenic isoform PrP^{Sc}. The remaining two α –helices and the disulfide bond need to be preserved for PrP^{Sc} to be infectious (Hornemann *et al.* 1997; Prusiner 1998; Wille *et al.* 2002).

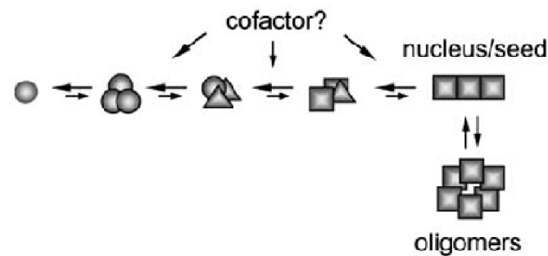
This structural "carry over" converts the normal cellular PrP to a pathogenic prion protein. However, C-terminal structures including α-helices and the disulphide bonds need to be preserved for PrP^{Sc} to be infectious (Prusiner, 1998; Hornemann *et al.*, 1997, Wille *et al.*, 2002). Yet, it has not been fully determined whether the disulfide bond remains intact during conformational change into PrP^{Sc}, or whether this bond must break and reform to permit structural rearrangement of the protein.

Conversion of PrP^c to PrP^{Sc} seems to be an at least two step process, where a direct interaction of PrP^c with PrP^{Sc} is required for the subsequent conversion into its

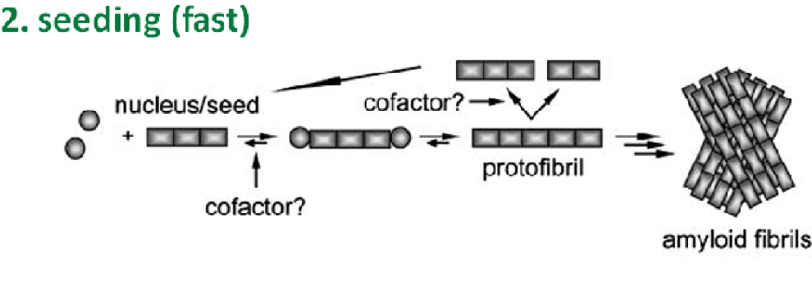
pathological isoform (Horiuchi *et al.*, 1999). The exact molecular mechanism of conversion is still subject of intensive research. One model describing the conversion process is called the heterodimer hypothesis proposed by Cohen and Prusiner (Prusiner *et al.*, 1990; Cohen *et al.*, 1994).

There is increasing evidence supporting a model called nucleation dependent polymerisation model in which aggregates are formed by a crystallization-like process, known as nucleated

1. nucleation (slow)



2. seeding (fast)



PrPC PrP* PrPSc

Figure 6. Conversion process according to the nucleation dependent polymerization model. Formation and propagation of amyloid may be divided into two steps. The initial step is crucial as the formation of 'nuclei' requires at least partially unfolded protein resulting in small oligomers. This rare and slow process is followed by a much faster elongation phase in which the nuclei act as seeds to recruit native proteins into the growing aggregate. During this process breaking of the elongating fibrils may be necessary to generate new seeds resulting in an exponential rise in amyloid formation. Spontaneous formation of PrP^{Sc} from normal PrP^{c} is a very rare event leading to sporadic forms, whereas mutations in the PRNP gene apparently render the protein more aggregation prone (heritable prion diseases). In acquired prion diseases the exogenous addition of PrP^{Sc} seeds induces conversion of the host-encoded protein.

polymerization (→Figure 6) (Come *et al.*, 1993; Gajdusek, 1994; Soto *et al.*, 2006). It was first defined by the group around Gajdusek in 1990 (Brown *et al.*, 1990) and then postulated by Landsbury together with Byron Caughey (Jarrett & Landsbury, 1993; Come *et al.*, 1993; Caughey *et al.*, 1995). This model suggests equilibrium between PrP^{c} and PrP^{Sc}. The crucial step for conversion is the so-called “nucleation” step responsible for the generation of a nucleation-seed, which consists of an infectious PrP^{Sc} oligomer. The “nucleation” step is very slow as the energy barrier is supposed to be very high and the

equilibrium lies for thermodynamic reasons on the side of PrP^c (Cohen *et al*. 1994). PrP^{Sc} is then able to generate nuclei (small oligomers) (Brown *et al*. 1990; Come *et al*. 1993; Jarrett and Lansbury 1993; Caughey *et al*. 1995). When the oligomer reaches an appropriate size, the process goes into the second, much faster "seeding" step. Here, the associated conversion becomes energetically favoured and PrP^{Sc} nuclei act as seeds to recruit native proteins into high molecular aggregates of PrP^{Sc} forming protofibrils. These protofibril might break up forming new PrP^{Sc} seeds resulting in an exponential rise in amyloid formation. It remains undeterminded, whether cofactors are needed for each or some steps in the PrP^{Sc} or amyloid formation, respectively.

Since the genesis of such a seed is energetically very unfavourable, its spontaneous occurrence is very rare, which explains why spontaneous formation of PrP^{Sc} from normal, physiological PrP^c leading to sporadic forms of prion disease is a very rare event. Exogenous addition of PrP^{Sc} seeds might induce conversion of the host-encoded PrP^c resulting in acquired prion disease. Mutations in *PRNP* make the prion protein more aggregate-prone leading to genetic prion disease

Newly converted PrP molecules are characterized by novel biochemical and biophysical properties that are used to distinguish the two isoforms. Recently evidence was provided that non fibrillar particles with masses equivalent to 14-28 PrP molecules are associated with both the highest PrP conversion activity and also infectivity (Silveira, *et al*., 2005).

1.1.4 Biochemical and structural characteristics of the prion protein isoforms

The charactersitics of the causative agent of TSEs became a matter of dispute, after the first occurrences of scrapie disease affecting sheep and goat in 1732. At first it was assumed to be a virus, but no viral particles or immunological response could be detected. In 1966 Tikvah Alper and colleagues found out that the scrapie agent resisted treatments that would inactivate nucleic acid. It was not affected by UV-inactivation or ionic radiation (Alper *et al.*, 1966; Alper *et al.*, 1967). Yet, infectivity was reduced by procedures that cause hydrolysis or modification of proteins, such as treatment with urea or NaOH. These findings gave rise to the assumption that the transmissible agent might be a protein (Griffith, 1967).

A breakthrough occurred when researchers led by of the University of Caifornia, San Fransisco, isolated a protease resistant glycoprotein which was the major constituent of

the infective fraction in brain homogenate of affected animals. It was confirmed that the infectious agent consisted mainly of this specific protein, which was able to form amyloid deposits in form of filamentous structures called "scrapie-associated-fibrils" (SAFs) or "prion rods" (Prusiner *et al.*, 1981; Prusiner, 1982; Hilmert and Diringer, 1984; Lehmann and Harris, 1996). Stanley Prusiner established the "protein-only" hypothesis. The proteinase-K (PK)-resistent PrP found in enriched fractions of scrapie-infected brains was called PrP^{Sc} (Sc for scrapie). The N-terminal sequence of PrP^{Sc} was accomplished by Edman degradation which finally led to the identification of the prion protein encoding gene: *Prnp/PRNP* (Prusiner *et al.*, 1984). The normal membrane-bound sialoglycoprotein was designated the cellular prion protein or PrP^{c}.

The amino acid sequence and covalent modifications of the two protein isoforms PrP^{c} and PrP^{Sc} are identical (Pan *et al.*, 1993). However, the prion model contradicts Anfinsen's (1973) rules and asserts that the two PrP isoforms share the same amino acid sequence but adopt two different confirmations. The two isoforms of the prion protein, the pathogenic and the cellular form strikingly differ in many of their properties as the conversion of PrP^{c} into PrP^{Sc} is associated with pronounced structural changes leading to distinct biochemical characteristics of the two PrP (Pan *et al.*, 1993) (→Table 1).

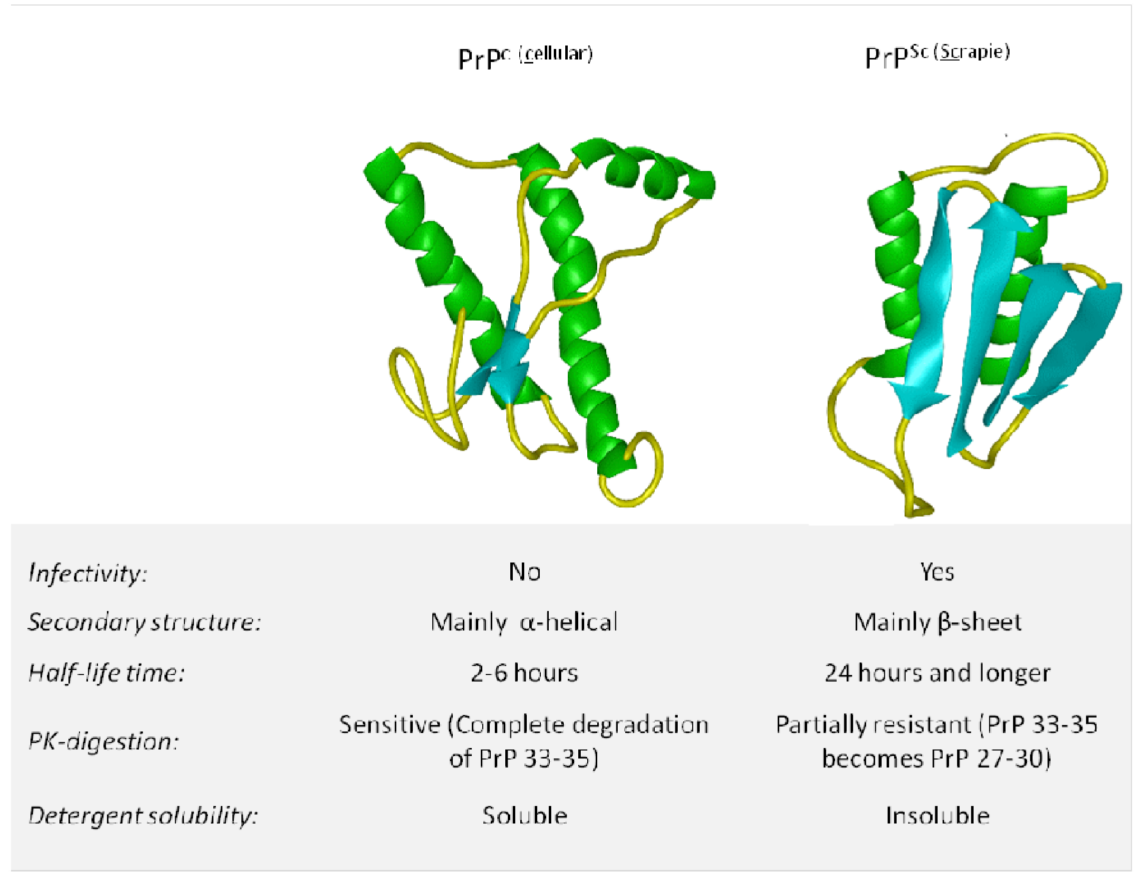
So far, no crystal structures are available, neither for PrP^{Sc} nor for PrP^{c}. However, the structure of PrP^{c} has been resolved by nuclear magnetic resonance (NMR) analysis of PrP23 - 231 resembling mature PrP^{c}, purified upon recombinant expression in *E. coli* (Riek *et al.*, 1996; 1997).

A defined structure of the N-terminal domain (aa 23-120) could not be identified due to its high flexibility, which might be altered upon binding of metal ions to the octarepeat region spanning about amino acid positions 50 – 90, depending on the species (Schätzl *et al.*, 1995; Wopfner *et al.*, 1999). In mammals it is known to contain a region formed by repetition of normally five consecutive peptides, consisting of eight residues long and rich in glycine, proline and histidine (octarepeats). Octarepeat insertions in this sequence can be pathogenic (Gilch *et al.*, 2000). Binding of this region *in vivo* to metal ions and other ligands might lead to a defined globular conformation.

NMR proposes for aa 121 – 231 a defined globular structure with 3 α-helices and two short antiparallel β- strands. Helix B and C are stabilised by an intramolecular disulfide bridge. Diverse investigations revealed that PrP^{c} contains about 42% of α-helices and 3%

β-sheet, whereas PrP^{Sc} is composed of about 30% α-helices and 45% β-sheet (Pan *et al.*, 1993; Gasset *et al.*, 1993; Pergami *et al.*, 1996).

Table 1. Structural and biochemical properties of PrP^{c} and PrP^{Sc}.



	PrP^{c} (cellular)	PrP^{Sc} (Scrapie)
Infectivity:	No	Yes
Secondary structure:	Mainly α-helical	Mainly β-sheet
Half-life time:	2-6 hours	24 hours and longer
PK-digestion:	Sensitive (Complete degradation of PrP 33-35)	Partially resistant (PrP 33-35 becomes PrP 27-30)
Detergent solubility:	Soluble	Insoluble

PrP^{Sc} lacks the N-terminal region (up to 90 aa) after PK-digestion, but the rest of the molecule is resistent, in contrast to PrP^{c}. The reason is supposed to be the structural difference between both isoforms (Prusiner, 1998). Electron microscopy and molecular modelling revealed that, PrP^{Sc} most probably forms trimers with left-handed β-helices between aa 89 – 175, whereas the C-terminal α-helices and the disulfide linkage are retained (→Figure 7) (Wille *et al.*, 2002; Govaerts, 2004). In addition, the proposed model matched the structural constraints of the 2D-crystals, positioning residues 141–176 and the N-linked sugars appropriately. The parallel left-handed β-helical model provides a coherent framework that is consistent with many structural, biochemical, immunological, and propagation features of prions. Since it has not been possible to produce 3-dimensional crystals of PrP so far, a final structure model could not be obtained by high-resolution analysis, only by computations.

Figure 7. PrP^{Sc} structure models determined by electronmicroscopy data. Modeling PrP residues 89–174 onto a left-handed β-helical fold. (A) The β-helical model of the N-terminal part of PrP 27–30 (B) Model of the monomer of PrP 27–30. The α-helical region (residues 177–227) as determined by NMR spectroscopy was linked to the β-helical model shown in A. (C) Trimeric model of PrP 27–30 (adapted from Govaerts *et al*., 2004).

Due to the described structural transitions, PrP^{Sc} differs from PrP^{c} in its resistance to proteolysis and its solubility in detergents (Meyer *et al.*, 1986). Limited digestion of PrP^{Sc} removes the N-terminal 67 amino acids from the larger molecule (33 to 35 kDa) to produce PrP 27-30 (Prusiner *et al.*, 1984; Oesch *et al.*, 1985), while PrP^{c} is completely degraded. Extensive studies showed that the conversion of PrP^{c} to PrP^{Sc} is accompanied by radical changes in structural and biochemical properties of the prion protein isoforms (→Table 1). This transformation displays the basic principle of prion pathogenicity (Prusiner, 1991; Cohen *et al.*, 1994; Clarke *et al.*, 2001; Weissmann *et al.*, 2001). These biochemical differences can be used for diagnostic purposes and research.

1.1.5 Prion strain typing and the "species barrier"

Transmission of prion diseases between different mammalian species is typically far less efficient than intra species; this is known as the "species barrier" (Pattison, 1965) (→Figure 8). Additionally, an altered neuropathological distribution is observed (Schätzl, 2003). Nevertheless, prions can adapt to another host by serial infections. Species barrier is mainly predicted by the degree of homology between the PrP sequences of two species and the position of the amino acid exchanges (Scott *et al*., 1989; 1992; 1993; Telling *et al*., 1994; Schätzl *et al*., 1995; Priola, 2001). In addition, species-specific co-factors interacting with PrP^{Sc} appear to play a role (Telling *et al*., 1995). For instance, hamster prions do not cause disease in mice and *vice versa* (Scott *et al*., 1989; 1992; 1993; Priola, 1999). If transgenic mice expressing hamster PrP in addition to endogenous mouse PrP are infected, species barrier can be overcome and mice generate disease with prion titers comparable to hamsters and equal infectivity. Thereby, incubation times and expression levels of hamster PrP were inversely correlated (Scott *et al*., 1989; 1992; 1993; Prusiner

et al., 1990). However, subclinical infection of wild-type mice with hamster prions was also demonstrated (Hill *et al.*, 2000). This fuels the discussion whether species barrier really is an absolute event or whether PrP^{Sc} is propagated but the host dies before symptoms can occur. In this case, possibly prions can be transmitted. Studies done with transgenic mice have stated that factors contributing to the species barrier are the differences in the PrP sequences between the donor and the recipient protein, the strain of prion and the species specificity of the postulated protein X, a still undetermined host factor eventually binding to PrP^{c} and promoting PrP^{Sc} formation (Telling *et al.*, 1995; Kaneko *et al.*, 1997b).

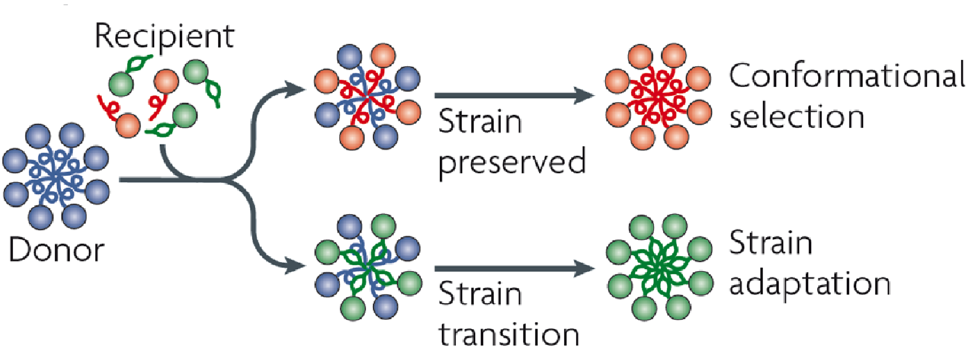


Figure 8. The species barrier. Incompatibility between donor and recipient prion proteins creates a barrier to interspecies transmission. This barrier may reflect an inability of the proteins to associate (not shown) or to adopt compatible conformations. For strains capable of crossing a species barrier, the recipient prion protein may adopt an identical conformation (conformational selection), leading to the conservation of strain identity on transfer. Alternatively, the recipient prion protein may adopt a partially compatible conformation that leads to a change in strain (strain adaptation) (Adapted from Tuite and Serio, 2010).

Different scrapie strains can be distinguished regarding their biological properties: distinct incubation periods and patterns of neuropathological targets (lesion profiles), glycoform profiling and fragment size of PrP^{Sc} upon PK digestion (Bruce *et al.*, 1994; Collinge *et al.*, 1996b, Safar *et al.*, 1998). Strain properties are obviously imprinted in the PrP^{Sc} conformation (Telling *et al.*, 1996; Scott *et al.*, 1997) and can be propagated (Dickinson *et al.*, 1968, Bruce *et al.*, 1991), in case of BSE strain even between many species (Will *et al.*, 1996; Collinge & Rossor, 1996). The fact that the species barrier can be overcome explains the zoonotic transfer of BSE to humans and the appearance of vCJD. Several human PrP^{Sc} strains have been identified which are linked to different phenotypes of CJD (Parchi *et al.*, 1996; Collinge *et al.*, 1996b). The different fragment size seen on Western blots after PK-treatment, the ratio of the three glycosylation forms (Collinge *et al.*, 1996b) and strain specific degrees of PK-resistance (Safar *et al.*, 1998) confirm the idea that there exist several variants of PrP^{Sc}.

1.1.6 Animal and human prion diseases and epidemiology

Prion diseases are noted in man and in a number of mammalian animals (→Figure 9). Under certain circumstances they can be transmitted within or between species (Weissman, 1996; Prusiner, 1998) causing transmissible spongiform encephalopathies (TSEs) or “prion diseases”. The epidemic occurrence of BSE (bovine spongiform encephalopathy) in Great Britain, later on in Europe, made TSEs win broad public attention: TSEs display an unusual group of disease that apparently are not caused by viruses or other nucleic acid-encoding agents. They are caused by prions (→ 1.1.3).

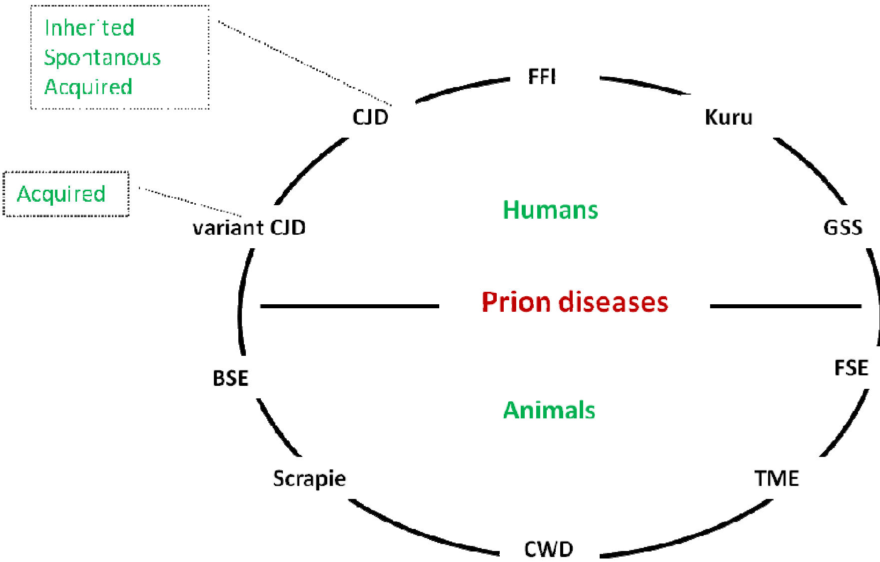


Figure 9. Animal and human prion diseases: an overview.

Animal prion diseases include scrapie of sheep and goats, chronic wasting disease (CJD) of elk and deer, and transmissible mink encephalopathy (TME). Bovine spongiform encephalopathy (BSE) first emerged in the United Kingdom in the mid-1980s and rapidly evolved to a major epizootic with an estimated number of more than 2 million UK infected cattle. BSE has also been reported from many other countries including most European Union states, the United States, Canada, and Japan. Many new animal prion diseases have since been identified, most resulting from infection with the BSE agent (Collinge, 2001). These diseases can be transmitted between species by inoculation or dietary exposure.

Scrapie has been identified in sheep and goats for more than 250 years ago and was described 1732 for the first time (McGoan, 1914; 1922). Along with progressive degenerative brain disease (which may manifest as a change in behaviour, lack of coordination or unsteady gait), the affected animal will scratch itself to the point of self-

mutilation. Hence, the disease was named "scrapie". In 1936 it was possible to experimentally transmit scrapie to goats and the possibility for infection as a cause was postulated (Cuille & Chelle, 1939).

Table 2. Animal prion diseases

Animal TSE agents		
Disease	**Natural host**	**Mechanism of transmission/infection**
Scrapie	Sheep, Goat	Vertical and horizontal infection in genetical susceptible sheep; oral transmission, sporadic
BSE (bovine spongiform encephalopathy)	Cattle	Infection with prion contaminated food, rarely sporadic
TME (transmissible mink encephalopathy)	Mink	Infection with contaminated meat from sheep & cattle
CWD (chronic wasting disease)	Deer, elk and moose	Unclear, possibly similar to scrapie
EUE (Exotic ungulate encephalopathy)	Ungulates	Infection with contaminated bovine tissue and food
FSE (feline spongiform encephalopathy	Cats	Infection with contaminated bovine tissue and food

Chronic wasting disease (CWD) was described in captive animals mainly in the USA and seems to primarily affect deer and elk in areas of Colorado and Wyoming (Williams and Young, 1980; Spraker *et al.*, 1997). Deer affected by the disease were originally thought to be suffering from a nutritional deficiency, but in 1978 lesions were discovered in the brain like those seen in TSE. Surveilling systems have been established to monitor the spread of CWD in captive and free deer and elk.

Bovine spongiform encephalopathy (BSE, more commonly known as "mad cow disease") was first identified in 1986 by British veterinarians (Wells *et al.*, 1987). Affected animals exhibit nervousness, heightened senses, weight loss and diminished milk production. Actually, the symptoms are very similar to scrapie, except of the missing pruritus (Schätzl, 2003). As the disease progresses, the animals have difficulty with walking and holding up their heads. 1992 was the year when the epidemic reached its peak; nearly 1,000 cases per week were reported in Britain (Hörnlimann *et al.*, 2001) (→Figure 12). In the 90s a number of cases have been reported in other European countries like Switzerland, Ireland and Portugal. A probable cause for the origin and spread of BSE are changes in the rendering processes introduced in the UK in the late 1970s which allowed

scrapie prions from sheep and bovine prions to survive (Wilesmith and Wells, 1991; Prusiner, 2001). It was passed to cattle in form of contaminated meat and bone meal (MBM) prepared from carcasses of sheep and cattle as a high nutritional supplement ("Neo-canibalism"). An alternative hypothesis is that epidemic BSE resulted from recycling of rare sporadic BSE into cattle by oral inoculation with food (Phillips *et al.*, 2000). The ban of food derived from animal carcasses in 1988 has led to a containment of the number of cases.

Prion diseases also affect domestic and wild cats (Lehmann and Harris, 1996) and a number of zoo animals (Jeffery and Wells, 1988; Kirkwood *et al.*, 1990). Several of these diseases are supposed to be caused by the BSE strain (Bruce *et al.*, 1994; Collinge *et al.*, 1996a) as they developed at the same time or following the emergence of BSE. However, many domestic animals, such as dogs, birds and pigs, show resistance to oral infection with BSE prions.

Table 3. Manifestations of human prion diseases. (Gilch & Schätzl, 2009)

Human TSE agents		
Manifestation	**Disease**	**Mechanism of transmission/infection**
Acquired *by environmental exposure to prions; exogenous*	Kuru (epidemic in the 1950s); iatrogenic CJD variant CJD (total so far>220 cases)	Acquired through cannibalistic rituals Acquired from contaminated tissue or neurosurgery instruments Infection by BSE contaminated food
Genetic *endogenous*	GSS (Gerstmann-Sträussler-Scheinker disease) FFI (fatal familia insomnia) familial/genetic CJD (~10%)	Mutation in the *PRNP* gene (more than 30 different types are known)
Sporadic *endogenous*	Sporadic CJD (~ 1 case per million per year worldwide, ~90%)	Apparently spontaneous formation of PrP^{Sc}; evtl. somatic mutation in PrP or spontaneous conversion.

Human prion diseases (→Table 3) are associated with a range of clinical presentations and are classified by both clinicopathological syndrome and aetiology with sub-classification according to molecular criteria (Collinge 1997, 2005; Collinge & Palmer 1997; Wadsworth *et al*. 2003).

Approximately 10 - 15 per cent of human prion disease are inherited and associated with autosomal dominant pathogenic *PRNP* mutations; to date over 30 mutations have been described (Collinge 2001, 2005;Kovacs *et al*. 2002; Wadsworth *et al*. 2003; Mead 2006)

(→Figure 10). Genetic change is transmitted to an individual's offspring. Diagnostic *PRNP* analysis allows recognition of over 20 distinct point mutations (Collinge, 1997). Thus, recognition of a range of atypical dimentias became possible (Collinge *et al.*, 1992; Gambetti *et al.*, 1995). Further human prion diseases are Fatal familial insomnia (FFI) (Lugaresi *et al.*, 1986) and Gerstmann-Sträussler-Scheinker disease (GSS) (Gerstmann *et al.*, 1936), which are almost invariably genetically determined TSEs, caused by a range of mutations within the open reading frame of the prion protein gene (PRNP) on chromosome 20.

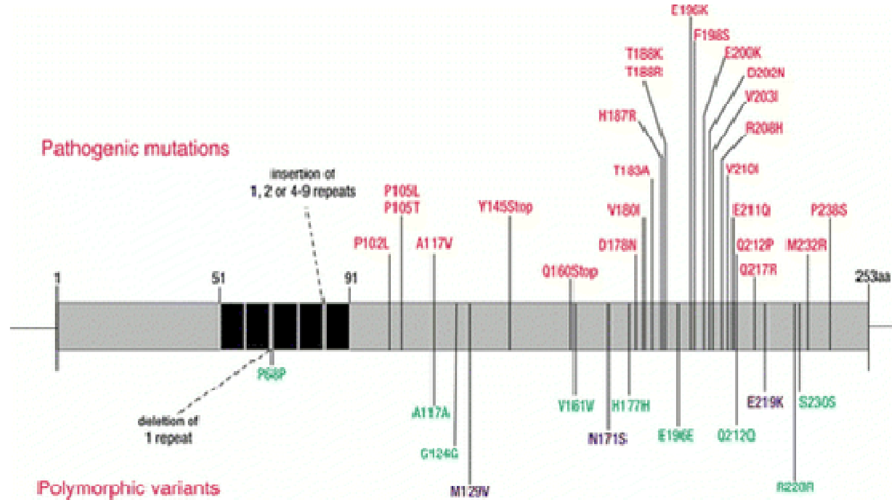


Figure 10. Pathogenic mutations and polymorphic variants of the human prion protein. In red: mutations that correlate with inherited prion diseases. In green & blue: polymorphisms, that might influence susceptibility for prion infections and the disease phenotype (from Collinge, 2001).

Approximately 85 – 95% per cent of cases occur sporadically as CJD (sporadic CJD) at a rate of 1–2 cases per million population per year across the world, with an equal incidence in men and women (Brown *et al*. 1987; Collinge 2001, 2005; Wadsworth *et al*. 2003; Collins *et al*. 2006).

Creutzfeldt-Jakob disease (CJD) is the most common, earliest described and best investigated human prion disease (Creutzfeldt, 1920; Jakob, 1921). However, it is still rare and only occurs in about one out of one million people. The classical form of CJD occurs sporadically and usually affects people aged 45–75, most commonly appearing in people between the ages of 60–65. 90% of CJD occurs sporadically. No pathogenic PrP mutations are present in sporadic prion diseases.

Acquired prion disease in humans due to a dietary origin has resulted in *Kuru*, an epidemic prion disease principally of the Fore linguistic group of the Eastern Highlands of Papua New Guinea, which was transmitted during mortuary feasts when deceased relatives were consumed by close relatives as a mark of respect and mourning (Alpers

1987; Collinge & Palmer 1997; Mead *et al*. 2003), and vCJD in the United Kingdom and other countries caused by human exposure to BSE prions from cattle (Collinge *et al*. 1996a; Bruce *et al*. 1997; Hill *et al*. 1997; Collinge 1999; Asante *et al*. 2002). Kuru demonstrates that incubation periods of infection with human prions can exceed 50 years (Collinge *et al*. 2006) and these data indicate that the parameters of any vCJD epidemic cannot yet be predicted with confidence (Collinge 1999; Frosh *et al*. 2004; Hilton *et al*. 2004; Collinge *et al*. 2006). After cession of cannibalism in the 1950s the number of cases decreased. As postulated (Hadlow, 1959), the transmissibility of Kuru was proven

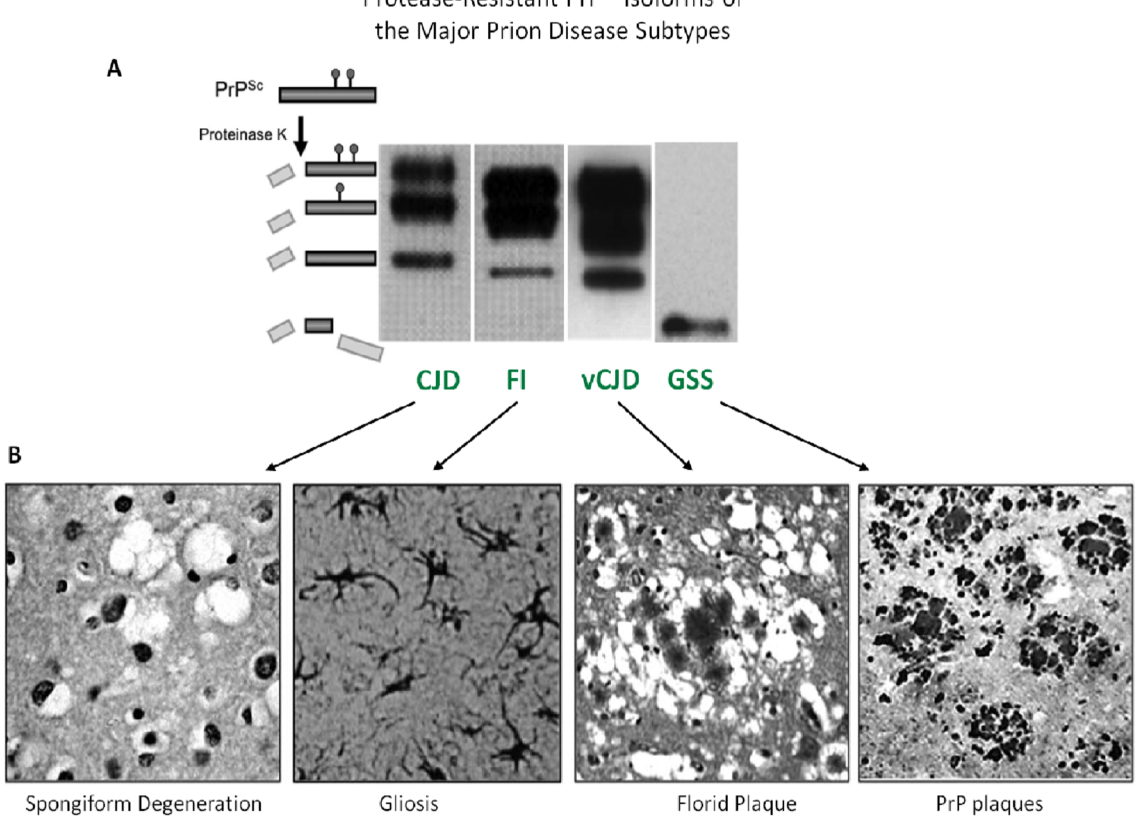


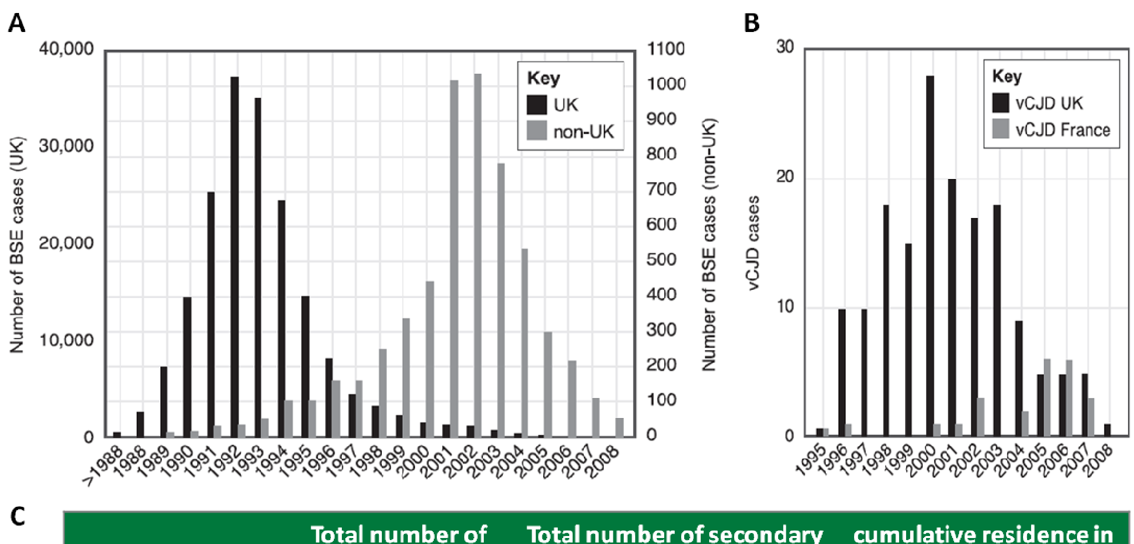
Figure 11. (A) Western blot comparing the major isoforms observed in the 4 principal subtypes of prion disease. To the left of the blot displays the prion protein (PrP) segment that is represented in the adjacent blot. The highest molecular weight of PrP is the diglycosylated fraction of PrP, whereas the monoglycosylated and unglycosylated fractions run faster in the gel, because of their lower molecular weight. In CJD, FI, and vCJD, proteinase-K cleaves the first *67 amino acids leaving the PK-resistant core, PrP90-231. In most cases of Gerstmann-Stra¨ussler-Scheinker syndrome (GSS), a second C-terminal cleavage that removes the glycosylated segment occurs endogenously, leaving a nonglycosylated central segment. **(B) The major Subtypes and their histopathologic features of** 1. Hemotoxylin and Eosin staining demonstrates typical spongiform degeneration (vacuolation) of the grey matter neuropil characteristic of Creutzfeldt-Jakob disease (**CJD**). This feature is less obvious in fatal insomnia (FI) and Gerstmann- Sträussler-Scheinker syndrome (GSS). 2. Glial fibrillary astrocytic protein (GFAP) antibodies demonstrate hypertrophy and proliferation of astrocytes. This feature is present in all prion subtypes. In **FI**, this is often found focally within the anterior nucleus and dorsomedial nucleus of the thalamus and brainstem, in combination with neuronal dropout. In GSS it may parallel PrP plaque pathology. 3. The florid plaques of variant CJD (**vCJD**) consist of dense core PrP amyloid deposits surrounded by vacuoles. 4. PrP-positive multicentric plaques are pathognomonic for **GSS**. These are mostly present within the molecular layer of the cerebellum but may be diffusely present throughout the cerebrum. Adapted from Brown and Mastrianni, 2010.

by intracereberal inoculation of brain homogenate to chimpanzees (Gajdusek *et al.*, 1966).

Iatrogenic forms of prion disease have occurred most frequently due to the transmission of CJD prions via contaminated growth hormone or gonadotropin products derived from human cadavers, or by implantation of contaminated dura mater grafts (Brown *et al*. 1992, 2000). Iatrogenic prion disease has also resulted from transmission of CJD prions during corneal transplantation, contaminated electroencephalographic (EEG) electrode implantation and surgical operations using contaminated instruments or apparatus (Davanipour *et al.*, 1985; Brown *et al*. 1992, 2000; Lueck *et al.*, 2000; Wadsworth *et al.*, 2001; Head *et al.*, 2002).

Variant Creutzfeldt-Jakob disease (vCJD, former *new variant Creutzfeldt-Jakob disease* ,nvCJD) extended the list of acquired TSEs and is a novel human prion disease that appeared in the UK (1995) and several other European countries (Will *et al.*, 1996; Collinge & Rossor, 1996) affecting teenagers and young people (→Figure 12). It is distinguished from the classical type by its early onset (Belay, 1999), slow progression rate with a prolonged clinical phase (1-2 years), and a predominance of ataxia and psychiatric disturbances and not dementia symptoms (Zeidler *et al.*, 1997; Hill *et al.*, 1999 a, b). Accumulation of characteristic floride PrP amyloid plaques in cerebral and cerebellar cortex is the most remarkable neuropathologic characteristic. Furthermore, PrP^{Sc} can be readily detected in tonsils and lymphoreticular system, which does not occur in other human prion diseases. Most vCJD cases reveal homozygosity for methionine at *PRNP* codon 129, however, reports of preclinical vCJD after blood transfusion in *Prnp* codon 129 heterozygous patients suggest that susceptibility to vCJD infection is not confined to the methionine homozygous *Prnp* genotype (Llewelyn *et al.*, 2004; Peden *et al.*, 2004; Bird, 2004).

Restricted geographical occurrence (over 95% of identified cases of vCJD are in Britain) and chronology of vCJD were the first indicators for a direct link to BSE, although there is no definite proof of this association as yet. It has been shown, however, that PrP^{Sc} accumulates in gastrointestinal lymphoid tissue in animals after oral infection (Maignien *et al* 1999; Beekes and McBride, 2000; Shmakov and Ghosh, 2001; Ghosh 2002). Furthermore, *in vitro* studies have shown the uptake of PrP^{Sc} particles by human gastrointestinal tract cells (Morel *et al*, 2005). From there infectivity seems to accumulate on FDCs in Peyer’s patches and subsequently spreads via the enteric nervous system



C

Country	Total number of primary cases (# alive)	Total number of secondary cases: blood transfusion (# alive)	cumulative residence in UK > 6 MONTHS during period 1980-1996
UK	171 (4)	3 (0)	174
France	25 (0)	-	1
Spain	5 (0)	-	0
Republik of Ireland	4 (0)	-	2
USA	3[†] (0)	-	2
Netherlands	3 (0)	-	0
Italy	2 (0)	-	0
Portugal	2 (0)	-	0
Canada	2 (1)	-	1
Saudia Arabia	1 (0)	-	0
Japan	1* (0)	-	0
Taiwan	1 (0)	-	1

Figure 12→ Bovine spongiform encephalopathy (BSE) and variant Creutzfeldt-Jakob disease (vCJD) cases reported worldwide. (A) reported cases of BSE in the United Kingdom (UK) (black) and in countries excluding the UK (gray). Non-UK BSE cases include cases from countries both within and outside of the European Union. **(B)** reported cases of vCJD in the UK (black) and in France (gray). Data are as of January 2009 (see http://www.oie.int). **(C)** In the table are reported vCJD cases worldwide. Data are as of March 2011. [†] the third US patient with vCJD was born and raised in Saudi Arabia and has lived permanently in the United States since late 2005. According to the US case-report, the patient was most likely infected as a child when living in Saudi Arabia.*the case from Japan had resided in the UK for 24 days in the period 1980-1996. [see the National Creutzfeldt-Jakob Disease Surveillance Unit Web site for vCJD data to March 2011 (http://www.cjd.ed.ac. uk)].

(ENS) to the central nervous system (→Figure 13). Molecular strain typing (Collinge *et al.*, 1996a) and *in vivo* experiments with mice (Bruce *et al.*, 1994; Hill *et al.*, 1997) and macaque monkey (Lasmezas *et al.*, 1996) confirmed the indications that vCJD was caused by transmission of bovine prions.

In March 2011, variant CJD cases have been reported from the following countries: 171 from the United Kingdom, 25 from France, 5 for Spain, 4 from Ireland, 3 each from the

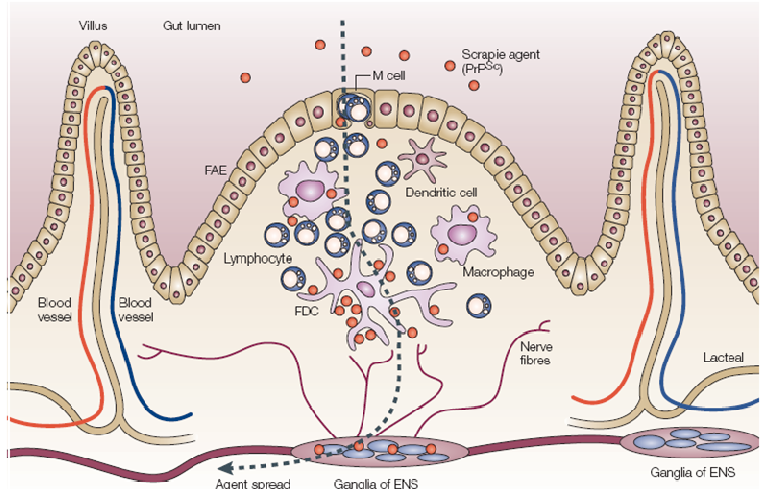


Figure 13→ Possible spread of scrapie infectivity from the gut lumen to the nervous system following oral infection (route indicated by dotted line). Soon after ingestion, the abnormal prion isoform (PrP^{Sc}) is detected readily within Peyer's patches on follicular dendritic cells (FDCs), within macrophages, within cells with morphology consistent with that of M cells and within ganglia of the enteric nervous system (ENS). These observations indicate that, following uptake of scrapie infectivity from the gut lumen, infectivity accumulates on FDCs in Peyer's patches and subsequently spreads via the ENS to the central nervous system. FAE, follicle-associated epithelium. (from Cashman and Caughey, 2004)

United States and Netherlands, 2 each from Italy, Protugal, Canada, and one each from Saudi Arabia, Japan, and Taiwan (reported from the Centre for disease control and prevention (CDC)). There has never been a case of vCJD that did not have a history of exposure within a country where the cattle disease, BSE, was occurring. Estimation of a possible future vCJD epidemic is difficult. Reports and statistics presume that the number of individuals developing vCJD in the next years range from hundreds to thousands (Donnelly *et al.*, 2002; Ghani *et al.*, 2002).

1.1.7 Mechanisms of neurodegeneration in prion diseases

In prion diseases, there is a long incubation time between challenge with the prion disease agent and the onset of symptoms. In the case of BSE this can be years, in human prion disease decades. Following onset of symptoms death from complications follows very rapidly. In humans with CJD this can be a matter of months.

All prion diseases are neurodegenerative conditions. They result in a significant loss of neurons in specific areas of the brain followed by a spongiform decay of these areas. The neuronal loss occurs late in the disease and precedes onset of neurological and behavioural symptoms.

Clinical symptoms of human TSEs vary, but they commonly include personality changes, psychiatric problems such as depression, lack of coordination, and/or an unsteady gait.

Patients also may experience involuntary jerking movements called myoclonus, unusual sensations, insomnia, confusion, or memory problems. In the later stages of the disease, patients have severe mental impairment and lose the ability to eat, move or speak.

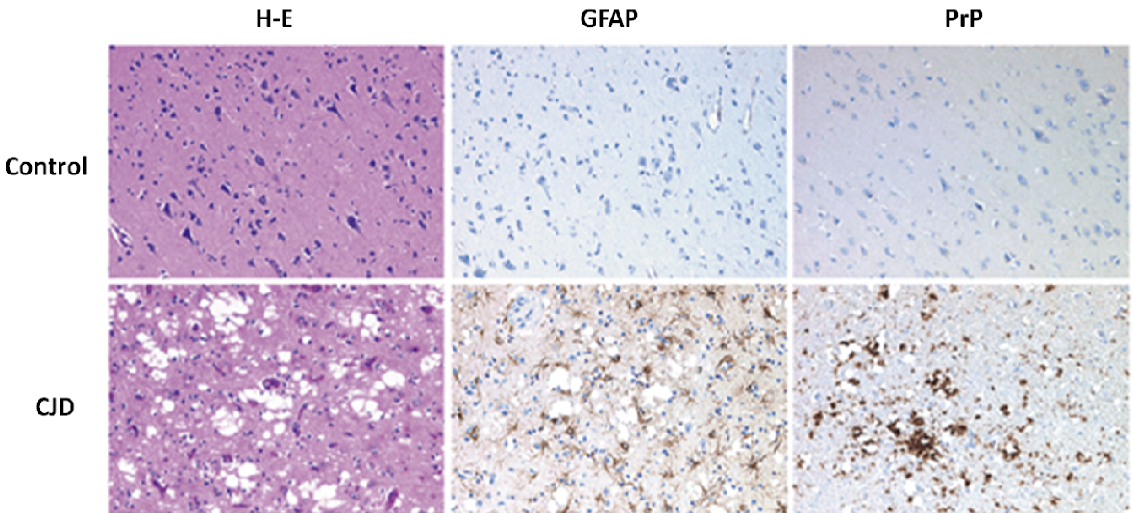


Figure 14→ Neuropathological features of transmissible spongiform encephalopathies. Histological and immunohistochemical analysis of frontal cortex samples from the brain of a patient who died of noncerebral causes (upper row) and of a patient suffering from Creutzfeldt–Jakob disease (CJD; lower row). Brain sections were stained with haematoxylin-eosin (H-E, left panels), with antibodies against glial fibrillary acidic protein (GFAP, middle panels) and with antibodies against the prion protein (PrP, right panels). In the H-E stain neuronal loss and prominent spongiosis are visible. Strong proliferation of reactive astrocytes (gliosis) and perivacuolar prion protein deposits are detectable in the GFAP and PrP immunostains of the CJD brain samples. (From Aguzzi *et al*., 2001a)

The brains of diseased individuals are highly abnormal and share the following histopathological hallmarks: spongiform vacuolation, severe neuronal loss, strong astrogliosis, mild microglia activation, and abnormal deposition of a misfolded β-sheet rich protein, called amyloid (DeArmond and Prusiner 1995; Prusiner 1998; Aguzzi and Polymenidou 2004; Weissmann 2004; Collinge 2005) (→Figure 14). In addition, amyloid plaques (consisting of the ordered proteinaceous deposits with high β-sheet content) characterize affected brains (Roberts & Wickner, 1992, Clinton *et al*. 1992; Bessen *et al*. 1997), though cases of TSEs lacking such plaques have been reported (Collinge *et al*. 1995a; Tateishi *et al*. 1995).

Prion pathology shares several profound similarities with other protein misfolding and neurodegenerative diseases like Alzheimer's, Huntington's and Parkinson's disease (Aguzzi and Haass 2003; Chiti and Dobson 2006). Nevertheless, prions are unique as they are not only able to replicate their conformation but are also naturally and experimentally transmissible within and to some extend between species (Weissmann *et al*.1996; Prusiner 1998).

Although it is known that transmission of prion diseases is mediated by prions which mainly consist of the pathological prion protein isoform PrP^{Sc} that accumulates in the brain, the mechanisms that lead to neuronal cell death, remain undetermined. Usually, PrP^{Sc} is equated with neurotoxicity, but the amount of PrP^{Sc} detected in brains of affected individuals did not necessarily correlate with the degree of neurodegeneration. Application of PrP^{Sc} into brains of $PrP^{0/0}$ mice was not able to induce neuronal cell death despite high amounts of inoculated PrP^{Sc} (Brandner *et al.*, 1996), which indicates that PrP^{c} is required for transmission of a neurotoxic signal or that prion conversion has to occur in order to produce toxic intermediates. In line with these data, it was observed, that postnatal depletion of neuronal PrP^{c} in prion-infected mice was able to prevent disease and revert neurodegeneration although in these mice PrP^{Sc} was present in high amounts within the brains and possibly was propagated by non-neuronal cell types (Mallucci *et al.*, 2003). Interestingly, upon expression of a secreted form of PrP^{c} lacking the GPI-anchoring signal deposition of high amounts of amyloid plaques was discovered upon inoculation with prions. Despite this, clinical manifestations were minimal but disease could be accelerated by coexpression of wild-type PrP^{c} (Chesebro *et al.*, 2005). It was also confirmed that small PrP^{Sc} oligomers that might be formed during the conversion process were more toxic to various cell types than amyloid fibrils composed of PrP^{Sc} (Novitskaya *et al.*, 2006, Simoneau *et al*, 2007). Such oligomers also seem to represent the most infectious unit in prion diseases, at least for the scrapie strain RML (Silveira *et al.*, 2005). All these data mentioned above point out that PrP^{Sc} is not necessarily neurotoxic. Critical factors for neurodegeneration appear to be the expression of PrP^{c} in neurons and probably the neuron-associated *de novo* conversion of PrP^{c} into PrP^{Sc}.

In addition, the innate immune system, mainly represented by microglial cells within the brain, might have a fundamental role in neurodegeneration observed in prion diseases. Also, release of reactive oxygen species (ROS) or pro-inflammatory cytokines may contribute to neuronal damage (Brown, 2001; Perry, 2004). However, also the role of microglia in neurodegeneration is controversial since these cells have the capacity to degrade PrP^{Sc}. All in all, these conflicting data do not suggest a clear mechanism how neurodegeneration occurs.

1.2 Therapeutic and prophylactic approaches

1.2.1 Therapeutic strategies for protein aggregation diseases

Protein misfolding and aggregation has been related to several human disorders, generally termed protein aggregation diseases. These diseases include neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's disease and peripheral disorders such as systemic amyloidosis and type 2 diabetes. Inappropriate aggregation of proteins is normally prevented by complex cellular quality control mechanisms. However, under certain circumstances, an unusual subset of proteins is able to aggregate within or around cells. Although the amino-acid sequences of this class of proteins are diverse, they all seem to adopt a similar, insoluble, highly ordered structure when aggregated known as the cross-β spine termed amyloid (Sawaya *et al.*, 2007; Virchow, 1853).

Nevertheless, in-depth research into the aggregation processes has recently yielded major insights into some key mechanisms of aggregation-mediated cell toxicity, cellular trafficking and degradation mechanisms offering new targets for drug development. In addition, recent findings in the field have identified similar features, revealing the possibility of shared mechanisms and hence potential common approaches for intervention.

On consideration of the aggregation process in principle, all the key steps in pathways of formation of pathological species can be considered good targets for blocking disease onset and/or progression (→Figure 15). Nevertheless, no specific target molecule that, once inhibited, will quantitatively block the progression of protein aggregation and no effective disease modifying treatments have yet been identified for any aggregation disease. The difficulties in effectively overcoming protein aggregation might be accounted for by the following factors: 1) complexity of the molecular mechanisms for protein aggregation 2) few promoting agents (either exogenous or endogenous) or events have been identified, 3) enzymes and chaperones involved in disease onset also have physiological roles, precluding their inhibition, 4) more than one single specific event could be involved in the initial and subsequent steps, and 5) few specific *in vitro* and *in vivo* assays suitable for mimicking the complex human pathological scenario and for monitoring the formation of different prefibrillar species are currently available (Bartolini and Andrisano, 2010).

Aggregation process	Therapeutic strategies	Potential application
Protein production	inhibit (anomalous) protein production or accumulation	AD, taupathies, polyQ diseases, amyloidosis, Hemodialysis-related disorders
	enhance alternative processing of protein precursors	AD, amyloidosis
Protein misfolding	inhibit conformational shift	AD, polyQ diseases, PD, type 2 diabetes, prion diseases
	inhibit inducing factors	AD, taupoathies
	enhance chaperone molecules	AD, taupoathies
Protein oligomerization (soluble forms)	inhibit oligomer elongation over accelerate fibril formation	AD, PD, type 2 diabetes, amyloidosis
	enhance clearance mechanisms	AD, prion diseases, amyloidosis
Ordered fibrils formation And deposition	activate/inhibit modulating factors	Taupoathies, amyloidosis
Amorphous aggregates Deposition	enhance deposit clearance	AD, polyQ diseases, taupoathies, prion diseases. ALS, amyloidosis
Cell toxicity	inhibit factors mediating cell toxicity or restoring cell damage	AD, polyQ diseases, taupoathies, ALS

Figure 15→ Therapeutic strategies and applications to interfere with protein aggregation

However, as a result of growing efforts to understand protein aggregation processes, related toxic events and involved cellular pathways, new potential targets have been discovered and characterized and several different strategies and new approaches are in discussion.

A growing number of reports have suggested that in most aggregation diseases a conformational transition from a soluble native conformer to β-sheet-rich oligomeric structures is the key event in the aggregation and fibrillation process. Misfolding has been suggested for prion protein ($PrP^{c} \rightarrow PrP^{Sc}$) (Horwich and Weissman, 1997; Caughey, 2001; Moore *et al.*, 2009) a-synuclein, (Uversky and Eliezer, 2009) polyQ proteins (Nagai and Popiel, 2008), Ab peptides, (Glabe, 2005; Flçck *et al.*, 2006) and amyloid-like proteins (Glabe, 2006; Thompson and Barrow; 2002). *Inhibition of the conformational shift* is a very promising strategy for blocking the aggregation process because it is the earliest event in the toxic cascade (Soto *et al.*, 2000; Gendron and Petrucelli, 2009; Chini *et al.*, 2009)

Inhibition of the accumulation of peptide/protein monomer and oligomer levels are a considered approach, as increase in template protein is a widely accepted riskfactor for the development of aggregation diseases (Postina *et al.*, 2008; Ghosh *et al.*, 2008; Henley *et al.*, 2009; Golde *et al.*, 2009; Simons *et al.*,2002; Bar-On *et al.*, 2008; Schneider and

Mandelkow, 2008; Selenica *et al.*, 2007; Hanger *et al.*, 2009; Liu *et al.*,2004; Sun *et al.*,2003.

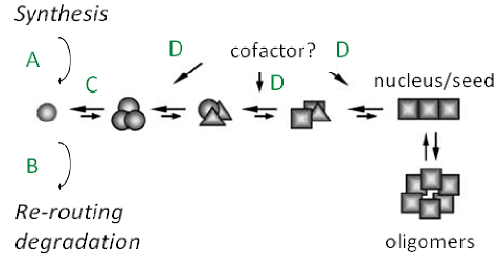
A major strategy against accumulation of pathological aggregates is *promotion of aggregate clearance*. This can be approached in different ways. One of them is regulation of cellular degradation pathways such as the ubiquitin-proteasome system and autophagy (Ravikumar *et al.*, 2002, Webb *et al.*, 2003; Nixon, 2006). Other ways to boost cellular aggregate clearance can be the activation of specific proteases or clearing enzymes (Bhutani *et al.*, 2007; Jacobsen *et al.*, 2008), inhibition of pathological chaperones, anti-immunotherapeutic approaches (Schenk *et al.*, 1999; Alexandrenne *et al.*, 2009). Moreover, *inhibition of aggregate-mediated cell toxicity* is also taken into consideration

1.2.2 Current knowledge of anti-prion therapeutics

The recognition of the novel human prion disease, variant CJD (vCJD), from the mid-1990s onward and the experimental confirmation that it is caused by the same prion strain as BSE raised major public-health concerns (Collinge, 1999). Prion infection is associated with prolonged, clinically silent incubation periods, which in humans can exceed 50 years, and secondary transmission of vCJD by blood transfusion appears to be efficient (Wroe *et al.*, 2006). In addition to ways of human TSE transmission described in →1.1.6, experimental airborne prion transmission was recently reported (Hayback *et al.*, 2011), which suggests a novel risk factor in cases of prion epidemiology. Although the number of human cases to date (around 220) has been relatively modest, key uncertainties, notably with respect to genetic effects on incubation period, allied with the widespread population exposure, suggest the need for caution and for an effective ant-prion therapy.

In recent years, a proliferation of research into the understanding and identification of therapeutics against prion diseases was initiated. *In vitro* and animal model studies have suggested a number of categories of drugs and biochemical tools as candidates for treatment, such as chemical compounds, nucleic acids and peptides, anti-PrP antibodies and antibody fragments and vaccination approaches (MacLeod *et al.*, 2003, Krammer *et al.*, 2009b). In →1.2.3 strategies for identification of anti-prion compound are described.

The prion conversion and accumulation process provides several targets for blocking disease onset and/or progression which are possible intervention points for drug application (→Figure 16). Possible points of interference are:



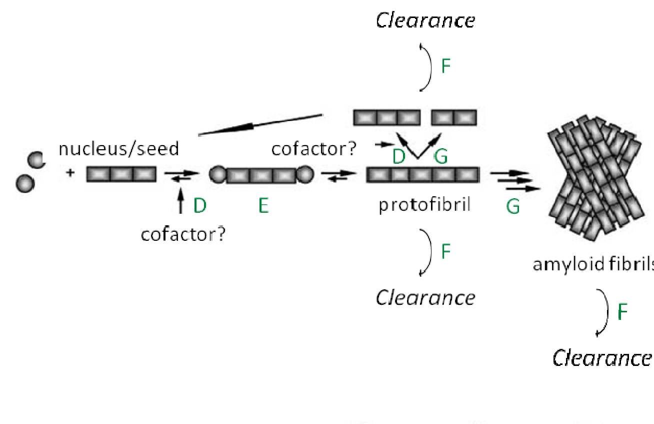


Figure 16. Putative intervention possibilities. Possible points of interference are blocking of PrP^c synthesis (**A**), intracellular rerouting or retention of PrP^c (**B**), overstabilization of PrP^c (**C**), preventing the binding of putative auxiliary factors postulated for prion conversion (**D**), interference with interaction of PrP^c and Pr PrP^{Sc} (**E**), increasing the cellular clearance for PrP^{Sc} (**F**), or sequestering incoming and nascent PrP^{Sc} (**G**). Additive or even synergistic effects might be obtained by rational combinations of targets (from Gilch *et al.*, 2008).

1) *Blocking of PrP^c synthesis.* One strategy to achieve related effects is the design of highly specific small interfering RNAs (siRNA) targeting PrP mRNA. Such siRNA has been successfully used to knock down PrP^c expression (Tilly *et al.*, 2003; Daude *et al.*, 2003) and to abrogate PrP^{Sc} accumulation in cell culture (Daude *et al.*, 2003). One advantage of this strategy is that antiprion effects are completely independent of the prion strain used for infection, which may not be the case when using molecules that target PrP^{Sc} or PrP^c – PrP^{Sc} interactions. However, in cell culture knock-down approaches for PrP^c were only transient, and for transfection of non-dividing cells like neurons methods have to be applied which allow efficient gene transfer. *In vivo* studies addressing siRNA approaches provided proof of principle (Pfeifer *et al.*, 2006; White *et al.,* 2008), however, the transgene delivery method is still highly artificial, and to make it applicable in

humans methods more practicable for vector transfer than those described have to be evaluated.

2) *Intracellular rerouting and inhibition of cell surface localization of PrP^c*, for example via suramin. The naphthylurea compound suramin, developed for the treatment of trypanosomiasis in humans, binds to PrP^c and induces its aggregation in the secretory pathway. PrP^c then bypasses the plasma membrane by rerouting to lysosomes, making it inaccessible for prion conversion (Gilch *et al*., 2001). Prolongation of incubation times in mice and hamsters upon peripheral prion infection were observed (Ladogana *et al*., 1992; Gilch *et al*., 2001).

3) *Overstabilisation of PrP^c or even PrP^{Sc}* and thereby impeding the nucleation process. Congo red is, like suramin, a polyanionic compound and a widely used dye for staining of amyloid deposits and is suggested to inhibit prion propagation by overstabilizing the conformation of PrP^{Sc} molecules (Caspi *et al*., 1998). Reduction of PrP^{Sc} in chronically infected cells and in *in vitro* conversion assays has been repeatedly reported (Caughey and Race, 1992; Caughy at al., 1993). *In vivo* studies in hamsters revealed a delay in onset of disease in intraperitoneally (i.p.) inoculated animals (Ingrosso *et al*., 1995; Poli *et al*., 2004) but the progression of disease was unaltered (Ingrosso *et al*., 1995). Another recent study was performed using RNA aptamers which recognize a peptide comprising amino acid residues of the human prion protein with high specificity. This domain of prion proteins is thought to be functionally important for the conversion of PrP^c into its pathogenic isoform PrP^{Sc} and is highly homologous among prion proteins of various species, including mouse, hamster, and man and were able to reduce levels of PrP^{Sc} (Proske *et al*., 2002). A further study showed that also peptide aptamers were able to interfere with endogenous PrP^{Sc} conversion upon expression in prion-infected cells (Gilch *et al*., 2007a; Gilch and Schätzl, 2009).

4) *The steric hindrance of the PrP^c – PrP^{Sc} interaction* and consequently an impaired conversion can for instance be achieved by incubation of prion-infected cell cultures with antibodies specific to PrP^c.

For this purpose, monoclonal antibodies (Peretz *et al*., 2001; Enari *et al*., 2001; Pankiewicz *et al*., 2006; Feraudet *et al*., 2005; Perrier *et al*., 2004) , mouse polyclonal antibodies elicited by auto-immunisation (Gilch *et al*., 2003; Oboznaya *et al*., 2007) targeting PrP^c , or antibodies recognising additionally PrP^{Sc} (Beringue *et al*., 2004) were used.

Table 4. Possible interventional approaches to prion diseases can be classified as curative, palliative and prophylactic (either pre-exposure or postexposure). (Adapted from Aguzzi *et al*., 2001b)

Therapeutic strategy	Target of intervention	Possible technology
Curative therapy	Eradication of prion infection; restoration of impaired function	Elimination of newly formed and residual PrP^{Sc} replacement of damaged cells
Palliative and life-prolonging therapies	Prion replication	Substances that interfere with PrP^{SC} build-up
		Partial suppression of PrP^{C} expression
	Neuroprotection	Inhibition of microgial and astrocytic reaction; interference with synaptic and neuronal loss
Post-exposure prophylaxis	Lymphoreticular organs	Depletion of follicular dendritic cells
		Depletion of B lymphocytes
		Depletion of complement factors
	Sympathetic nervous system	Immunological or chemical sympathectomy
Pre-exposure prophylaxis	Immune system	Induction of active immunity
		Passive antibody transfer

5) *Interference with cofactors postulated in the conversion process*. Sulfated glycans like pentosan polysulfate (PPS) or dextran sulfate 500 (DS500) interfere presumably with binding of PrP to glucosaminoglycans, known to act as co-receptors for prion protein and/or stimulate endocytosis of PrP^{c} (Shyng *et al*., 1995; Horonchik *et al*., 2005; Hijazi *et al*., 2005). Both PPS and DS500 prolong the incubation time of prion disease in mice or hamsters, depending on prion strain, inoculation route, and time point of drug administration (Diringer and Ehlers, 1991; Farquhar and Dickinson, 1986; Ladogana *et al*., 1992).

6) *Increasing the cellular degradation of prions*. A study performed with imatinib (STI571, Gleevec) (Ertmer *et al*., 2004) opened the door for a novel class of antiprion compounds, namely inhibitors of signal transduction. For example imatinib impedes the tyrosine kinase c-abl and can lead to induction of autophagy (Ertmer *et al*., 2007).

7) *Absorption of PrP^{Sc} in the periphery* is a further approach studied. Certain cyclic tetrapyrroles such as phthalocyanines (PcTS) have been shown to inhibit the *in vitro* formation of PrP^{Sc} and treatment of TSE-infected animals showed increased survival times. Mechanistically, it is suggested that PcTS, interacts directly with the infectious

agent in peripheral tissues to slow disease onset (Priola *et al.*, 2000; Priola *et al.*, 2003). Additive or even synergistic effects might be obtained by rational combinations of targets.

Clinically, possible interventional approaches to prion diseases can be classified as curative, palliative and prophylactic, either pre-exposure or postexposure (→Table 1).

1.2.3 Strategies for identification of anti-prion compounds

In the past years numerous efforts aimed at the identification of compounds useful against prion diseases and a variety of test systems have been established. The most commonly used model is to select novel drugs by treatment of persistently prion-infected cells and subsequent analysis of the amount of PrP^{Sc}, which serves as a surrogate marker for prion infectivity. The advantage of this system is that it is the most physiological compared to other systems, such as high-throughput screen utilizing scanning for intensively fluorescent targets (SIFT) (Bertsch *et al.*, 2005), semiautomated assays that monitored the accumulation of amyloid recombinant PrP by thioflavin T staining (Breydo *et al.*, 2005) and others (Legname *et al.*, 2004; Riesner, 2003).

In a well established cell culture model, cellular requirements for prion conversion, in addition to the physical interaction between both PrP isoforms, are considered. These include, for example, the proper subcellular localisation and turn-over of PrP^{c} as well as the degradation kinetics of PrP^{Sc}. With this system, library screening in a 96-well format is possible if PrP^{Sc} amounts are measured e.g., by dot blot analysis (Kocisko *et al.*, 2003). Different cell lines infected with various prion strains have been used (Kocisko *et al.*, 2003; 2006). A new and more economic screening method was established by Bach and colleagues (Bach *et al.*, 2003) by using budding yeast to screen for antiprion compounds. This also indicates that the mechanism of prion propagation is at least to some extent conserved from yeast to humans. However, antiprion activity in persistently infected cell cultures does not necessarily ensure benefits in an *in vivo* situation, mainly due to inadequate bioavailability or toxicity of the drugs and insufficient availability within the central nervous system. Very recently, a cell culture system has been generated employing primary cerebellar granular neurons derived from transgenic mice overexpressing PrP^{c} of different origin, including human PrP (Cronier *et al.*, 2007). In this study the first system useful for testing substances for inhibition of biosynthesis of human CJD prions was described. With regard to anti-prion activity of substances tested with these cells, results might be more closely related to the *in vivo* situation than upon

testing in established cell lines like the neuroblastoma line ScN2a. Nevertheless, any compound selected by cell culture screens needs to be further evaluated in bioassays (Kocisko *et al.*, 2006; Larramendy-Gozal *et al.*, 2007) . Here, the readout is usually incubation time to prion disease. However, the activity of drugs depends on parameters like route of prion inoculation, prion strain or timing and duration of drug administration (→Figure 17).

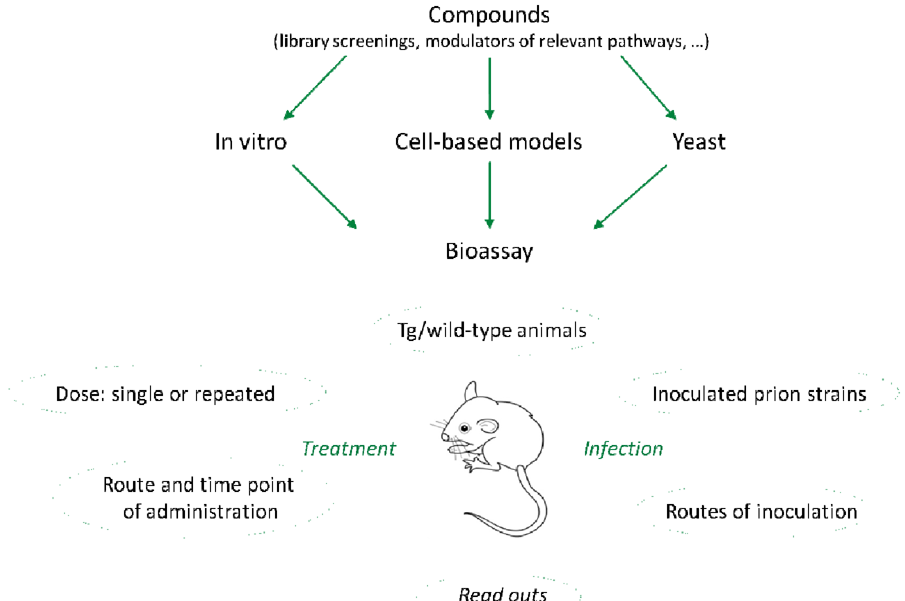


Figure 17. Screening strategies for anti-prion compounds. There are several ways to identify novel anti-prion agents. The effects of identified substances need to be evaluated in bioassays. The outcome of bioassays is significantly influenced by numerous experimental parameters. Tg: transgenic. (adapted from Gilch *et al.*, 2008)

Although the exact mechanism of prion conversion has not been elucidated to date, the current models offer several points of intervention in this process (→1.2.2). It can be distinguished between strategies targeting *de novo* synthesis and degradation of PrP^{Sc}, respectively. One of the tools successfully used for this purpose, the possibility to express 3F4-tagged PrP^{c} and to monitor specifically its de novo conversion (Vorberg *et al.*, 2004a, b).

A number of drugs have been isolated as active against mammalian prion (Trevitt and Collinge, 2006). For most of these molecules, the mode of action and targets remain largely unknown and are a subject of research. In addition to their direct interest as potential therapeutic agents, these molecules could be used as original research tools to understand prion propagation (Tribouillard *et al.*, 2007). Global genetic and/or biochemical approaches aim to identify the intracellular target(s) and mechanism of action of the drugs. Once those are known, the biological activity of the compounds can be optimized on a rational basis, their potential side effects understood and minimized.

1.3 Autophagy

Degradation of organelles or cytoplasmic proteins can be mediated by an intracellular bulk degradation process called macroautophagy (referred to hereafter as autophagy). One can imagine autophagy, or cellular self-digestion, in its simplest form as single cell's adaptation to starvation: if there is a lack of nutrition in the surroundings, a cell is forced to break down parts of its own reserves to stay alive until the situation improves. During autophagy, portions of the cytosol are engulfed by a membrane sac resulting in a double-membrane vesicle, called autophagosome/ autophagic vacuole, which delivers cytoplasmic cargo to lysosomes (→Figure 18). After fusion with lysosomes, the protein and organelle contents of the autophagosome are degraded by acidic lysosomal hydrolases and recycled (Klionsky and Ohsumi, 1999; Yoshimori, 2004). In single-cell organisms such as yeasts, this starvation response is one of the primary functions of autophagy, but in fact this role extends up to humans. For example, even on a day-to-day basis, autophagy is activated between meals in organs such as the liver to maintain its metabolic functions, supplying amino acids and energy through catabolism (Kuma *et al.*, 2004; Mizushima and Klionsky, 2007).

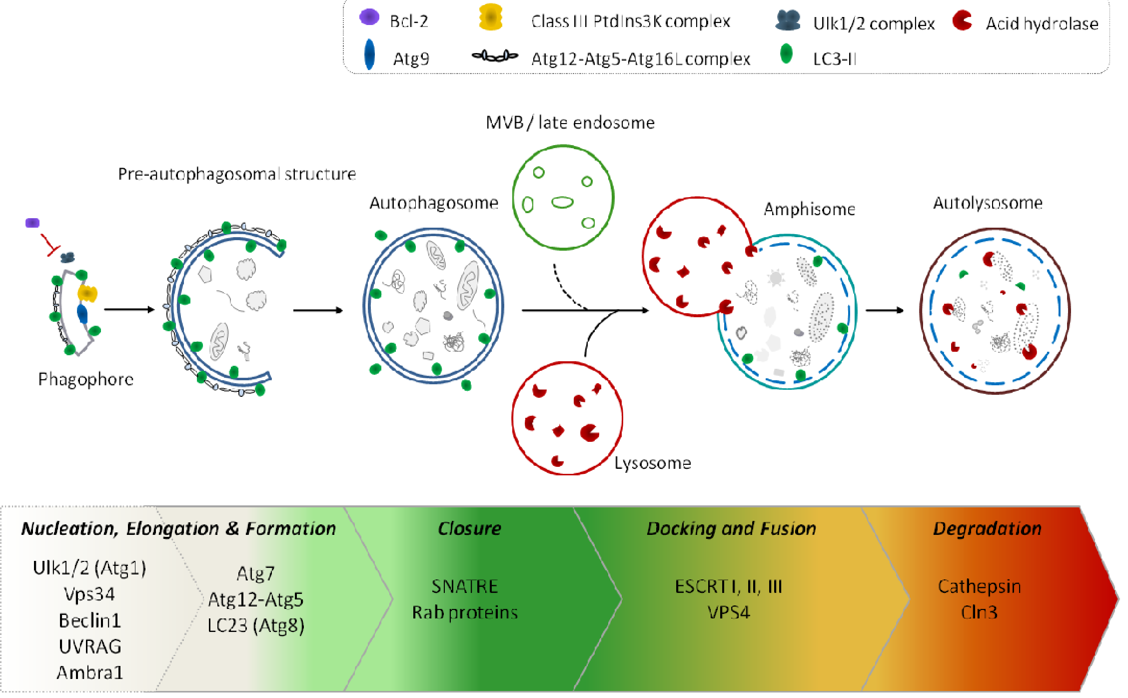


Figure 18. Schematic representation of main cellular steps in macroautophagy. Autophagosome nucleation starts with an isolation membrane in the cytosol which matures to an autophagosome vesicle. By docking and fusion to lysosomes the content of this autophagolysosome gets access to the lysosomal degradation machinery.

1.3.1 The autophagy process: Types and signalling

In most mammalian cells, autophagy occurs by three different means that distinguish the subtypes of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) due to their characteristics, regulation and main molecular components (Klionsky, 2007; Mizushima *et al.*, 2008; He & Klionsky, 2009) (→Figure 19). Briefly, macroautophagy and microautophagy involve the direct sequestration of whole areas of the cytosol by invaginations at the lysosomal membrane (in the case of microautophagy), or by a membrane that seals to form a double-membraned vesicle, or autophagosome (in macroautophagy). Microautophagic vesicles at the lysosomal membrane 'pinch off' into the lysosomal lumen, and cargo is degraded by the lysosomal hydrolases upon digestion of the vesicles' limiting membrane (Mortimore, 1989). In the case of macroautophagy, fusion between autophagosomes and lysosomes mediates the delivery of the autophagic cargo into the lysosomal lumen (Mizushima *et al.*, 2008; He & Klionsky, 2009). In the third common type of autophagy, CMA, cargo is not sequestered but is instead selectively recognized by a complex of cytosolic chaperones that mediate its delivery to a receptor at the lysosomal membrane (Dice, 2007; Cuervo, 2010).

In addition, there are also other specialized forms of autophagy for partial sequestration and degradation of the nucleus (Roberts *et al.* 2003), pexophagy for selective degradation of peroxisomes (Dunn *et al.*, 2005), xenophagy for degradation of intracellular bacteria and viruses (Levine, 2005), reticulophagy for selective autophagy of the ER (Bernales *et al.*, 2007), mitophagy for direct targeting of mitochondria to lysosomes (Kanki and Klionsky, 2008) and macrolipophagy for regulation of cellular lipid content by autophagy (Singh *et al.*, 2009). In this thesis the main focus is macroautophagy; this is why it will be referred to as autophagy.

Autophagy is conserved among eukaryotes and has been characterized from yeast to man (Reggiori and Klionsky, 2002). Basal levels of autophagy are important for maintaining normal cellular homeostasis. As the autophagic process has the capacity for large scale degradation, unregulated degradation of the cytoplasm is likely to be deleterious. Thus, a tight cellular regulation of the autophagic process is important so that it is induced when needed, but otherwise maintained at a basal non-deleterious level. Several protein kinases regulate autophagy contributing to a complex molecular machinery. The best characterized is the mammalian target of rapamycin, mTOR, which is the major inhibitory signal that shuts off autophagy in the presence of growth factors and nutrients

(Kamada *et al.*, 2000). Downstream of mTOR, numerous proteins encoded by *Atg* genes are essential for the execution of autophagy (Levine and Klionsky, 2004). Some of the other regulatory molecules that control autophagy include 5′-AMP-activated protein kinase (AMPK), BH3-only proteins, the inositol 1,4,5-trisphosphate receptor (IP3R), Erk1/2 and calcium (Criollo *et al.*, 2007; Maiuri *et al.*, 2007; Meijer and Codogno, 2006; Rubinsztein *et al.*, 2007). Autophagy can also be pharmacologically induced by inhibiting negative regulators such as mTOR via the compound rapamycin (Rubinsztein *et al.*, 2007) or by mTOR-independent inducers of autophagy such as trehalose and lithium (Sarkar *et al.*, 2007a; Sarkar *et al.*, 2005). Pharmacological inhibitors of autophagy are for instance 3-methyladenine (3-MA), Wortmannin and LY294002 (Blommaart *et al.*, 1997; Seglen and Gordon, 1982).

Although a complete picture of autophagy regulation is not yet available, breakthroughs in yeast genetics and analysis of mammalian homologues of the Autophagy-related (Atg) proteins identified in yeast (Harding *et al.*, 1995; Suzuki and Ohsumi, 2007; Tsukada and Ohsumi, 1993) have greatly improved the understanding of autophagy and its regulation. Several aspects of regulation mechanisms have recently been reviewed in great detail (Botti *et al.*, 2006; Gozuacik and Kimchi, 2007; Meijer and Codogno, 2006; Yorimitsu and Klionsky, 2007).

Autophagy generally undergoes four processes: 1) formation of autophagosomes (induction, nucleation, cargo recognition, elongation and completion of the autophagosomes), 2) maturation and closure 3) docking and fusion of autophagosomes with lysosomes, and 4) degradation of the cargo in autolysosomes (Klionsky, 2007). Several autophagy-related genes involved in these processes have been characterized in yeast and mammals and identified as highly conserved (Kroemer and Levine, 2007; Levine and Klionsky, 2004).

Autophagy is either constitutively active at basal levels or upregulated under certain conditions such as starvation, development or stress. It has been shown that autophagy is induced by mTOR-dependent or mTOR-independent pathways (Scarlatti *et al.*, 2009; Rubinsztein *et al.*, 2009), orchestrated by the molecular machinery mentioned above. Once autophagy is induced, isolation membranes (also called phagophores) are formed (vesicle nucleation) that initially sequester organelles. These become the autophagosomes, which mature into autolysosomes by fusing with endosomes or

lysosomes. Finally, cytosolic components sequestered by these autolysosomes are degraded (→Figure 18). During

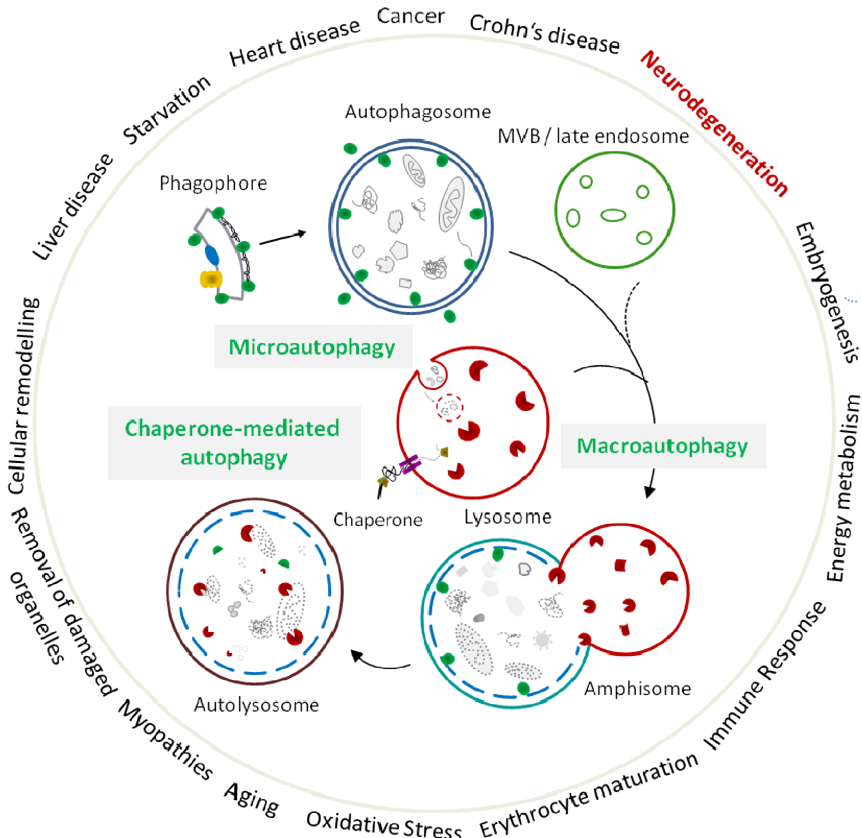


Figure 19. Different types of autophagy: chaperone-mediated autophagy (cytosolic chaperones target proteins destined for degradation to the lysosome), microautophagy (lysosome deforms and engulfs cytosol for degradation), and macroautophagy (autophagosomes containing substrate for degradation fuse with lysosomes) play a role in health and disease.

vesicle nucleation, Ulk1/2 (Yeast Atg1), a serine/threonine kinase, works cooperatively with Vps34/PI3K complexes, which are composed of Beclin1, ambra1 and UVRAG (Wand and Klinonsky, 2003; Klionsky, 2007). The other core molecular machinery is comprised of two ubiquitin-like conjugation systems that mediate elongation of pre-autophagomal structure: One is the conjugation of atg5 with atg12 and the other is the covalent linkage of atg8 to phosphatidylethanolamine (PE) (→Figure 18). In particular, LC3 (Microtubule-associated Light Chain1), a mammalian homolog of yeast *atg8*, is used as a marker of autophagosomes based on the fact its lipidated form is specifically localized to autophagosomes. After autophagosomes are formed they undergo a maturation step by fusing with endosomes or multivesicular bodies (Kovacs *et al.*, 1998), followed by another fusion event with lysosomes to become autolysosomes. SNARE, Rab proteins, ESCRT complexes, Vps4 and Fab1 mediate these fusion events (Eskelinen, 2008). Finally, autolysosomal degradation of cytosolic proteins and organelles is

mediated by lyosomal hydrolases. Therefore, each step in the autophagy pathway is tightly regulated and crucial for efficient execution; any disruption can impair autophagy and lead to disease.

1.3.2 Role of autophagy in health and disease - with a focus on CNS and neurodegenerative disorders

The pleiotropy of autophagy is striking. Beyond its classical role in nutrient supply under starvation and turnover of organelles and proteins, autophagy contributes to various physiological processes such as intracellular cleansing, differentiation, development, longevity, elimination of invading pathogens and antigen transport to the innate and adaptive immune systems or counteracting endoplasmic reticulum stress and diseases characterized by the accumulation of protein aggregates (Levine and Kroemer, 2008; Levine and Yuan, 2005; Lum *et al.*, 2005; Maiuri *et al.*, 2007; Mizushima *et al.*, 2008; Yorimitsu and Klionsky, 2007). However, in the context of cancer, potentially this pro-survival function seems to be maladaptive (Mathew *et al.*, 2007). This takes one to the other face of autophagy and its connections to pathophysiology and disease. Besides cancer, autophagy plays a role in a number of infectious and inflammatory diseases and in protein ‘unfolding and misfolding’ diseases that lead to neuronal, muscle and liver degeneration or heart failure (reviewed in Levine and Deretic, 2007; Levine and Kroemer, 2008; Mizushima *et al.*, 2008) (→Figure 19).

With respect to the importance of tight regulation of autophagy, perhaps the most fundamental point is that either too little or too much autophagy can be deleterious, a complex balance resulting in its dual role in survival and adaptation or cell death. However, in response to most forms of cellular stress, autophagy plays a cytoprotective role, because *Atg* gene knockdown/knockout accelerates rather than delays cell death (Levine and Yuan, 2005; Maiuri *et al.*, 2007). Within the cell death research field, autophagy has long been defined as a form of non-apoptotic, or type II, programmed cell death (Clarke, 1990; Kovacs *et al.* 1986). However, due to the recent findings a consensus is emerging that autophagy might be a cell death impostor which, in reality, functions primarily to promote cellular and organismal health (Kroemer and Levine, 2008).

The CNS and neurodegenerative diseases provide an interesting example for autophagy and its possible dual roles. Cellular quality control through autophagy is particularly relevant in neurons, where the total content of altered proteins and damaged organelles

cannot be reduced by redistribution to daughter cells by means of cell division. Neuronal surveillance mechanisms must identify these malfunctioning structures and assure their autophagic degradation before their intracellular buildup gives rise to neurotoxicity (Nixon *et al.*, 2008; Pickford *et al.*, 2008) (→Figure 20). Delivery of autophagic subcellular components to the damaged structures must accommodate the unique neuronal architecture, whereby the cytoplasm can extend long distances through the many projections from the cellular body and accommodate the dynamic traffic to and from polarized neuronal projections. Besides neuronal homeostasis, autophagy is also used for the continuous remodeling of neuronal terminals that is required to support neuronal plasticity (Wang *et al.*, 2006; Komatsu *et al.*, 2007b; Fimia *et al.*, 2007). Neuronal autophagy in axonal terminals may play an important role in the homeostasis of synaptic vesicles or membrane-bound structures abundant in axons. In particular, synaptic vesicles are interesting candidate organelles because they are highly regulated in axonal terminals by synaptic activity. Autophagy in axonal terminals may also be involved in growth cone remodeling during axonal development or regenerative processes after injury (Lee, 2009). On the basis of these prior observations, it is not surprising that alterations in the autophagic system would be intimately linked to different neuronal diseases.

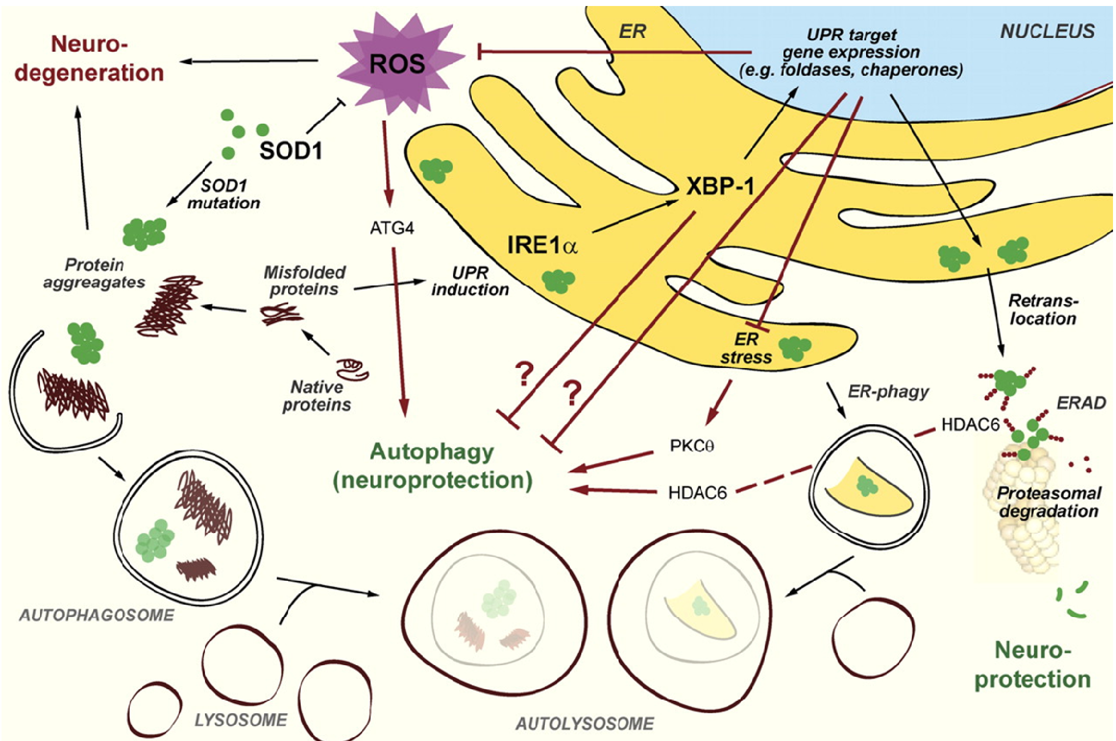


Figure 20. Schematic view of autophagy and UPR interactions with relevance to neuroprotection. Unfolded or misfolded protein aggregates accumulate during neurodegenerative diseases within the cytosol or the ER of neurons. Several cellular defense mechanisms can be activated in order to resolve protein aggregates, thus serving as neuroprotective processes. This applies to the UPR, the ERAD pathway, and the lysosomal degradation pathway or autophagy. However, the exact mechanism of how autophagy interacts

with the other pathways remains to be investigated and might involve several nonexclusive ways of interaction, illustrated by red colored arrows. Question marks indicate hypothetical interrelations. (Madeo *et al*., 2009)

As already mentioned, the importance of basal autophagy in intracellular quality control is tissue-specific. In liver and other tissues where cells, such as neurons and myocytes, do not divide after differentiation basal autophagy is of great relevance (Hara *et al*., 2006; Komatsu *et al*., 2006; Komatsu *et al*., 2005; Komatsu *et al*., 2007a; Nakai *et al*., 2007). Despite the important function of basal autophagy in healthy individuals, the requirement for autophagy is even more evident under disease conditions and levels of autophagosomes can be dramatically increased in injured neurons and accumulation of autophagosomes in diseased brains is an event highly associated with the progression of many neurological disorders or degenerating neurons (Petersen *et al*., 2001).

Several studies confirm a crucial role of autophagy in neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, tauopathies and polyglutamine expansion diseases like Huntingon's disease (Berger *et al*., 2006; Iwata *et al*., 2005b; Mizushima and Hara, 2006; Nixon *et al*., 2005; Qin *et al*., 2003; Ravikumar *et al*., 2002; Rubinsztein, 2006; Rubinsztein *et al*., 2005; Ventruti and Cuervo, 2007; Webb *et al*., 2003).

As described in →1.3.1, autophagic processes are composed of several steps. A number of studies have shown that alteration of any of these steps, such as the disruption of autophagosome formation, maturation or clearance, can impair autophagy and lead to neurodegenerative phenotypes. Here are some examples of related phenotypes aligned with the altered autophagic step:

The disruption of autophagosome formation: Autophagosome formation was disrupted following the impairment of autophagy by the loss of genes involved in induction or vesicle formation. Disruption of ULK1 (Atg1) leads to impairment of endocytosis, abnormal axonal branching and growth in mice and *c.elegans* (Zou *et al*., 2007; McIntire *et al*., 2008). Knockout mice deficient in the tumor suppressor BECN1 show neurodegeneration and lysosomal abnormality (Pickford *et al*., 2008). Recent work shows that loss of *Ambra1* induces cell death and neural tube defects (Fimia *et al*., 2007). The depletion of *atg5* or *atg7* can cause accumulation of ubiquitinated proteins and ultimately neuronal cell loss (Hara *et al*., 2006; Komatsu *et al*., 2006).

The disruption of autophagosomal maturation: Autophagosomes with defects in maturation rapidly accumulate in the cytosol due to their inability to be degraded.

Interestingly, the ESCRT (Endosomal sorting complex required for transport) has been presented as a key regulator of autophagosome maturation (Lee *et al*., 2007). Loss of CHMP4b (a component of ESCRT-III) results in the accumulation of ubiquitin-positive proteins as well as neuronal cell loss in flies and mice (Lee *et al*., 2007). Interestingly, mutation of CHMP2B, another ESCRT-III subunit, is associated with chromosome 3 (FTD3)-linked frontotemporal dementia. Based on a cell culture study using primary cortical neurons, the mutant form of CHMP2B can also cause neurodegeneration by disrupting the fusion of autophagosomes with lysosomes, which is one possible explanation of FTD-3 pathogenesis (Lee *et al*., 2007, Filimonenko *et al*., 2007). A recent study showed that loss of Hrs (a component of ESCRT-I) caused neurodegeneration and abnormal protein accumulation, thereby providing evidence that ESCRT components play essential roles in autophagy (Tamai *et al*., 2008). However, further investigation is needed to identify how ESCRT regulates the fusion process.

The disruption of autophagosomal clearance: The final step of autophagy is the digestion of sequestered materials in autolysosomes. A defect in autophagic clearance is also observed in many neurological diseases whose main feature is a deficiency of lysosomal hydrolases. Loss of *cln3* or *cathepsinD* presents neuronal ceroid lipofuscinosis (Cao el al., 2006; Koike at al., 2005; Shacka *et al*., 2007). Moreover, loss of *cathepsinB/cathepsinL* causes severe brain atrophy and enhanced apoptosis, indicating their crucial roles in autophagic degradation (Felbor *et al*., 2002; Koike at al., 2005).

During the last years, important *in vivo* studies (some of them were mentione above) showed that conventional autophagy knockout mice die during embryogenesis or the neonatal period (Fimia *et al*., 2007; Komatsu *et al*., 2005; Kuma *et al*., 2004; Qu *et al*., 2003; Takahashi *et al*., 2007; Yue *et al*., 2003). Mice with neural-tissue-specific knockouts of these genes survive the postnatal starvation period. However, these mice develop progressive motor deficits and display abnormal reflexes, and ubiquitinpositive inclusion bodies accumulate in their neurons (Hara *et al*., 2006; Komatsu *et al*., 2006). Studies showed that the CNS, in contrast to other organ systems, displays only low levels of autophagosomes under normal conditions and even after starvation, but it was also demonstrated that constitutive turnover of cytosolic contents by autophagy is indispensable, even in the absence of expression of any disease-associated mutant proteins (Mizushima *et al*., 2004; Nixon *et al*., 2005).

Like two sides of the same coin, autophagy can be protective or detrimental to neuronal cell survival and degeneration. Thus, understanding the detailed mechanism of autophagy in specific disease conditions will help identify therapeutic targets for neurodegenerative diseases. This work, will focus on the impact of the autophagic pathway and its alterations on the neurodegenerative prion diseases.

1.3.3 Methods for monitoring autophagy

As increasing evidence suggests that the deregulation of autophagy may contribute to a broad spectrum of mammalian diseases (Levine and Kroemer, 2008; Mizushima *et al*., 2008), there is a growing need among scientists to be able to accurately detect autophagy and to study its function in diverse biological processes, especially in mammalian systems.

With the rapidly advancing research in the autophagy field a range of biochemical and morphological methods has been developed to monitor autophagy (Klionsky *et al*., 2007; Mizushima, 2004). These methods are useful and reliable to monitor autophagy in yeast, however, there is some confusion regarding some methods to measure autophagy in higher eukaryotes. A key point in monitoring autophagy is that there is a difference between measurements that monitor the numbers of autophagosomes versus those that measure flux through the autophagic pathway. Thus, a block in autophagic flux, for example due to disturbance in lysosomal function, results in autophagosome accumulation which needs to be differentiated from fully functional autophagy that includes delivery to and degradation within lysosomes (in most higher eukaryotes) or the vacuole (in plants and fungi). Recently, a number of studies about the selection and interpretation of the methods that can be used to examine autophagy and related processes were published, recommending the use of multiple assays to verify an autophagic response (Kawai *et al*., 2006; Klionsky *et al*., 2008; Mizushima and Yoshimori, 2007, Barth *et al*., 2010; Mizumshuma *et al*., 2010).

The methods described below detect different stages of the autophagy pathway (e.g., early autophagosome, autolysosome, autophagic degradation products) and should be used coordinately with each other to determine whether an increase in intermediates in the pathway represents a true increase in autophagic degradation or rather, a block in the completion of the autophagic pathway. The term “autophagic flux” is used to denote the dynamic process of autophagosome synthesis, delivery of autophagic substrates to the

lysosome, and degradation of autophagic substrates inside the lysosome and is a relevant indicator of autophagic activity completing measurements of autophagosome levels.

Three principal methods are presently used for *monitoring autophagosome numbers*, including a) conventional electron microscopy, b) fluorescence microscopy detection of levels and subcellular localization GFP-LC3 or endogenous LC3 (also called ‘puncta formation assay’ as it measures average levels/numbers of punctate structures per cell) and c) biochemical detection of the conversion of LC3-I (cytosolic form) to LC3-II (membrane-bound lipidated form) by immunoblotting. Methods for *monitoring autophagic flux* are for example d) the LC3 turnover assay, where degradation of LC3-II inside the autolysosome is estimated by the comparison of two samples with and without lysosomal inhibitor treatment, e) detection of degradation of autophagy-selective substrates such as LC3 (LC3 is part of the autophagy machinery rather than a true substrate but is selectively degraded by autophagy) and p62 is detected by immunoblotting, f) Detection of autophagosomes labeled with a yellow signal via double-tagged LC3(mRFP/mCherry-GFP-LC3) and their maturation into autolysosomes labeled with a red signal (after quenching of GFP fluorescence in the lysosome), g) Detection of the GFP fragment generated by the degradation of GFP-LC3 inside autolysosomes by immunoblotting with an anti-GFP antibody or h) Measurement of long-lived protein degradation that is suppressed by autophagy inhibitors.

Several different assays are presently available to monitor autophagy and the autophagic flux, however, the utility and limitations of each of these assays may vary somewhat in different cell types and in different experimental contexts. Therefore, it is recommended that the choice of assays should be “custom-tailored” accordingly. As the limitations of the individual assays are largely nonoverlapping, it is recommended to use a combination of different assays to monitor autophagy. In this manner, it should be possible to reliably monitor autophagic activity and flux in most mammalian tissue culture settings. A more difficult, but extremely important, challenge will be to develop assays to measure autophagic flux in patients (and in blood and tissue samples from patients). For this purpose, novel methods of molecular recognition, such as Alternative non-antibody scaffolds (Skerra, 2007; Gebauer and Skerra, 2009), need to be developed.

1.4 Objective of the thesis

Alterations in the autophagic pathway are suggested to play a key role in the CNS and in neurodegenerative diseases. In this work, the role of autophagy in TSEs should be

addressed, with a focus on altered autophagy. This work should provide interesting insight into 1) How non-cytosolic aggregated proteins might be influenced by autophagy and 2) whether autophagy and its modulation provide an interesting target for prion therapeutics.

Autophagy modulators, inducers and inhibitors of autophagy, shall be used as research tools to analyze the effect of autophagic alteration on prion infection. Approaches for autophagic manipulation shall be established on the pharmacological and genetical (siRNA) level to provide profound evidence. Furthermore, autophagic modulators should also be analyzed for potential therapeutic purposes. Thus, after identification of autophagic modulators that are able to counter-act cellular prion accumulation, their anti-prion effects shall be characterized in detail. A well established and in the prion field widely used mouse neuroblastoma cell line (N2a) model was chosen for the investigations. As it should be determined whether and when autophagic acitivity is beneficial or harmful in prion infections, the potential of selected autophagy modulators should be investigated in diverse prion infection conditions, including primary, persistent and *in vivo* infection scenarios.

Findings obtained from this work should add to the knowledge of the role of autophagy in prion disease and other neurodegenerative disorders.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Acetic acid 100%	Roth GmbH & Co, Karlsruhe
Agarose	Invitrogen, Paisley, Schottland, UK
Ammonium chloride (NH_4Cl)	Roth GmbH & Co, Karlsruhe
Ammonium peroxodisulfate (APS)	Roth GmbH & Co, Karlsruhe
Bafilomycin A1	Sigma-Aldrich Chemie GmbH, Steinheim
Bacillol® plus	Bode Chemie, Hamburg, G
Bromphenolblau	Merck, Darmstadt
ß-Mercaptoethanol	Sigma-Aldrich Chemie GmbH, Steinheim
Coomassie Protein Assay Reagent	Pierce, IL, USA
Chloroform	Roth GmbH & Co, Karlsruhe
Diethyl pycarbonate	Sigma-Aldrich Chemie GmbH,
Dimethylsulfoxid (DMSO)	Sigma-Aldrich Chemie GmbH, Steinheim
Ethanol 99,8%	Roth GmbH & Co, Karlsruhe
Ethylendiamintetraacetic acid (EDTA)	Roth GmbH & Co, Karlsruhe
Gelatine 40% solution	Sigma-Aldrich Chemie GmbH, Steinheim
Glycerol	Roth GmbH & Co, Karlsruhe
Glycine	Roth GmbH & Co, Karlsruhe
Guanidine hydrochloride (Gnd HCl)	Roth GmbH & Co, Karlsruhe
Hoechst 33342, trihydrochloride, trihydrate	Sigma-Aldrich Chemie GmbH, Steinheim

HCl	Roth GmbH & Co, Karlsruhe
Insulin	Sigma-Aldrich Chemie GmbH, Steinheim
Imatinib	Novartis Pharmaceuticals Corpororation, Basel, Switzerland
Isoamylalcohol	Roth GmbH & Co, Karlsruhe
Isopropanol	Roth GmbH & Co, Karlsruhe
Lithium chloride (LiCl)	Sigma-Aldrich Chemie GmbH, Steinheim
Methanol	Roth GmbH & Co, Karlsruhe
N,N,N',N'- Tetramethylenediamine (TEMED)	Sigma-Aldrich Chemie GmbH, Steinheim
N-Lauroyl-Sarcosin (Sarkosyl)	Sigma-Aldrich Chemie GmbH, Steinheim
Pefabloc SC (AEBSF)	Roche, Mannheim
Permafluor	Beckman Coulter, Marseille, Frankreich
Phosphate Buffered Saline (PBS)	Invitrogen (Gibco), Paisley, UK
Polybrene	Sigma-Aldrich Chemie GmbH, Steinheim
Protogel ultra pure	National Diagnostics, Atlanta, USA
Rapamycin	Sigma-Aldrich Chemie GmbH, Steinheim
Re-blot Plus Strong Solution	Chemicon Int., Carrigtwohill, Ireland
Roti-Histofix	Roth GmbH & Co, Karlsruhe, G
Roti-Phenol	Roth GmbH & Co, Karlsruhe, G
Saponin	Roth GmbH & Co, Karlsruhe
Skim Milk Powder	Vitalia Reformhaus, G

Sodium acetate	Roth GmbH & Co, Karlsruhe
Sodium chloride (NaCl)	Roth GmbH & Co, Karlsruhe
Sodium deoxycholate (DOC)	Roth GmbH & Co, Karlsruhe
Sodium dodecylsulfate (SDS)	Roth GmbH & Co, Karlsruhe
Sodium hydroxide (NaOH)	Roth GmbH & Co, Karlsruhe
Tamoxifen	Sigma-Aldrich Chemie GmbH, Steinheim
Trehalose	Sigma-Aldrich Chemie GmbH, Steinheim
Tris-Hydroxy-Methyl-Amino-Methan (Tris)	Roth GmbH & Co, Karlsruhe
Triton X-100	Sigma-Aldrich Chemie GmbH, Steinheim
Tween 20	Roth GmbH & Co, Karlsruhe
Wortmannin	Sigma-Aldrich Chemie GmbH, Steinheim
3-Methyladenine	Sigma-Aldrich Chemie GmbH, Steinheim

2.1.2 Buffers and solutions

Buffers and solution are listed in their applied concentration in the context of the described method (→0).

2.1.3 Cell culture media and additives

Fetal calf serum (FCS, Inactivated at 56°C for 30 min before use)	Invitrogen GmbH, Karlsruhe
OptiMEM with GlutaMAX	Invitrogen GmbH, Karlsruhe
Penicillin	Invitrogen GmbH, Karlsruhe
Streptomycin	Invitrogen GmbH, Karlsruhe

2.1.4 Enzymes

Proteinase K (30 U/mg) Roth GmbH & Co, Karlsruhe

Trypsin 0,25 % with EDTA Invitrogen, Karlsruhe

2.1.5 Antibodies

Antibodies used for Western blotting (WB) and immunoflourescence analysis (IF) are listed in →Table 5 and →Table 6.

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Table 5. Primary antibodies used in this work.

Denotation	Source	Specifity	Reference	Dilution
Anti-LC3	Mouse monoclonal	LC3 (microtubule associated protein 1 light chain 3) I and II	NanoTools Antikörpertechnik GmbH & Co, KG, Teningen	WB 1:2500
Anti-LAMP-1	Rat (reactive with mouse) monoclonal	Lysosome associated membrane protein I Mouse (LAMP-1)	(Santa Cruz Biotechnolgy; Santa Cruz, CA, USA)	IF 1:100
Anti-β-Actin	Mouse monoclonal	β-Actin	Sigma-Aldrich Chemie GmbH, Steinheim	WB 1:10000
Anti-Atg5	Mouse monoclonal	Atg5 (Autophagy related gene 5),	NanoTools Antikörpertechnik GmbH & Co, KG, Teningen	WB 1:800
Anti-Beclin 1	Rabbit polyclonal	Beclin 1	Cell Signalling Technology, Inc., Danvers, MA, USA	WB 1:1000
4H11	Mouse monoclonal	PrP of various species, including mouse and hamster; no linear epitope defined	Ertmer *et al.*, 2004	WB 1:1000 FACS 1:10
3F4	Mouse monoclonal	Epitope in Syrian hamster and human PrP; aa 109-112 (MKHM)	(Barry *et al*. 1986; Barry and Prusiner 1986; Kascsak *et al*. 1987; Rogers *et al*. 1991); Kindly provided by Michael Baier (Robert Koch Institut, Berlin, Germany)	WB 1:5000

Table 6. Secondary antibodies used in this work.

Denotation	Source	Specifity	Reference	Dilution
Horseradish peroxidase (HRP)-conjugated anti-IgG	Sheep	Mouse IgG	Dianova, Hamburg	WB 1:10000
HRP-conjugated anti-IgG	Goat	Rabbit IgG	Cell Signalling Technology, Inc., Danvers, MA, USA	WB 1:2000
Cy2™- conjugated anti-IgG	Donkey	Mouse IgG	Dianova, Hamburg	IF 1:100 FACS 1:100
Cy3™- conjugated anti-IgG	Donkey	Rat IgG	Dianova, Hamburg	IF 1:400

2.1.6 Kits and Standards

Coomassie Protein Assay Reagent	Pierce, Bonn
ECLplus Western Blot Detection Kit	Amersham Biosciences, Freiburg
FuGENE® 6 Transfection Kit	Roche, Mannheim
Page Ruler Plus Prestained Protein Ladder	Fermentas GmbH, St. Leon-Rot
Lipofectamine™ 2000	Invitrogen, Karlsruhe

2.1.7 Plasmids

The mammalian expression vector pEGFP-LC3 was kindly provided by Tamotsu Yoshimori. It was constructed by insertion of rat LC3 cDNA into the *Bgl*II and EcoRI sites of pEGFP-C1 (GeneBank accession number U55673) and encodes a fusion protein of enhanced green fluorescent protein and LC3 (Kabeya *et al*., 2000).

2.1.8 siRNA

Table 7. siRNA used in this work

Denotation	Source	Target Sequence
HP GenomeWide siRNA (5nmol) Mm_Apg5l_5	Qiagen, Hilden, G	CAG AAG GTT ATG AGA CAA GAA *Sense* r(GAA GGU UAU GAG ACA AGA A)dTdT *AntiSense* r(UUC UUG UCU CAU AAC CUU C)dTdG

HP GenomeWide siRNA (5nmol) Mm_Apg5l_5	Qiagen, Hilden, G	ACA GTT TGT ATT TCT GAT TAA *Sense* r(AGU UUG UAU UUC UGA UUA A)dTdT *AntiSense* r(UUA AUC AGA AAU ACA AAC U)dGdT
HP GenomeWide siRNA (5nmol) Non silencing	Qiagen, Hilden, G	NonSense
On-Target-Plus anti-Beclin SMART Pool	Thermo Fisher Scientific, Dharmacon	A mixture of 4 siRNA provided as a single reagent; providing advantages in both potency and specificity
On-Target-Plus SMART Pool control	Thermo Fisher Scientific, Dharmacon	A mixture of non-sense siRNA provided as a single reagent

2.1.9 Cell lines

Neuronal and non-neuronal cells lines used in this work are listed in → Table 8.

Table 8. Cell lines used in this work.

Cell line	Description	Reference
N2a	Murine neuroblastoma cell line	ATCC® CCl-131™; Klebe & Ruddle, 1969
ScN2a	N2a cells both persistently infected with RML prion strain and overexpressing 3F4-epitope-tagged murine PrP	Butler *et al.*, 1988 Scott *et al.*, 1992
Kl17; Kl21	PrPSc-susceptible N2a clones (cells were obtained by curing prion-infected ScN2a cells using pentosan polysulphate; subsequently cells were sub-cloned and tested for PrP^{Sc}-susceptibility)	Kindly provided by Gloria Lutzny (Institute of Virology, TU München)

2.1.10 Instruments & Consumables

Autoklav V95	Systec, Wettenberg
Autoradiography films	
- Amersham Hyperfilm™ MP	GE Healthcare, Freiburg
- Autoradiography film full blue	Lab Scientific, New Jersey, USA
Cell scraper (blade length 1,7 cm)	Sarstedt AG & Co, Nümbrecht
Coverslips	Marienfeld, Bad Mergentheim
Centrifuges	
- Eppendorf 5417R	Eppendorf-Nethaler-Hinz GmbH, Köln
- Sigma 4K15	Sigma-Aldrich, Schnelldorf
- Beckmann TL-100 ultracentrifuge	Beckmann Coulter GmbH, Krefeld
Counting chamber Fuchs-Rosenthal	Roth GmbH & Co, Karlsruhe
CO_2-Incubator	W.C. Heraeus GmbH, Hanau
ECL Semi-Dry Tansfer unit (TE77)	Amersham Biosciences, Freiburg, G
Eagle eye processor	Stratagene, The Netherlands
Electronic balance	
- Electronic balance	Kern, Bailingen
- Electronic balance Sartorius Research	INXS Inc., Delray Beach, USA
FACS-polystyrene tubes	Becton Dickinson, USA
Flowcytometers	
- EPICS XL	Beckman Coulter GmbH, Krefeld
- BD FACS Canto II	BD Bioscience, Heidelberg
Gloves	
- Roti® protect-VINYL	Roth GmbH & Co, Karlsruhe
- SafeSkin Satin Plus (M)	Kimberly-Clark GmbH, Weinheim
- SemperCare®- NITRILE	Sempermed, Austria
Magnet stirrer RCT basic	IKA Werke Staufen

Midi-protein gel-chamber	Peqlab Biotechnologie GmbH, Erlangen
Microscopes	
- Axiovert 40C microscope	Carl Zeiss Jena GmbH, Göttingen
- EpiFluorescence-Mikroscope CK X41	Olympus, Hamburg
- LSM510 confocal laser microscope	Carl Zeiss Jena GmbH, Göttingen
- FluoView® FV10i	Olympus, Hamburg, Germany
Object slides	Marienfeld, Bad Mergentheim
Optimax X-Ray Film Processor	PROTEC Medizintechnik GmbH & Co-KG, Oberstenfeld
Perfect Blue™ vertical double gel system	Peqlab Biotechnologie GmbH, Erlangen
Photoimaging unit	Labortechnik GmbH&CoKG, Wasserburg
pH-Meter	WTW, Weilheim
Pipets	
- Eppendorf 10 µl	Eppendorf-Nethaler-Hinz GmbH, Köln
- Eppendorf 100 µl	Eppendorf-Nethaler-Hinz GmbH, Köln
- Eppendorf 1000 µl	Eppendorf-Nethaler-Hinz GmbH, Köln
Pipet tips	
- SafeSeal-Tips 20 µl	Biozym Scientific GmbH, Vienna, Austria
- SafeSeal-Tips 100 µl	Biozym Scientific GmbH, Vienna, Austria
- SafeSeal-Tips 1000 µl	Biozym Scientific GmbH, Vienna, Austria
- Stripette 5 ml	Corning Incorporative, Corning, USA
- Stripette 10 ml	Corning Incorporative, Corning, USA
- Stripette 25 ml	Corning Incorporative, Corning, USA
Pipetus	Hirschmann Laborgeräte

Power Supplies	
- Electrophoresis Power Supply EPS 3500XL	Pharmacia Biotech, Sweden
- Electrophoresis Power Supply E835	Consort, Belgium
- Blotting Power Supply Power pac 200	BIO-RAD Laboratories GmbH, München
- Blotting Power Supply Model 200/2.0	BIO-RAD Laboratories GmbH, München
- Blotting Power Supply Model 1000/500	BIO-RAD Laboratories GmbH, München
PVDF-membrane (Hybond™-P)	GE Healthcare, Freiburg
Reaction tubes	
- Cryotubes (2ml)	Corning Incorporative, Corning, USA
- Eppendorf tubes (1,5 ml; 2 ml)	Eppendorf-Nethaler-Hinz GmbH, Köln
- Falcon tubes (15ml; 50 ml)	Becton Dickinson, USA
- Ultracentrifuge tubes (1,5 ml)	Beckmann Coulter GmbH, Krefeld
Sunrise ELISA Reader	Tecan, Maennedorf, Switzerland
Tissue Culture dish 100x 22 mm	Beckton Dickinson, USA
Tissue Culture dish 60x 15 mm	Beckton Dickinson, USA
Tissue Culture plate (96-, 48-, 24-, 12- well)	Beckton Dickinson, USA
Blotting paper (46 x 57 cm)	Schleicher & Schnell, Dassel
Thermomixer compact	Eppendorf-Nethaler-Hinz GmbH, Köln
Trans-Blot SD Semi-dry Transfer Cell	BIO-RAD Laboratories GmbH, München
Vortex Mixer	NeoLab Migge, Heidelberg, USA
Whatman paper (46 x 57 cm)	Schleicher & Schnell, G
Waterbath	GFL, Burgewede
X-ray films	Kodak Biomax XS

2.1.11 Computing programs and internet portals

Adobe Acrobat 7.0	Adobe System Inc., USA
Adobe Photoshop 7.0	Adobe System Inc., USA
Cell AnalySIS image processing software	Olympus, Hamburg, G Cell^F imaging Olympus, Hamburg
Expo32 ADC	Beckmann Coulter GmbH, Krefeld
FACS Diva Software	BD Bioscience, Heidelberg
FlowJo	Tree Star, USA
ImageQuant TL	GE Healthcare, Freiburg
Medline http://www.ncbi.nlm.nih.gov/sites/entrez	
Microsoft Office XP	Microsoft, USA
Microsoft Windows XP	Microsoft, USA
Prism 4 software	Graphpad Software, San Diego, CA, USA
Zeiss AIM Software	Carl Zeiss Jena GmbH, Göttingen
Zeiss LSM Image Viewer	Carl Zeiss Jena GmbH, Göttingen

2.2 Methods

2.2.1 Biological safety

Genetic engineering work was carried out in a biosafety level 2 laboratory and according to the guidelines of the german genetic engineering law of 01.01.2004. Biologically contaminated material and solutions were separately collected, inactivated and disposed accordant to the operating instructions.

2.2.2 Molecular and cell biological methods

All the work with mammalian cells was accomplished under a *laminar flow hood*. Sterile one-way plastic pipettes and latex gloves were used.

2.2.2.1 Thawing of cells

Thawing was achieved by incubating frozen cells at 37° C for 10-15 min. Cells were resuspended in 10 ml culture medium with 10 % FCS and centrifuged for 10 min at 1000 rpm at 22° C in order to remove toxic DMSO. The supernatant was removed and the cell pellet was resuspended in 10 ml culture medium and plated on a culture dish.

2.2.2.2 Cultivation and passaging of cells

Cells were cultivated on appropriate culture dishes and kept in an incubator in a 5 % CO_2, 90 % humidity atmosphere at 37° C. Medium (OPTIMEM + 10% FCS + 1% P/S (Penicillin / Streptomycin)) was changed every two days. If not differently stated, cell culture medium was always supplemented with 10 % fetal calf serum (FCS) and 1 % Pen/Strep. Passaging of cells was carried out using 6 or 10 cm culture dishes. Confluent adherent cells were rinsed once with 5 ml PBS and then detached using 500 µl Trypsin/EDTA solution. Cells were subsequently suspended in 5 or 10 ml appropriate culture medium and a desired volume of this cell suspension was transferred to a new 6 or 10 cm culture dish supplemented with 5 or 10 ml appropriate cell culture medium.

2.2.2.3 Determination of cell number

Cells were grown to confluency and cells were washed with PBS and detached from the cell culture plate either by trypsin treatment or by simple rinsing (→2.2.2.2). Cells were resuspended in 10 ml new culture medium and transferred to a 15 ml Falcon. A 1:20 dilution was prepared from this suspension and 20 µl of the sample was analysed in a Fuchs-Rosenthal-chamber. The counting chamber consists of cubes with a defined total volume. Cells of 4 diagonal big squares were counted. The following equation was used to determine the total number of cells *(N_{total})* per ml:

$$N_{total} = \frac{Cells_{counted}}{Squares_{counted}} \times F_{diltuion} \times F_{volume},$$

with $F_{dilution}$ representing the dilution factor (here 1 or 20) and F_{volume} representing the volume factor (here 5000).

2.2.2.4 Cryoconservation

For storage of mammalian cells, 75-80 % confluent adherent cultures were subjected to mild treatment with trypsin and suspended in culture medium with addition of 10 % FCS (= 20 % total FCS) and 10 % DMSO. 1 ml aliquots of the suspension were transferred to

1.5 ml cryotubes and frozen to –80° C for 24 h. Tubes were then transferred to liquid nitrogen for long-term storage.

2.2.2.5 Transient transfection of mammalian cells

<u>Background</u>

The transient transfection method allows the introduction of foreign nucleic acid into eukaryotic cells. Plasmid DNA and siRNA were introduced into mammalian cells by lipofection, a special form of transfection. Lipofection is carried out by mixing the plasmid DNA or siRNA with a cationic lipid to form liposomes that fuse with the cell membrane and so free their cargo inside the cell.

2.2.2.5.1 Transfection with plasmid DNA

For Immunofluorescence analysis transient transfection of cells with recombinant plasmids was done by means of lipofection using FuGENE® reagent that contains a blend of different lipids and other components.

2,5 x 10^5 cells were plated in a culture dish (6 cm Ø) and grown for 24 h such that they were 10-20 % confluent at the time of transfection. For transfection, 6 µl FuGENE® reagent and 2 µg plasmid DNA were added to 100 µl OptiMEM without additives and incubated for 20 min at room temperature to allow for forming of transfection complexes. Cells were rinsed once with PBS and covered with 3 ml fresh culture medium before drop-wise addition of transfection complexes. Culture medium was exchanged and treatment started after 6 or 24h. Cells were analyzed 24 and 48 h after treatment start as detailed below (→2.2.2.10).

Successful transfection was monitored in an epifluorescence microscope by autofluorescence of cells due to expression of plasmid encoded EGFP. Transfection efficiency was determined by optical evaluation of the ratio of non-fluorescent to autofluorescent cells.

2.2.2.5.2 Transfection with siRNA

The transfection reagent Lipofectamine 2000 (Invitrogen, Karlsruhe, D) was used for all siRNA transfection experiments according to the manufacture's manual. Briefly: cells were plated in 6 cm dishes for each experiment. 2 µl of siRNA and 2.5 µl of Lipofectamine 2000 were separately incubated in 100 µl OptiMem + GlutaMAX without FCS or Pen/Strep for 5 min at room temperature. Samples were mixed together and further incubated at RT for 30 min. Meanwhile cells were washed twice with the same

medium and 1.8 ml medium was added to the cells. 200 µl of transfection mixture was added stepwise to the cells and gently mixed by pivoting the plate. Cells were incubated for 24 hours in the incubator at 37°C and the medium was subsequently replaced by fresh medium.

2.2.2.6 Pharmalogical modulation of the autophagy pathway in mammalian cells

Cells were incubated with autophagy inducing or inhibiting chemical compounds in culture media in the indicated concentrations →Table 9.

For treatment start, culture media was removed and replaced with fresh culture medium containing chemical compounds. Treatment lengths and detailed experimental setup is demonstrated for respective experiments in → 4.

Table 9. Autophagy modulators and their applied concentrations

Autophagy inducer	Applied concentration
Tamoxifen	3 µM
Trehalose	100 mM
Rapamycin	200 nm
Imatinib (Glivec)	10 µM
LiCl	10 mM
Autophagy inhibitor	**Applied concentration**
Wortmannin	1 µM
3-Methyladenine	10 mM
Insulin	100 nm
Lysosomal inhibitor	**Applied concentration**
Bafilomycin A1	200 nM

2.2.2.7 Genetic modulation of the autophagy pathway in mammalian cells via siRNA

Background

The transient transfection of mammalian cells with short interference RNA (siRNA) is a powerful tool to knock-down one or more proteins in cells and thus modulate cellular

pathways. The siRNA machinery is a host encoded system activated by dsRNA complexes to protect cells for example against viral dsRNA genomes. In order to activate this machinery *in vitro* the artificial siRNA normally consists of 21 to 23 nucleotides with a sequence complementary to the mRNA of the protein of interest and must be introduced into the cytoplasm of the cells. In this work the lipofection method was used (→2.2.2.5.2). In the cytoplasm siRNA binds selectively to the complementary mRNA resulting in double stranded (ds) RNA. This dsRNA is recognized by an enzyme called DICER which cleaves the dsRNA and transfers it to RISC (RNA induces silencing complex). One strand of the dsRNA is degraded while the other strand serves as a template for RISC to detect and cleave additional mRNA coding for the same protein. The cellular siRNA machinery therefore leads to a sequence-specific cleavage of perfectly complementary mRNA resulting in a knock-down of the protein. The effect of the knock-down is transient and time dependent as the siRNA itself gets cleaved by cellular nucleases

Procedure:

To knock-down expression of autophagy essential genes (*Atg5* or *Beclin 1*), gene specific siRNAs (→Table 7) and one non-silencing (ns)-siRNA were utilized. Cells were plated in 6 cm dishes for each experiment and transfected with Lipofectamine 2000 (Invitrogen, Karlsruhe, D) and the respective constructs under RNase free conditions for 24 hours (2.2.3.9). Cells were subsequently cultured in serum-free OptiMEM medium supplemented with antibiotics. Further treatments are depictet in →Figure 28, Figure 36 and Figure 39. Cells were then lysed allowing detection of the knocked down gene, ß-Actin and PrP (PrP^{Sc} and PrP^{c}).

2.2.2.8 Infection of mammalian cells with prions

2.2.2.8.1 Preparation of brain homogenate

The mouse-adapted scrapie strains RML and 22L were a kind gift of Prof. Dr. M. Groschup (Friedrich-Loeffler-Institut, Bundesforschungsinstitut für Tiergesundheit (FLI), Insel Riems) and were propagated in CD1 or C57B1/6 mice. To prepare brain-homogenates, brains were homogenized in PBS prepared using a glass douncer. 10 % (wt/vol) homogenate was stored at -80° C. Brains were stored at -80° C.

2.2.2.8.2 Infection of cells with prions

Cells were plated in a 12-well culture plate (2,5 x 10^5/plate). 24h later, culture media were removed and 300 µl of brain homogenate in medium (270 µl of fresh culture medium + 30 µl brain homogenate thawn at room temperature were previously mixed) were added giving a final concentration of 1 % brain homogenate. The brain homogenate was incubated for 24 hours and was then removed. Cultures were washed 3 times with PBS to remove residual brain homogenate. Fresh culture medium was added and cells were cultivated until further analysis. In transiently transfected cells, infection was performed one day after transfection.

2.2.2.9 Trypane blue exclusion assay

In order to determine cell viability and to exclude toxic effects of trehalose and tamoxifen Trypane blue exclusion assay was performed in the same experimental settings. Trypane blue is a membrane impermeable dye. Only dead cells or cells with damaged cell membranes can be stained. The percentage of stained cells can be determined after counting stained and unstained cells and represents a measure for toxicity.

Cells were cultured for 48, 72 or 96 h in medium containing 100 mM Trehalose, 3 µM Tamoxifen or control medium. Then cells were mixed in a 1:2 ratio with trypan blue. After incubation for 2 min at room temperature, the cell suspension was loaded on a Fuchs-Rosenthal chamber and number of colored and uncolored cells was determined.

Percentage of viable cells was calculated by determining the ratio of unstained (viable) cells to stained (not viable) plus unstained cells.

2.2.2.10 Confocal Laser Microscopy (Immunofluorescence (IF)) analysis

Background

Immunofluorescence is a technique allowing the localization of specific proteins in cells by specifically binding antibodies. There are two major types of immunofluorescence staining methods: 1) direct immunofluorescence staining in which the primary antibody is labelled with fluorescence dye, and 2) indirect immunofluorescence staining in which a secondary antibody is labelled with a fluorescence dye and is used to detect a primary antibody. Immunofluorescence staining can be performed using cells fixed on slides or tissue sections. Immunofluorescence stained samples are examined with a fluorescence microscope or a confocal laser-scanning microscope (CLSM).

Material:

- Roti-Histofix	10 %	Solution
- Quenching-solution	50 mM	NH_4CL
	20 mM	Glycine in PBS
- Permeabilizing solution	0.3 %	Triton X-100 in PBS
- Blocking solution	0,2 %	Gelatine in PBS
- Denaturing solution	6 M	GdnHCl

▪ *For LC3-puncta assay:* Sterile coverslips were inlayed into the 6-well plates and cells were plated and transfected →2.2.2.5 with GFP-LC3 →2.1.7 one day later. 6 or 24 h later cells were treated with autophagy inducers. After 24 h or 48 h of treatment, cover slips were removed and transferred into a 12-well plate, washed three times with PBS, and fixed in 500 µl Roti-Histofix (10 %) for 30 min at RT. Subsequently, cells were quenched using NH4Cl/glycine solution, permeabilized with 0.3 % Triton X-100 and blocked by incubation with blocking solution. Each step was performed for 10 min at RT and terminated by three times rinsing steps with PBS. Then, cells were incubated with the primary antibody anti-lamp-1 (→Table 5) in a humid chamber for 45 min at RT, washed three times with PBS and incubated with the secondary antibody Cy3 (→Table 6) in a dark, humid chamber for 30 min at RT. Again, cells were rinsed three times with PBS and nuclei were stained by incubation with Hoechst DNA staining solution for 10 min at RT in the dark. Finally, cover slips were overlaid with mounting medium (Permafluore) on the slides and dried over night at 4 °C. Analysis was carried out using a LSM 510 confocal laser microscope (Zeiss).

▪ *For inocculum tracking:* For scrapie-specific confocal microscopy, sterile coverslips were inlayed into the 6-well plates before cells were plated and incubated with brain homogenate and with autophagy inducers 24h later. Then the coverslips were removed at defined time-points (→3.2.3) and transferred into a 12-well plate and washed twice with PBS. Cells were fixed in 500 µl Roti-Histofix (10 %) for 30 min (max. 2 days) and were quenched with 50 mM NH_4Cl/ 20 mM Glycin for 10 min and permeabilized with 0,3 % Triton X-100 for 10 min. For PrP^{Sc} staining, cells were incubated in 500 µl 6M Gnd HCl for 7 min. Unspecific binding of the antibodies was blocked by incubation in 0,2 % Gelatine (10 min). Between each incubation step cells were washed 3 x with PBS. Afterwards, cells were incubated with 1st antibody at 1:100 (4H11) in PBS, washed 3 x with PBS and then incubated with Cy3-conjugated secondary antisera (1:200 in PBS) for 30 min and washed again. Used antibodies are listed in → Table 5 and →Table 6. Finally,

coverslips were overlaid with mounting medium (Permafluore) on the slides and dried overnight at 4° C. Analysis occured with a FluoView® FV10i.

2.2.2.11 Fluorescence activated cell sorting (FACS) analysis

Background

Fluorescence-activated cell-sorting (FACS) is a type of flow cytometry (FCM), a method for investigating cells in suspension with respect to different parameters and is based upon specific light scattering and fluorescent characteristics of each cell. However, FACS is a trademarked term that is owned by Becton-Dickinson and is not a general term. The cell suspension is entrained in the center of a narrow, rapidly flowing stream of liquid. The flow is arranged in a way that there is a separation between cells relative to their diameter. A vibrating mechanism causes the stream of cells to break into individual droplets. The system is adjusted so that there is a low probability of more than one cell being in a droplet. Just before the stream breaks into droplets the flow passes through a fluorescence measuring station where the fluorescent character of interest of each cell is measured. When the laserlight focussed on the cell particles meets a cell it is scattered and absorbed by fluorescent dyes (in the cell inner and on its surface) and is emitted in a lower wave length. The change of the excitation light is detected by different photomultipliers. The photodiode of the forward scatter (FCS) detects the size and cell surface property of the measured particle. The photomultiplier of the side scatter (SSC) registers scattered light in a 90° angle to the excitation light. The signal allows conclusions about the granularity of the particle and the analysed cells can be counted. Up to 4 fluorescence signals can be detected.

EDTA solution	1mM	EDTA in PBS
FCM buffer	2.5 %	FCS
	0.05 %	NaN_3 in PBS
Saponin buffer	0.1 %	Saponin in FCM buffer
Quenching solution	50 mM	NH_4Cl
	20 mM	Glycine in PBS

For analysis of total PrP^c level, cells were grown to 80 % confluence, rinsed in PBS and detached by incubation in 1 ml EDTA solution for 10 min. Cells were suspended in FCM buffer and transferred to FACS-polystyrene tubes (~10^6 cells per approach). One unstained control sample was kept on ice during the following staining procedure. For

staining, cells were sedimented by centrifugation (1200 rpm, 5 min 4 °C, Sigma 4K15 centrifuge, rotor 11150, buckets 13350). The supernatant was discarded and the cell sediment resuspended in 100 µl Roti-Histofix. After incubation for 10 min at room temperature, cells were washed once in PBS. This and all following centrifugation and washing steps were performed at 1200 rpm for 2 min at 4 °C (Sigma 4K15 centrifuge, rotor 11150, buckets 13350) in a volume of 500 µl each. Autofluorescence was quenched by incubation of cells in 100 µl quenching solution for 10 min at room temperature. Cell membranes were permeabilized by subsequent incubation in 500 µl saponin buffer for 10 min at room temperature. Cells were washed once in saponin buffer and resuspended carefully in 100 µl solution of first antibody (→Table 5, dilution in saponin buffer). Samples were incubated on ice for 45 min and subsequently washed three times with saponin buffer. For fluorescent labelling of PrP^c, cells were carefully resuspended in 100 µl solution of the fluorescent labeled secondary antibody (→Table 6), dilution in saponin buffer) and incubated for 45 min on ice in the dark. As negative control, samples without primary antibody were prepared simultaneously to adjust the gate that included measured cells. Finally, cells were washed three times in saponin buffer, resuspended in FCM buffer and kept on ice in the dark for 45-60 min, until analysis in the flow cytometer.

2.2.3 Protein biochemical methods

2.2.3.1 Preparation of postnuclear lysates

<u>Material</u>

- Lysis buffer	100 mM	NaCl
	10 mM	Tris-HCl; pH 7.5
	10 mM	EDTA
	0.5 %	Triton X-100
	0.5 %	DOC

Adherent cells were washed once with PBS and were subsequently covered with 1 ml lysis buffer at room temperature for 10 min. Total lysates were transferred to 1.5 ml reaction tubes for separation of cell debris by centrifugation for 1 min at 14.000 rpm at 4° C (5417R, rotor FA-45-24-11, Beckman). The supernatants were transferred into a fresh 1.5 ml reaction tube and immediately prepared for Western blot analysis (→2.2.3.3) or stored at -20° C preparation.

3.2.3.2 Sample preparation for Western blot analysis

Material:

- Pefabloc SC (AEBSF)	1 %	stock solution
- Proteinase K (PK)	1 mg/ml	stock solution in $H_2O_{dest.}$
- TNE	50 mM	Tris-HCl pH 7.5
	150 mM	NaCl
	5 mM	EDTA
- N-lauryl sarcosine	10 %	stock solution

Treatment of postnuclear lysates with PK-digestion and solubility assay before Western blot analysis depended on conditions and aim of the experiment. When only few cells were available (e.g. immediately after exposure to brain homogenate) or when statements about protein aggregation were needed, samples were subjected to solubility assay.

3.2.3.2.1 Proteinase K (PK) digestion

Aliquots of post-nuclear lysates (→2.2.3.1) were incubated for 30 min at 37 ° C with 20 µg/ml PK; the digestion was stopped by addition of the protease inhibitor Pefabloc. Samples were precipitated with methanol (5 x volume) overnight at -20° C. After 25 minutes centrifugation (3500 rpm, 4° C, Sigma centrifuge) pellets were resuspended in an adjusted volume of TNE. 3 x SDS- sample buffer (1/2 x volume) was added and samples were heated to 95° C for 10 min and placed on ice or stored at -20° C till Western blot analysis. Instead of methanol precipitation some samples were subjected to a solubility assay.

3.2.3.3.2 Detergent solubility assay

Post-nuclear cell lysates (→2.2.3.1) were supplied with N-lauryl sarcosine to 1 % and ultracentrifuged (1 h at 100 000 g, 4° C, Beckman TL-100). Soluble fractions (supernatant) were precipitated with methanol as described above. Insoluble fractions (pellet) were resuspended in adjusted volumes of TNE. Samples were analysed on 12.5 % SDS-PAGE. 3 x SDS-sample buffer (1/2 x volume) was added and samples were heated to 95° C for 10 min and placed on ice or stored at -20° C till Western blot analysis.

2.2.3.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

<u>Background</u>

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a technique used to separate proteins according to their size (length of polypeptide chain or molecular weight).

The solution of proteins to be analyzed is first mixed with SDS, an anionic detergent which denatures secondary and non–disulfide–linked tertiary structures, and applies a negative charge to each protein in proportion to its mass. SDS linearizes the proteins so that they may be separated strictly by length (primary structure, or number of amino acids). The SDS binds to the protein giving an approximately uniform mass: charge ratio (1.4:1) for most proteins, so that the distance of migration through the gel can be directly related to only the size of the protein. The smaller the protein, the larger the distance it can travel through the pores of the cross linked gel. Besides the addition of SDS, proteins may optionally be boiled in the presence of a reducing agent, such as dithiothreitol (DTT) or 2-mercaptoethanol (beta-Mercaptoethanol/BME), which further denatures the proteins by reducing disulfide linkages, thus overcoming some forms of tertiary protein folding, and breaking up quaternary protein structure (oligomeric subunits). A tracking dye may also be added to the protein solution to allow tracking the progress of the protein solution through the gel during the electrophoretic run.

The “discontinuous" buffer system according to Lämmli (1970) consists of 2 gels that vary in pH-value and size of pores. An ion gradient is formed in the early stage of electrophoresis that causes all of the proteins to focus into a single sharp band. This occurs in the stacking gel (larger pores, 5 % acrylamide). Then the proteins are subsequently separated by the sieving action in the lower, "resolving" region of the gel (smaller pores, 8-15 % acrylamide). This technique significantly enhances the sharpness of the bands within the gel.

- 4x Resolving gel solution (Lower)	1,5 M	Tris/HCl pH 8,8
	0,4 % (w/v)	SDS in A. dest
- 4 x Stacking gel solution (Upper)	0,5 M	Tris pH 6,8
	0,4 % (w/v)	SDS in A. dest

- Ammoniumpersulfate solution (APS)	10 % (w/v)	Stock solution in A. dest
- 10 x SDS electrophoresis buffer	250 mM	Tris
	2,5 M	Glycin
	1 % (w/v)	SDS in A. dest
- 3x SDS sample buffer	90 mM	Tris/HCl, pH 6,8
	7 % (w/v)	SDS
	30 % (v/v)	Glycerol
	20 % (v/v)	ß-Mercaptoethanol,
	0,01 % (w/v)	Bromphenole blue
- Acrylamide solution	30 % (w/v) acrylamide/	
	0,8 % bisacrylamide (37.5:1)	

<u>Protocol for SDS-PAGE gels (for 4 gels):</u>

- Resolving gel (12.5 %)	acrylamide solution	51,8 ml
	resolving solution	30,8 ml
	H_2O_{dest}	40,6 ml
	TEMED	180 µl
	10% (w/v) APS in H_2O_{dest}	384 µl
- Stacking gel (5 %)	acrylamide solution	5,6 ml
	stacking solution	8,4 ml
	H_2O_{dest}	19,8 ml
	TEMED	60 µl
	10% (w/v) APS in H_2O_{dest}	336 µl

Electrophoretic separation of proteins was carried out on denaturing SDS gels containig 12.5 % acrylamide (Laemmli, 1970). Glass plates were rinsed with ethanol, separators were positioned at the plates' both edges, then the set was put in a plastic bag into the casting chamber. The resolving gel was mixed, added and overlaid with isopropanol.

After polymerisation of the resolving gel, isopropanol was completely removed; stacking gel was mixed and added. The gel combs were inserted immediately. After polymerisation was completed, the glass plates containing stacking and resolving gels were mounted in an electrophoresis chamber and covered with electrophoresis buffer. Samples (15-35 µl) and 5 µl molecular weight marker were loaded on the gel. Constant power (30 mA for 20 min, then 45 mA) was applied for mass separation until the tracing dye (represented by the tracking dye) had reached the bottom of the resolving gel.

2.2.3.3 Western Blot

Background

The Western blot (also called immunoblot) is a technique to identify proteins that were separated by SDS-PAGE (→2.2.3.2) using specific anti-sera. Applying the "Semidry"-method, proteins are electrically transferred out of the gel and onto a nitrocellulose or PVDF (polyvinyl-difluoride) membrane. Then the membrane is blocked and incubated with a 1st antibody solution. The primary antibody specifically binds to one or more epitopes of the target protein and can be recognized by a secondary (2nd) peroxidase-linked anti-immunoglobulin G (IgG) - antibody. The peroxidase catalyzes the conversion of chromogenic substances. The resulting light reaction can be detected by exposure to an X-ray film.

Material

- Blotting buffer	20 % methanol
	3 g Tris
	14.5 g glycine
	Ad 1000 ml $H_2O_{dest.}$
- 10 x TBST	0,5 % Tween-20
	100 mM NaCl
	10 mM Tris-HCl (pH 8,0)
- Skim milk buffer	5 % (2.5 g) in 50 ml TBST buffer
- Detection kit ECL^{PLUS}	solution B: solution A (1:40) 100 µl:4 ml

After electrophoresis mass separation, proteins were electro-transferred from polyacrylamide gels onto a Hybond™-P PVDF membrane using a "Semi-dry" method. The membrane was activated with methanol and then equilibrated with $H_2O_{dest.}$. Gel was placed in contact with the membrane. They were then sandwiched together between 6 layers of Whatman paper which had been soaked in blotting buffer. A "Semi-Dry" transblot electrophoresis apparatus was used for the transfer. When the "Semi-Dry PowerPac 200" (Biorad Laboratories) was used, it was run at constant voltage (18V) for 30 min (60 min for 2 gels). When the ECL "Semi-Dry transfer unit" (GE Healthcare) was used, it was run at constant power (x cm^2 of gel (total) X 0,8 = needed mA) for 60 minutes. The membrane was then incubated in 5% skim milk buffer for 30 min at room temperature for saturation of unspecific binding sites. Then the specific primary antibody (→ Table 5) in 1x TBST was incubated overnight with constant shaking at 4° C. The membrane was washed 6 times (5 min each) with TBST and the secondary antibody (peroxidase conjugated) (→Table 5) was subsequently added for 30 min. The membrane was washed again 6 times with TBST. The antibody-protein complex was visualised with an enhanced chemoluminescence ECL-plus kit by incubating the membrane for 3 min in the ECL-solution (solution B: solution A = 1:40) and drying it with Whatman paper. Then it was wrapped in a transparent foil, exposed to X-ray films which were developed in the film processor machine.

A membrane can be used several times; if e.g. 2 different proteins should be investigated in the same approach to compare the amounts. Therefore, the membrane was washed twice for 10 min in $H_2O_{dest.}$., then it was washed twice for 30 min in "Re-blot Plus Strong" solution (diluted 1:10 in $H_2O_{dest.}$) in order to break the bonds between protein and antibody. Afterwards the membrane was washed 3 x 10 min in 1x TBST and the incubation process with 1st and 2nd antibodies was repeated (→described above).

2.2.3.4 Coomassie Blue staining of SDS-polyacrylamide (SDS-PAGE) gels

SDS-PA gels were submerged in Coomassie solution and stained for 30 min with gently rocking at room temperature. Coomassie solution was removed and the gels were discoloured by washing with discoloring buffer until protein bands of interest were clearly visible and no Coomassie blue background staining was present.

- Coomassie solution	Coomassie Brillant blue G-250	1,5 g
	Methanol	455 ml

	cetic acid 99 %	80 ml
	A. dest ad	1000 ml
- Discoloring buffer	Methanol	45 %
	Acetic acid	99 % 10 % in A. dest

2.2.3.5 Protein Quantification

2.2.3.5.1 Determination of protein concentration by Bradford assay

Five µl of protein solution diluted 1:20 in A. dest or protein standard dilutions were mixed with 250 µl Coomassie Brilliant Blue reagent in a 96well plate and incubated for 5 min at room temperature. By binding to proteins the absorption maximum of Coomassie Blue is shifted from a wave length of 465 nm to 595 nm. Absorption of protein samples was measured at a wave length of $\lambda = 595$ nm in an ELISA reader. Since protein concentrations are in linear relation to the extinction measured, a standard curve using the extinction of the protein standard dilutions was created that allowed to determine the protein concentration of the samples.

2.2.3.5.2 Band intensity quantification by ImageQuant TL

Intensities of signals detected by Western blot analysis were quantified using a ScanJet 4100C scanner (Hewlett-Packard) and the Image Quant TL software (GE Healthcare).

2.2.3.6 Statistical Analysis

Densitometric analysis of immunoblots was done by ImageQuant software TL v2005 (Amersham Biosciences, Freiburg, Germany) from three independent experiments (n=3). Quantification of immunoblot signals was done by means of the ImageQuant Program (Image Quant Analysis, Molecular Dynamics). Statistical analysis was performed with Prism software (Graphpad Software, San Diego, CA) using the unpaired two-tailed t-test for pairwise comparisons, where the control condition was set to 100%. The *y*-axis values are shown in percentage (%), and error bars denote S.E. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; NS, non significant. LC3-II was measured relative to actin signals to determine endogenous LC3-II levels.

2.3 Animal Experiments

- *For the trehalose study:*

Intraperitoneal infections of mice were performed as previously described in Mok *et al.*, 2006). Trehalose or the control disaccharide sucrose was thereafter continuously administered as 4% solutions via the drinking water (*ad libitum*). Spleens of individual mice were analysed in immunoblot at different time points for PrP^{Sc} and incubation times to clinical prion infection monitored.

- *For the tamoxifen study*

5 to 6 weeks old female CD1-mice were intracerebrally (i.c.) inoculated with 20ml of a 10-5 diluted brain homogenate prepared from a terminally diseased CD1-mouse infected with the scrapie strain 139A (courtesy of R.H. Kimberlin, Edinburgh, UK) as previously described (Mok *et al*. 2006, Riemer *et al*. 2008). Tamoxifen-citrat (Sigma-Aldrich, Taufkirchen, Germany) was administered as ingredient of the chow pellet (Ssniff, Soest, Germany). 5% Sucrose was added to enhance drug-acceptance. Tamoxifen-treatment started 50 days post infection (dpi) at a concentration of 66,6 mg/kg bodyweight (calculated for mice weighing 30 g and a medium need of food of 5 g per day) which is the equivalent for 400mg/kg chow pellet. Mice were sacrificed at 125 dpi or at the terminal stage of disease, at which animals would naturally succumb to disease within next 48 hours. Survival times were monitored. The animal experiments and care protocols were approved by the institutional review committee Landesamt für Gesundheit und Soziales (Berlin, Germany).

Animal experiments were carried out in collaboration with Dr. Michael Baier (Project Neurodegenerative Diseases, Robert-Koch- Institut, Berlin, Germany).

3 Results

3.1 Analysis of interplay between autophagy induction and cellular prion infection

Several studies suggest a crucial role of autophagy in neurodegenerative diseases, including Alzheimers´s disease, Parkinson´s disease, tauopathies, and polyglutamine expansion diseases like Huntingon´s disease. Upregulated autophagy seems to act as a cellular defense mechanism by degrading aggregate-prone proteins causing disease. Recent studies showed that treatment of prion-infected cells with Imatinib activates lysosomal degradation of PrP^{Sc} and that Imatinib can induce the formation of autophagosomes (Ertmer *at al.*, 2004, Ertmer *at al.*, 2007). These two studies, however, did not determine whether the activation of the autophagic pathway *per se* has an anti-prion effect as a consequence. Therefore, this study focused on elucidating the interconnection between prion infection and autophagic activation via trehalose, an autophagy inducer that in fact has been seen to accelerate the clearance of other aggregate-prone proteins, e.g. of mutant huntingtin and α-synuclein, by activating autophagy in an mTOR independent manner (Sarkar *at al.*, 2007a). Here, the impact of autophagic induction on cellular PrP^{Sc} is studied (Aguib *et al.* 2009).

3.1.1 Autophagy induction and reduction of PrP^{Sc} by trehalose

Based on the finding that clearance of mutant huntingtin and α-synuclein is accelerated by activating autophagy in an mTOR independent manner (Sarkar *et al.*, 2007a), it was investigated whether trehalose-activated autophagy has the potential to reduce cellular levels of PrP^{Sc}. Therefore, neuronal cells persistently infected with prions were treated with rising concentrations of trehalose, varying from 0 to 150 mM. In immunoblot analysis of prion-infected mouse cells, the un-, mono- and di-glycosylated forms of PrP^{c} show signals at ~25, ~30 and ~35 kDa, while PrP^{Sc}, already N-terminally truncated by the cell, provides signals at 19, 24 and 27-30 kDa after proteinase K (PK) digestion. A reduction of PrP^{Sc} when cells were treated with 100 mM trehalose was observed (→Figure 21 A). To further study induction of autophagy by trehalose, levels of microtubule-associated protein 1 light chain 3 (LC3-I and -II) were measured. Cytosolic LC3-I, which is generated by post-translational processing of endogenous LC3, is converted into LC3-II upon induction of autophagy. LC3-II is associated with

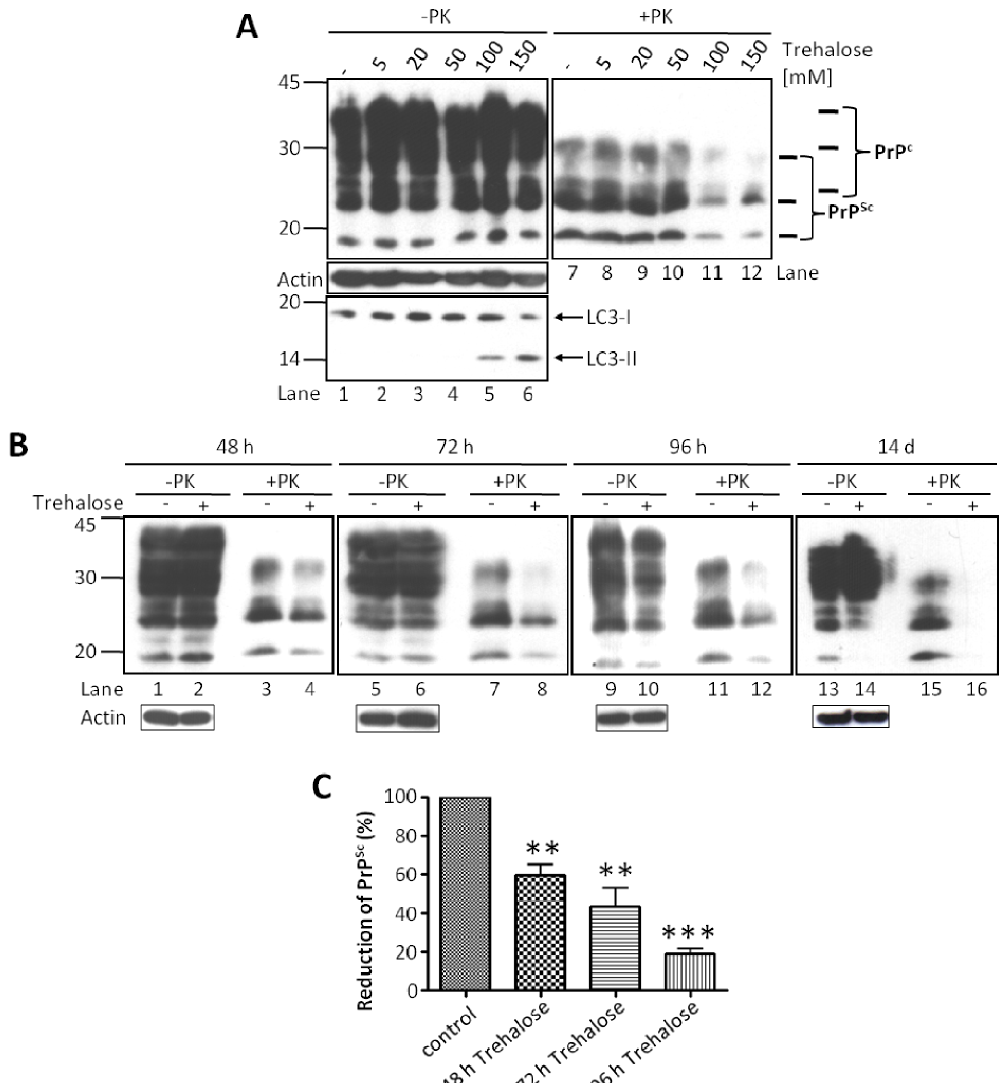

Figure 21. Dose- and time-dependent reduction of PrP^{Sc} and induction of autophagy by trehalose. (A) ScN2a cells were either mock-treated or treated with different concentrations of trehalose. Cells treated for 72 hours (h) were PK digested and PrP was visualised by immunoblotting using anti-PrP mAb 4H11 (upper panels). Lysates of ScN2a cells treated for 48 h with trehalose were probed with anti-LC3 mAb in immunoblot to analyse induction of autophagy (lower panel). The un-, mono- and di-glycosylated forms of PrP^{c} show signals at ~25, ~30 and ~35 kDa (lanes 1 to 6). PrP^{Sc}, already N-terminally truncated by the cell, provides signals at 19, 24 and 27-30 kDa after proteinase K (PK) digestion (lanes 7 to 12). Trehalose induces cellular PrP^{Sc} reduction and induces autophagy in a dose-dependent manner. **(B)** ScN2a cells were either mock-treated (control) or treated with 100 mM trehalose for 48, 72, 96 h and 14 days (d). Upon PK digestion, PrP was visualised by immunoblotting using anti-PrP mAb 4H11. **(C)** PrP^{Sc} signals in cells treated with 100 mM trehalose (for 48, 72 and 96 h) are expressed as percentage of control and represent the mean ± S.E. of three independent experiments (**: $p<0.01$; ***: $p<0.001$). Trehalose induces time-dependent reduction of cellular PrP^{Sc}. Published in (Aguib *et al.* 2009).

autophagosome membranes and the amount of LC3-II correlates with the extent of autophagosome formation (Kaybeya *et al.*, 2000). Thus, increase in the level of LC3-II can be used as a marker for autophagy induction. Similar to the reduction of PrP^{Sc} an increase in LC3-II levels was observed at trehalose concentrations of 100 mM or higher (→Figure 21 A, lower panel, lane 5 and 6). As 100 mM trehalose treatment was

necessary for both activating autophagy and reducing PrP^{Sc}, this strongly indicated a correlation between autophagy induction and reduction of cellular PrP^{Sc}. This feature was also observed in other neuronal and non-neuronal cell lines persistently infected with RML prions or the 22L prion strain, respectively (*data not shown*).

Next, it was analysed whether 100 mM trehalose reduces PrP^{Sc} in a time-dependent manner. ScN2a cells were treated with 100 mM trehalose for 48, 72 and 96 h and analysed by immunoblotting (→Figure 21 B). Quantification revealed a time-dependent reduction of PrP^{Sc} upon trehalose treatment (→Figure 21 C). Long-term treatment of cells with 100 mM trehalose (14 days) showed that reduction of PrP^{Sc} is not a transient phenomenon (→Figure 21 B, right-hand panel).

Taken together, these results indicate that activation of cellular autophagy by trehalose leads to a time- and dose-dependent reduction of PrP^{Sc} in prion-infected cells.

3.1.2 Characterization of trehalose-induced autophagy

So far it was observed that reduction of PrP^{Sc} by trehalose treatment can only be detected when in parallel autophagy is induced (i.e. with a concentration of 100 mM trehalose; see Figure 21). To characterize the observed induction of autophagy in more detail, cells were treated with 100 mM trehalose for 48 h and subsequently analysed by immunoblotting (→Figure 22 A**,** lane 1 and 2). Increase amounts of LC3-II were clearly detectable. However, increased amounts of LC3-II do not necessarily indicate total autophagic flux but can also result from impaired autophagosome-lysosome fusion. Therefore, it is important to measure and compare the amounts of LC3-II in the presence and absence of bafilomycin A1, which is supposed to block autophagosome-lysosome fusion (Yamamoto *et al.*, 1998), in the same experimental set. First, it had to be established that at the dosis used for bafilomycin A1 has achieved a ceiling effect in terms of its ability to induce LC3-II. Only then can one deduce that the addition of trehalose may be acting not on the pathway of autophagosome-lysosome fusion, but that of autophagosome formation. In (→Figure 22 B) a dose kinetic experiment with bafilomycin A1 is shown. LC3-II was assayed in the presence of rising concentrations of bafilomycin A1 (0, 50, 100, 150, 200 and 250 nM) in ScN2a cells. Bafilomycin A1 resulted in the expected dose-dependent increase of LC3-II. Concentration higher than 200 nM results in no increase in LC3-II indicating that bafilomycin A1 treatment has achieved a ceiling effect. This demonstrated that the dose of bafilomycin A1 used in the following assay is saturating for LC3-II levels. For this assay, ScN2a cells were either mock-treated or treated with 100 mM

trehalose for 48 h with simultaneous bafilomycin A1 treatment and analysed by immunoblotting (→Figure 22 C). Treatment of ScN2a cells with 100 mM trehalose increased LC3-II levels about 7-fold, which is typically achieved upon induction of autophagy (Kaybeya *et al.*, 2000). Co-treated cells show a significantly increased amount of LC3-II compared to cells treated with bafilomycin A1 or trehalose alone, confirming that trehalose induced LC3-II increase results from increased autophagosome formation and not from impaired autophagosome-lysosome fusion (→Figure 22 D).

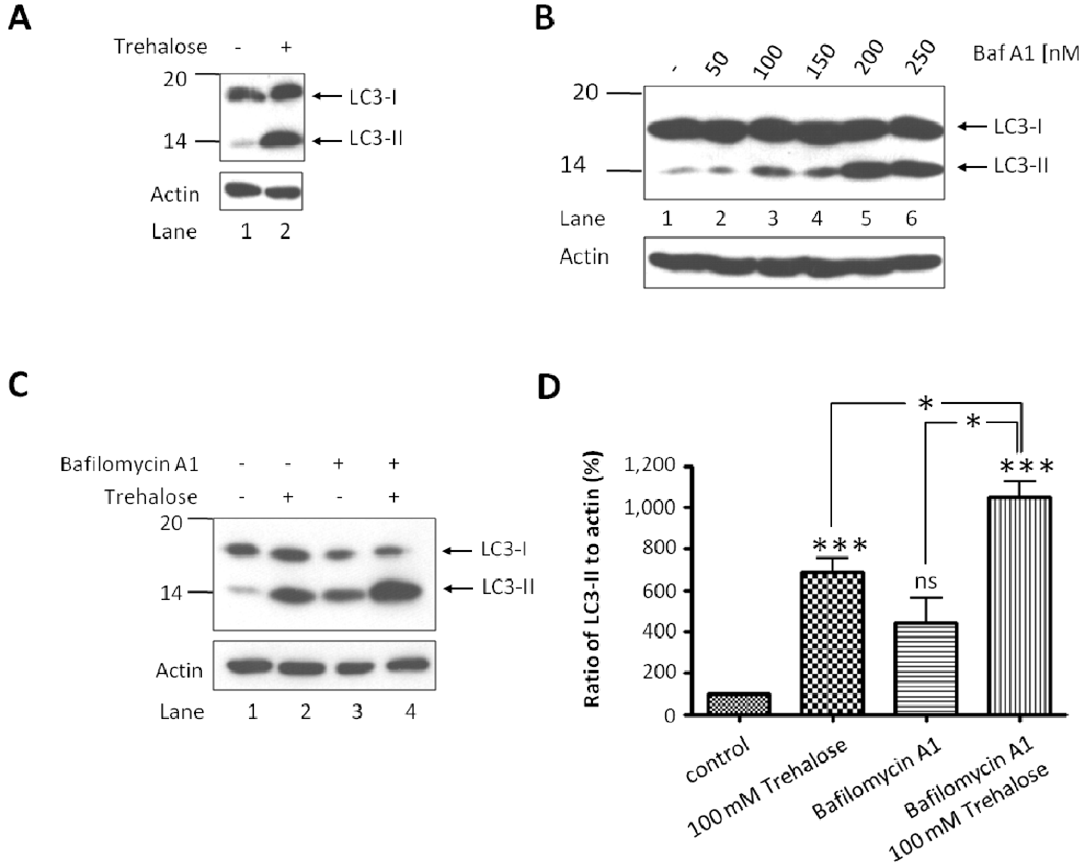

Figure 22. Characterisation of trehalose-activated autophagy. (A) ScN2a cells were either mock-treated (control, lane 1) or treated with 100 mM trehalose for 48 h (lane 2). Cells were analysed by immunoblotting using anti-LC3 mAb. **(B)** Dose kinetic experiment with bafilomycin A1. LC3-II in the presence of rising concentrations of bafilomycin A1 (0, 50, 100, 150, 200 and 250 nM) in ScN2a cells. Concentration higher than 200 nM results in no increase in LC3-II. **(C)** ScN2a cells were either mock-treated (control, lane 1), treated with 100 mM trehalose for 48 h (lane 2), with bafilomycin A1 (200 nM) for 4 h prior to lysis of cells (lane 3), or treated with 100 mM trehalose for 48 h with simultaneous bafilomycin A1 (200 nM) treatment for 4 h prior to lysis of cells (lane 4). Cells were analysed by immunoblotting using anti-LC3 mAb. **(D)** Levels of endogenous LC3-II in compound-treated cells are expressed as percentage of control and represent the mean ± S.E. of three independent experiments (ns: not significant; *: $p<0.05$; ***: $p<0.001$). Trehalose induces an increase (~7-fold) of endogenous LC3-II and activates autophagy beyond autophagosome-lysosome fusion. Published in (Aguib *et al.* 2009).

To further investigate induced autophagosome formation by trehalose, confocal laser microscopy was performed with N2a and ScN2a cells stably over-expressing LC3 fused

to green fluorescent protein (N2aGFP-LC3 and ScN2aGFP-LC3) (→Figure 23). Cells treated with trehalose or imatinib, which served as positive control for autophagosome formation (Ertmer *at al*., 2007), clearly showed punctuate GFP staining, indicating the association of GFP-LC3 with autophagosomal membranes as a result of induction of autophagy. Co-staining with lamp-1 revealed that GFP puncta observed upon trehalose treatment partially co-localised with lysosomes, indicating autophago-lysosome formation.

Taken together, these results reveal that trehalose treatment strongly enhances the autophagic flux in prion-infected and uninfected neuronal cells.

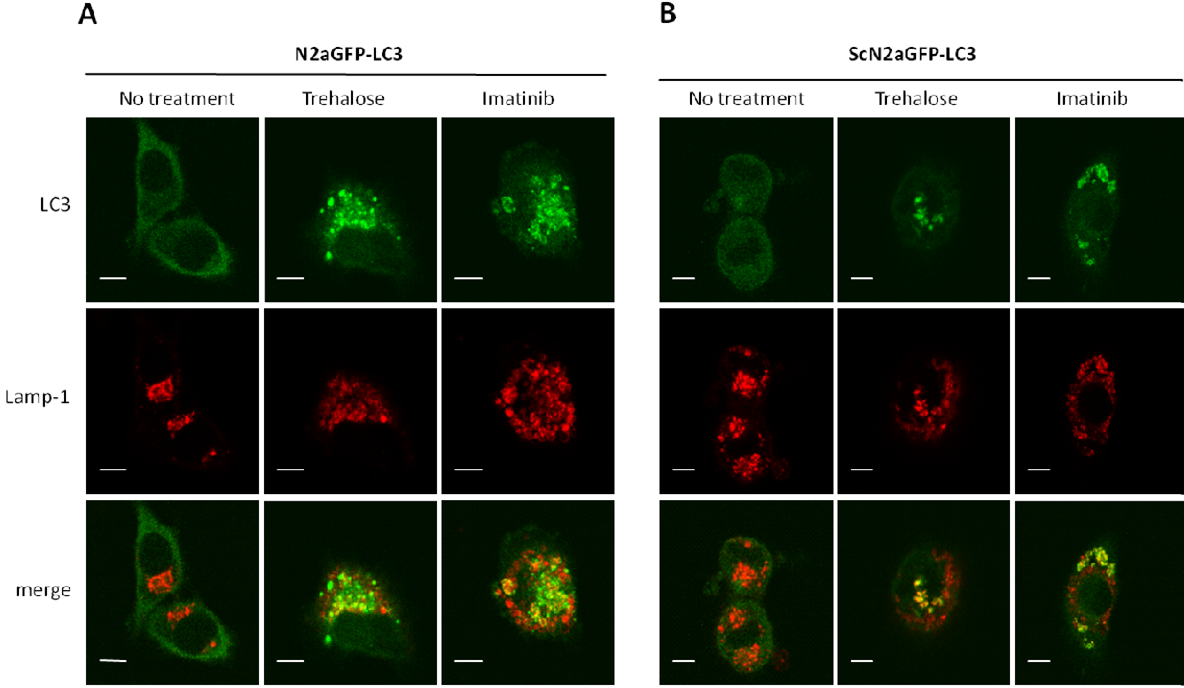

Figure 23. Autophagosome formation following trehalose treatment. N2a **(A)** and ScN2a **(B)** cells, which stably over-express GFP-LC3 (N2aGFP-LC3 and ScN2aGFP-LC3), were either mock-treated (left-hand column), treated with 100 mM trehalose (middle column) or 10 µM imatinib (right-hand column) for 48 h and subsequently analysed by confocal microscopy. Cells treated with the tyrosine kinase inhibitor imatinib served as positive control for autophagosome formation.1 An even distribution of GFP-LC3 over the entire cytoplasm (left-hand column of each figure, upper panel) was observed in mock-treated cells. In comparison, cells treated with either trehalose or imatinib (middle and right-hand column of each figure, upper panel) clearly showed punctuate GFP staining, indicating the association of GFP-LC3 with autophagosomal membranes as a result of induction of autophagy. Co-staining with lamp-1 (middle and lower panels) revealed that GFP puncta as observed upon trehalose or imatinib treatment partially co-localise with lysosomes, indicating autophago-lysosome formation. GFP auto-fluorescence (LC3) is shown in the upper panels, Cy3-labelled lysosomes (lamp-1) in the middle panels, and the overlay in the lower panels (merge). Bars in the lower left of each panel correspond to 5 µm. Published in (Aguib *et al.* 2009).

3.1.3 Exposition of post-nuclear lysates of prion-infected cells to trehalose

In light of the previously described role of trehalose as a molecular chaperone, it was important to discard the possibility that the effect of the drug is not directly on the protein. Analysis of the effect of trehalose on the resistance to proteinase K of protein in a cell-free system would strengthen the study. For this purpose, post-nuclear cell lysates of untreated cells were prepared first and then incubated for 96 hrs with 100 mM trehalose and analyzed for PrPSc-levels (→Figure 24). No alteration in signal intensities of PrP^{Sc} could be observed in comparison to control cell lysates (lanes 3 and 4). This confirmed that trehalose does not directly mediate the reduction of PrP^{Sc} (by e.g. destabilizing the PrP^{Sc} oligomer via chemical interactions), rather active cellular mechanisms are necessary for tamoxifen to exert its effect on PrP^{Sc}.

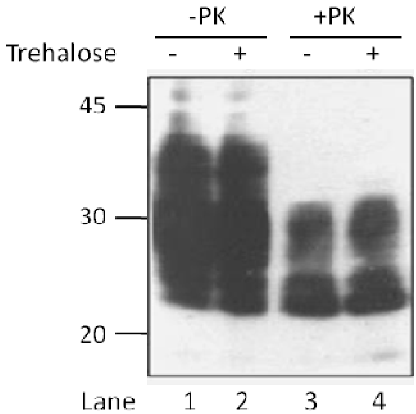

Figure 24. Trehalose treatment of cell lysates. Post-nuclear lysates from untreated cells were incubated either with control- treatment or with tamoxifen at room temperature for 96 hrs with 100mM trehalose, respectively. Samples were analyzed by immunoblotting using anti-PrP mAb 4H11.

3.1.4 Cell viability and level of PrP^c upon trehalose treatment

Cell viability and levels of PrP^c are factors that influence levels of cellular prion infection. These two aspects were studied, to analyse whether they are influenced upon treatement with trehalose.

To study whether treatment of ScN2a cells with 100 mM trehalose affects viability of cells, a trypan blue assay was performed. Reduction in viability or toxic effects upon trehalose treatment was not detected under the used conditions (→Figure 25).

To analyse whether trehalose-induced autophagy is involved in the degradation of the total pool of PrP (PrP^c and PrP^{Sc}) or only of the PK-insoluble component, a control experiment was performed first and uninfected N2a cells were treated with 100 mM trehalose for 96 hours (→Figure 26). In immunblot analysis the un-, mono- and di-

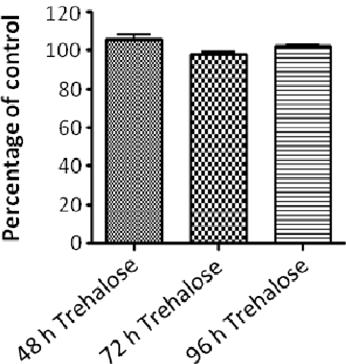

Figure 25. Cell viability upon trehalose treatment. Trypan blue assay to analyse whether 100 mM trehalose is toxic for cells. The percentage of viable cells treated with 100 mM trehalose (for 48, 72 and 96 h) was calculated and is expressed as percentage of viable, mock-treated cells (control) for each time point. Values represent the mean ± S.E. of three independent experiments.

glycosylated forms of PrP^c were not influenced by trehalose treatment. In a further approach ScN2a cells were treated with 100 mM trehalose for 96 hours followed by a solubility assay via ultracentrifugation. Levels of soluble PrP^c in the supernatant fraction [S] of trehalose-treated cells are equal compared to un-treated cells (→Figure 26B, lanes 1 and 2). On the other hand, trehalose treatment caused a reduction of insoluble PrP^{Sc} detected in the pellet [P] of the same cell lysates (→Figure 26 B, lanes 3 and 4). This confirms the statement that trehalose-induced autophagy leads to the degradation of insoluble PK-resistant PrP^{Sc} aggregates and that the observed PrP^{Sc} reduction cannot be related to a toxicity effect or an effect of trehalose on the proteinase K resistance of the protein.

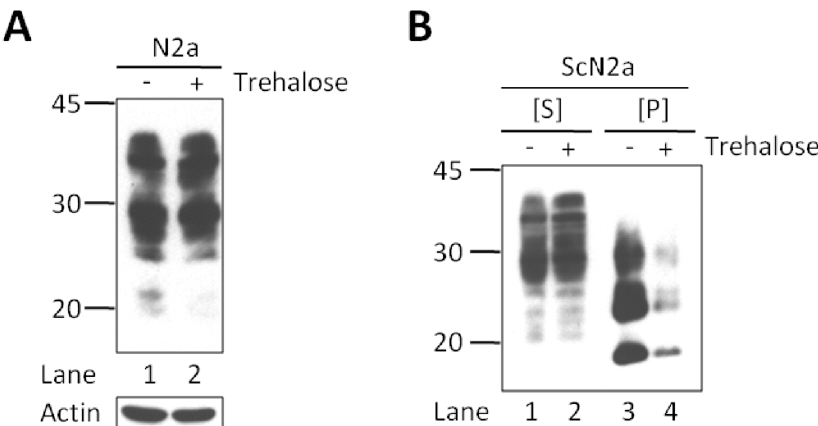

Figure 26. Analysis of trehalose-induced autophagy on degradation of PrP^c and PrP^{Sc}. **(A)** Uninfected N2a cells were treated with 100 mM trehalose for 96 h. Cell lysates were analysed by immunoblotting using anti-PrP mAb 4H11. Level of PrP^c was not affected upon trehalose treatment. **(B)** Solubility analysis of PrP^{Sc} upon treatment with trehalose. ScN2a cells were treated with 100 mM trehalose for 96 h followed by solubility assay via ultracentrifugation. Levels of soluble PrP^c in the supernatant fraction [S] of trehalose-treated cells were comparable to that of mock-treated cells. In contrast, trehalose treatment caused a clear reduction of insoluble PrP^{Sc} detected in the pellet fraction [P] (lanes 3 and 4). This indicates that trehalose-induced autophagy leads to reduction of insoluble PK-resistant PrP^{Sc} aggregates while not influencing PrP^c.

3.1.5 Pharmacological impairment of autophagy and the effect of trehalose

In order to further confirm the connection of trehalose-induced autophagy and the resulting degradation of PrP^{Sc}, the effect of 3-MA, an inhibitor of phosphatidylinositol-3-kinase (Blommaart *et al*., 1997) known to inhibit cellular autophagy, on the anti-prion effect of trehalose was investigated. ScN2a cells were either mock-treated, treated with 100 mM trehalose, or co-treated with 100 mM trehalose and 10 mM 3-MA for 48 h and analysed by immunoblotting (→Figure 27 A and B). Compared to cells treated with trehalose alone (lane 2), induction of autophagy by trehalose was strongly impaired by co-treatment with 3-MA (lane 3). The same approach was used to analyse the effect of 3-

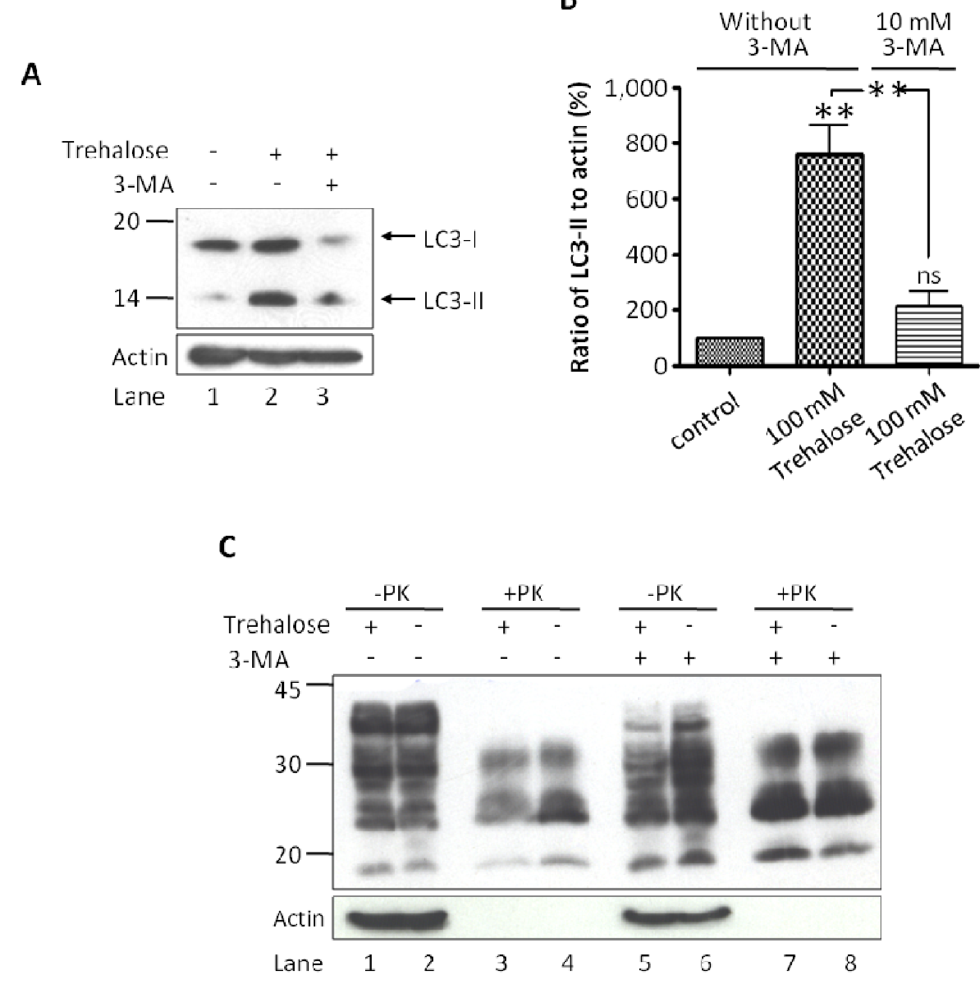

Figure 27. Effect of trehalose is attenuated by pharmacological impairment of autophagy activity. (A) ScN2a cells were either mock-treated, treated with 100 mM trehalose or co-treated with 100 mM trehalose and 10 mM 3-MA for 48 h. Cells were analysed by immunoblotting using anti-LC3 mAb. **(B)** The level of endogenous LC3-II in trehalose treated cells, with or without co-treatment with 10 mM 3-MA, is expressed as percentage of mock-treated cells (control) and represents the mean ± S.E. of three independent experiments (**: $p<0.01$; ns: not significant). **(C)** ScN2a cells were either mock-treated, treated with 100 mM trehalose, 10 mM 3-MA or co-treated with 100 mM trehalose and 10 mM 3-MA for 48 h. Upon PK digestion, PrP was visualised by immunoblotting using anti-PrP mAb 4H11. Reduction of PrP^{Sc} is not observed when cells are co-treated with trehalose and 3-MA. Published in (Aguib *et al.* 2009).

MA on the anti-prion effect of trehalose. In fact, ScN2a cells co-treated with 100 mM trehalose and 10 mM 3-MA showed no reduction of PrP^{Sc} (→Figure 27 C, compare lane 7 and 8). These results demonstrate that inhibition of the autophagic pathway impairs the PrP^{Sc} reducing effect of trehalose.

3.1.6 Genetic impairment of autophagy (Atg5 knock-down) and the effect of trehalose

As pharmacological inhibition of autophagy might be affected by non-warranted side effects of used drug, siRNA designed to down-regulate the expression of Atg5 was additionally used to knock down the *Atg5* gene, an essential gene implicated in the autophagic pathway. This experiment would give the missing evidence about the connection beween autophagy and the observed effect on PrP^{Sc}.

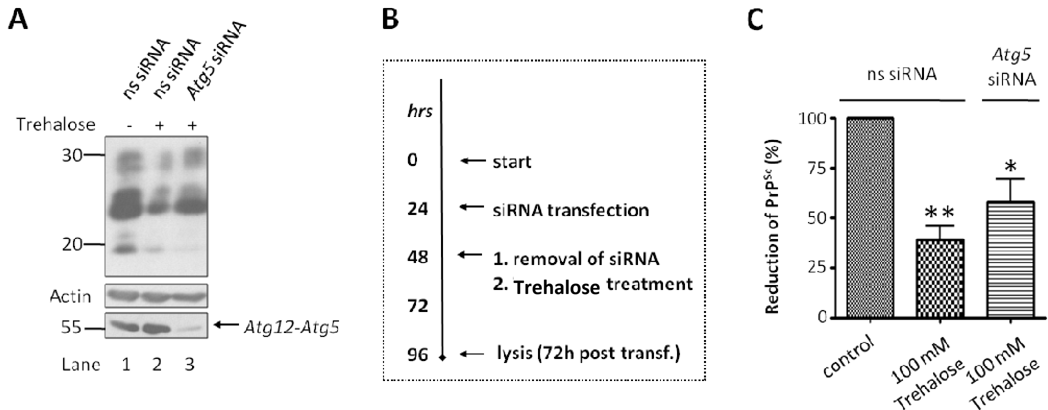

Figure 28. Effect of trehalose is attenuated by genetic impairment of autophagy activity. (A) ScN2a cells were either transfected with non-silencing (ns) siRNA (lane 2) or siRNA targeting *Atg5* (lane 3) and then treated with 100 mM trehalose. As a control, cells were transfected with ns siRNA and left un-treated (lane 1). Chronological course of the experiment is schematically shown on the right. To confirm successful knock down of Atg5, lysates were probed with anti-Atg5 mAb by immunoblotting (lower panel). PrP^{Sc} was visualised by immunoblotting using anti-PrP mAb 4H11. **(B)** Chronological course of the experiment. **(C)** PrP^{Sc} signals in trehalose treated cells either transfected with ns siRNA or *Atg5* siRNA are expressed as percentage of mock-treated cells transfected with ns siRNA and represent the mean ± S.E. of three independent experiments (ns: not significant; *: $p<0.05$; **: $p<0.01$). Transfection of trehalose-treated cells with *Atg5* siRNA reproducibly counteracts the anti-prion effect when compared to trehalose-treated cells transfected with ns siRNA. Published in (Aguib *et al.* 2009).

ScN2a cells were treated with non-silencing siRNA, with non-silencing siRNA and 100 mM trehalose, or co-treated with anti-*Atg5* siRNA and 100 mM trehalose and analyzed by immunoblotting (→Figure 28 A). Chronological sequence of the experiment is displayed in →Figure 28 B. Trehalose treatment led to the expected reduction of PrP^{Sc} (compare lanes 1 and 2). The reduction of PrP^{Sc} was strongly impaired by co-treatment with anti-*ATg5* siRNA (lane 3). Densitometric quantification was performed and analysed in →Figure 28 C.

This provides evidence that PrP^{Sc} reduction upon trehalose treatment depends on the autophagic activity in the cell.

3.1.7 Trehalose-treatment of prion-infected mice

Whether trehalose had the potential to interfere with prion infection in *in vivo* models, was still not addressed. This was studied (*in collaboration with Prof. M. Baier, RKI, Berlin*) by continuously applying trehalose, and sucrose as a control, to the drinking water of mice which were intraperitoneally infected with a high dose of prions. Individual mice were sacrificed and tested for the amount of PrP^{Sc} in the spleen (→Figure 29). Immunoblot analysis of spleens obtained from trehalose- or

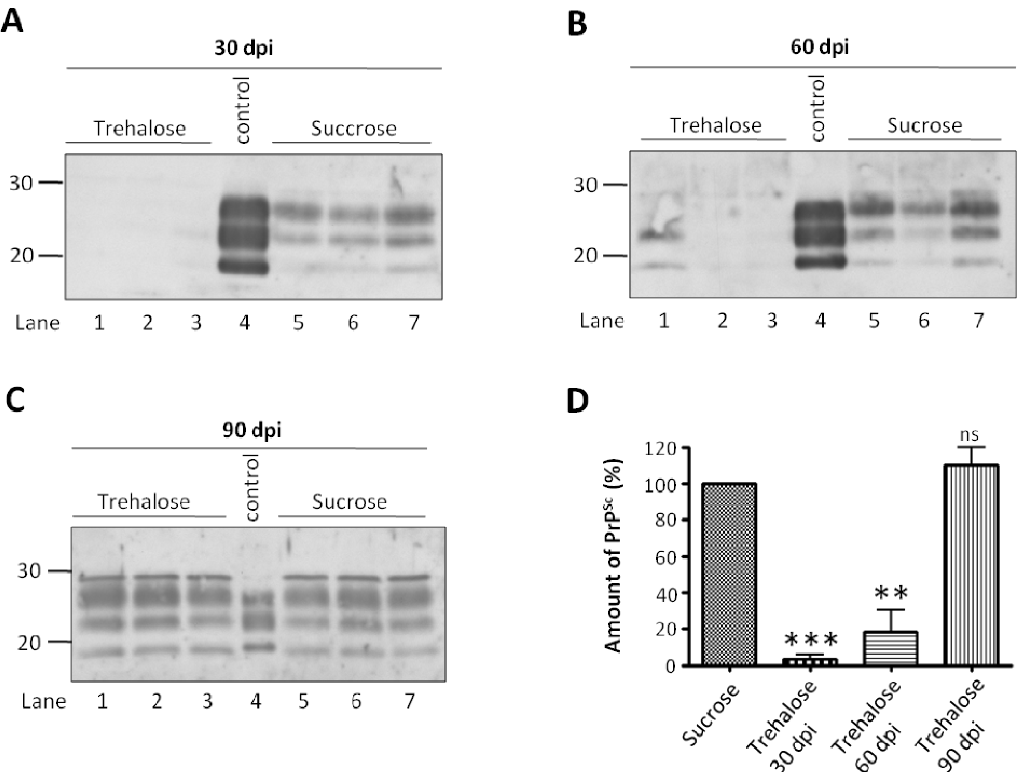

Figure 29. PrP^{Sc} in spleens of trehalose- and sucrose-treated mice. Immunoblot detection of PrP^{Sc} in spleens of trehalose- and sucrose-treated mice 30 **(A)**, 60 **(B)** and 90 **(C)** days post infection (dpi), respectively. Lanes 1-3 denote trehalose-treated mice, lane 4 is a positive control for PrP^{Sc}, lanes 5-7 show sucrose-treated mice. **(D)** PrP^{Sc} signals in spleens of trehalose-treated mice are expressed as percentage of sucrose-treated mice and represent the mean ± S.E. of three independent experiments (ns: not significant; **: $p<0.01$; ***: $p<0.001$). Reduced levels of PrP^{Sc} were detected both after 30 and 60 dpi, whereas equal PrP^{Sc} levels were detected after 90 dpi. Published in (Aguib *et al.* 2009).

sucrose-treated mice revealed reduced PrP^{Sc} deposition in the trehalose-treated group at time points 30 and 60 days post infection (→Figure 29 A, B and D). However, at day 90 post infection PrP^{Sc} levels were equal between sucrose- and trehalose-treated animals (→Figure 29 C and D). In addition, the survival rates of trehalose-treated mice were not

significantly prolonged when compared to sucrose-treated mice (*data not shown*). Although these results show that trehalose treatment *in vivo* has the potential to reduce PrP^{Sc} accumulation in the periphery, it also suggests that levels of trehalose obtained in the central nervous system are probably not high enough to be effective.

3.2 Analysis of the anti-prion potential of SERM and autophagy inducer Tamoxifen

The study performed with trehalose confirmed that drug-induced activation of autophagy accelerates clearance of PrP^{Sc} in persistently prion-infected cells. However, the *in vivo* efficacy of trehalose seemed to be limited. In order to investigate a candidate with high *in vivo* potential, in respect to its ability to induce autophagy as well to its suitability for future therapies, the widely used anti-cancer drug tamoxifen was chosen. In previous studies, Tamoxifen was reported to induce autophagy in breast cancer cells (Bursch *et al*., 1996; Bilir *et al*., 2001; Pattingre *et al*., 2009; de Medina *et al*., 2009b). While the previous studies provide the evidence of a role of autophagy in persistent prion infection, the next study goes further to confirm a general role of autophagy in prion disease scenarios based on a study with the well characterized and established drug Tamoxifen.

3.2.1 Attenuation of primary prion infection upon tamoxifen-treatment

The previous study revealed and confirmed that drug-induced autophagy can accelerate clearance of PrP^{Sc} in persistently prion-infected cells. To further study the impact that activation of the autophagic pathway can have on prion infection, a further scenario of prion infection was addressed, namely, primary prion infections. For this purpose, the possibility to overexpress 3F4-tagged PrP^{c} was used to monitor specifically de novo formation of PrP^{Sc} (Vorberg *et al*., 2004a, b) and how it is influenced upon modulation of autophagy.

3F4-PrP expressing N2a cells (3F4-N2a) were treated with 10 µM imatinib, 100 mM trehalose, 10 mM lithium, 200 nM rapamycin, 3 µM tamoxifen and 100 nM insulin for 24 h or with serum deprived medium (-FCS), 10 mM 3-MA, 1 µM wortmannin and 200 nM bafilomycin A1 for 4 h. Cells were then incubated with brain homogenate (1 %) derived from terminally ill mice infected with prion strain 22L for 24h. Immunoblots using anti-PrP monoclonal 3F4 (→Figure 30, lanes 1-11) and 4H11 antibody (lanes 12-23) show that levels of de novo converted PrP^{Sc} are attenuated in cells treated with imatinib, trehalose, lithium and tamoxifen, while tamoxifen shows the most pronounced effect.

Interestingly, treatment with bafilomycin A1 showed reduced PrP^{Sc} amounts, too. To conclude, it was obvious that tamoxifen treatment also restricts primary cellular prion infections. The ability to restrict acute prion infection was a common feature of diverse autophagy inducers, of which tamoxifen had the strongest effect.

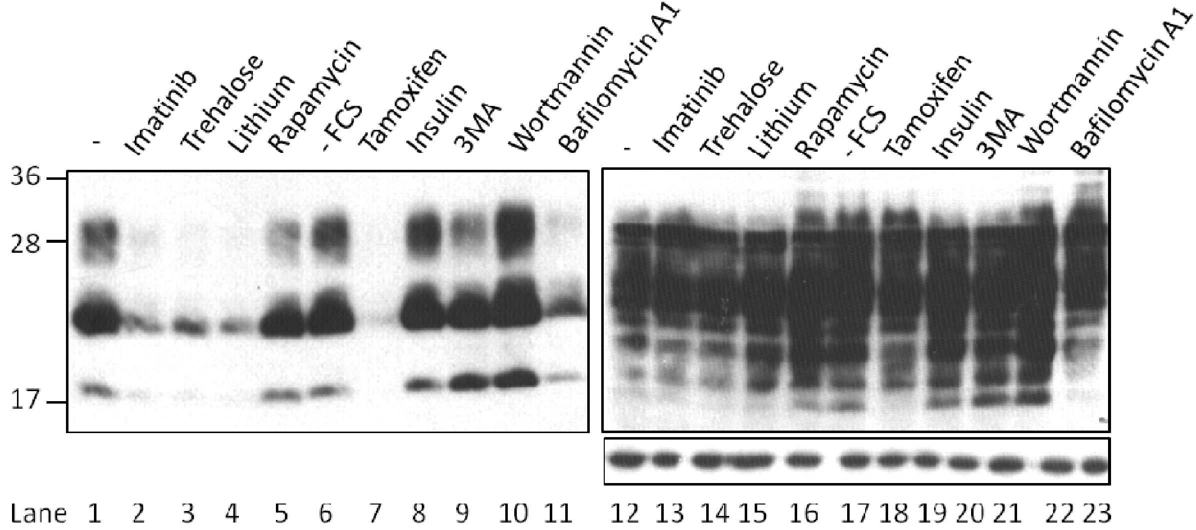

Figure 30. Tamoxifen & other autophagy modulators have an effect on primary prion infections. 3F4-PrP expressing N2a cells (3F4-N2a) were treated with 10 µM imatinib, 100 mM trehalose, 10 mM lithium, 200 nM rapamycin, 3 µM tamoxifen and 100 nM insulin or with serum deprived medium (-FCS), 10 mM 3-MA, 1 µM wortmannin and 200 nM bafilomycin A1 for 4 h. Cells were incubated with brain homogenate (1 %) derived from mice infected with prion strain 22L for 24h After removal of brain homogenate cells were passaged and postnuclear lysates of the cells were prepared (+/- PK-digestion) and subsequently analyzed by SDS-PAGE and immunoblot (here: passage1) using anti-PrP monoclonal 3F4, which detects primary formed PrP^{Sc} and not the inocculated PrP^{Sc} (lanes 1-11) and 4H11 antibody which detects both (lanes 12-23). Levels of primary converted PrP^{Sc} are reduced in cells treated with imatinib, trehalose, lithium, tamoxifen and bafilomycin A1.

3.2.2 Time kinetics of tamoxifen-treatment and progress of infection

To further analyze the effect of tamoxifen on prion infection, tamoxifen-treatment and *de novo* prion infection kinetics were performed. 3F4-N2a cells were treated with 3 µM tamoxifen at different time-points of de novo prion infection with prion strain 22L (→Figure 31**,** time scheme). Levels of newly converted PrP^{Sc} were reduced in cells treated with tamoxifen during or/and after incubation with the prion-infected brain homogenate (lanes 3-5). No changes were observed when treatment occurred only before incubation (lane 2). In summary, these short-term time kinetics' experiments visualize that inspite of tamoxifen treatment, PrP^{Sc} is internalized by the cells. Autophagic induction is of importance after prions are taken up by the cell and can strongly interfere

with de novo prion infections restricting the accumulation of intracellular aggregated prion protein.

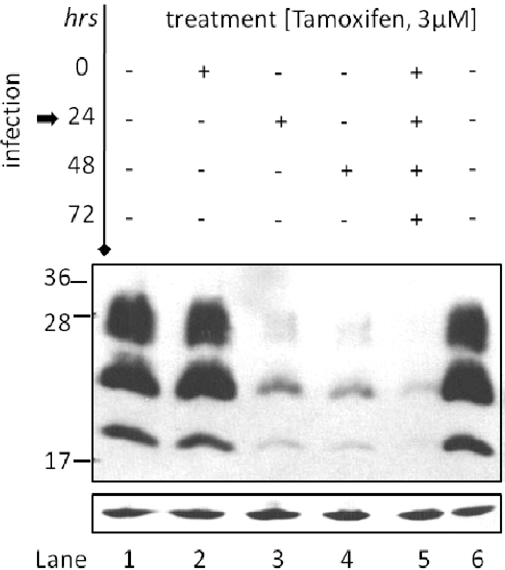

Figure 31. Time kinetics: Tamoxifen-treatment and primary prion infection. 3F4-N2a cells were treated with 3 µM tamoxifen at different time-points of primary prion infection (→ time scheme). For infection, cells were incubated for 24 hours with brain homogenate (1 %) derived from mice infected with prion strain 22L. After removal of brain homogenate cells were passaged and postnuclear lysates of the cells were prepared and subsequently analyzed by SDS-PAGE and immunoblot using anti-PrP monoclonal 3F4 (lanes 1-6). Levels of primary converted PrP^{Sc} are reduced in cells treated with tamoxifen during or after incubation with the prion-infected brain homogenate. No changes were observed when treatment occurred before incubation.

3.2.3 Tracking of PrP^{Sc} in tamoxifen-treated cells during primary prion infection

Initial time-points of cellular prion infection within hours upon infection are of big relevance, as alterations occurring to internalized PrP^{Sc} during primary infection can influence the progress of prion infection. In this experiment, the effect of PrP^{Sc} reduction via tamoxifen leading to clearance of prion infection was to be visualized via confocal microscopy. To trace uptake and degradation of PrP^{Sc}, N2a cells, treated with tamoxifen or left untreated, were incubated with 22L infected brain homogenate. Cells were then stained for immunofluorescence analysis under conditions specific for the detection of PrP^{Sc} (→Figure 32). Confocal fluorescence microscopy after 24 h of exposure to infected brain homogenate of both, N2a cells treated or untreated with tamoxifen, showed fluorescent aggregates inside a subset of cells (→Figure 32**,** 24h). At this early time-point, directly after inocculation, PrP^{Sc} was distributed in vesicular shapes. 72h after

infection a strong reduction of detectable PrP^{Sc} fluorescence in tamoxifen-treated cells is observed (→Figure 32**,** 72h). In control cells, the number of cells with detectable fluorescent particles increased and larger aggregates were visible. PrP^{Sc} was also located to the cellular membrane. In contrast, in tamoxifen-treated cells this progress seems to be constrained.

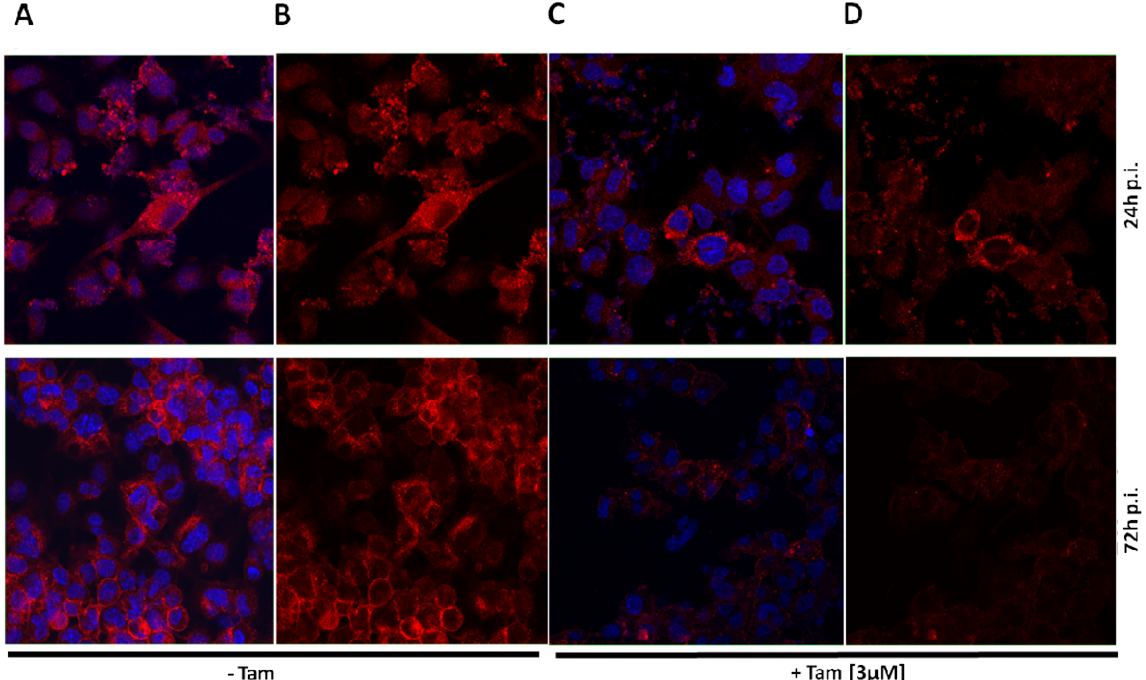

Figure 32. Immunofluorescent detection of PrP^{Sc} in tamoxifen-treated cells during primary prion infection. ScN2a cells were exposed to 22L-infected brain homogenate for 24h and treated with 3µM tamoxifen. Following removal of the brain homogenate, an immunofluorescence assay including a denaturation step with guanidinium chloride (6 M) to allow specific detection of PrP^{Sc} was performed at different time points: 24 h & 72 h after removal of brain homogenate. mAb 4H11 was used for staining. Mock-treated Cells were also exposed to infected brain homogenate were used as a negative control. Nuclear staining was performed with Hoechst 33342. Confocal microscopy images show merged PrP^{Sc} and nuclear staining (panels A and C) or PrP^{Sc} staining alone (panels B and D).

Compared to earlier time-points, only a weak punctuate intracellular fluorescence was visible in these cells.

These results go in line with previous immunoblot analysis revealing a significant decrease of PrP^{Sc} 72h days after exposure of tamoxifen-treated cells to prion- infected brain homogenates.

In summary, these stainings show that tamoxifen treatment did not hinder internalization of PrP^{Sc} by the cells, however, intracellular dissemination and propagation of PrP^{Sc} was changed.

3.2.4 The effect of tamoxifen treatment in persistent prion infections

It was now necessary to consolidate the observed effect of tamoxifen's ability to reduce PrP^{Sc} in primary infections, in the context of persistent prion infection and with the monitoring tools and procedures established in the first part of this work. The results presented in this paragraph are partially performed and reproduced within the frame of a Master thesis (Schäffler, 2009) that was accomplished under my supervision.

3.2.4.1 Time-dependent and robust clearance of PrP^{Sc} by tamoxifen.

The first experiments on tamoxifen and its effect on already persisting prion accumulation were performed in N2a cells persistently infected with prions (ScN2a). The outcome of dose finding experiments and viability examination showed the optimal dose-effect at an applied concentration of 3µM tamoxifen. When cells were treated with 3µM Tamoxifen a strong effect of tamoxifen on PrP^{Sc} reduction and no toxicity were observed (*data not shown*). As tamoxifen showed a strong effect already upon treatment for four days, kinetics of the tamoxifen-mediated effect were performed i.e. whether the effect is time-dependent and how rapidly reduction of prion infection level occurs. ScN2a cells were then treated with 3µM tamoxifen for 24h, 48h, 72h and 96 h and 120h analyzed by immunoblotting (→Figure 33 A). Control cells were treated with equal volumes of the solvent vehicle of tamoxifen, a mixture of DMSO and ethanol in the ratio 1:1. This allows for exclusion of effects solely caused by the solvent. Quantification revealed a time-dependent reduction of PrP^{Sc} upon tamoxifen treatment (→Figure 33 B). When cells were treated for 120h or up to 30 days, no PrP^{Sc} signals were detectable.

To rule out the possibility that PrP^{Sc} was present in cells after treatment with tamoxifen, but kept below the detection level of immunoblotting, treatment with tamoxifen was ended after 30 days and cells were cultivated without treatment for more than further 54 days. PrP-levels were monitored in the meanwhile. The absence of any signals in PK digested samples of previously tamoxifen treated cells indicates robust clearance of PrP^{Sc} by tamoxifen (→Figure 33 C, lanes 4 and 8). In contrast, control cells (treated with vehicle) still showed high infection level by that point in time (lanes 3 and 7). No recurrence of prion infection has occurred upon termination of treatment.

In order to determine whether the tamoxifen-mediated effect on PrP^{Sc} is dependent on cellular mechanisms or not, lysates of untreated cells were prepared first and then incubated with tamoxifen and analyzed for PrP^{Sc}-levels (→Figure 33 D). For this purpose, cells were grown to confluence. Post-nuclear lysates were prepared and then

incubated with tamoxifen for 24 h or for 96 h at room temperature. For tamoxifen treatment of lysates, same amounts of compound were used per sample as was used for treatment of living cells. Upon incubation of cell lysates with tamoxifen for 1 day (lane 6) or for four days (lane 8), no alteration in signal intensities of PrP^{Sc} could be observed in comparison to control cell lysates (lanes 5 and 7). This confirmed that tamoxifen does not

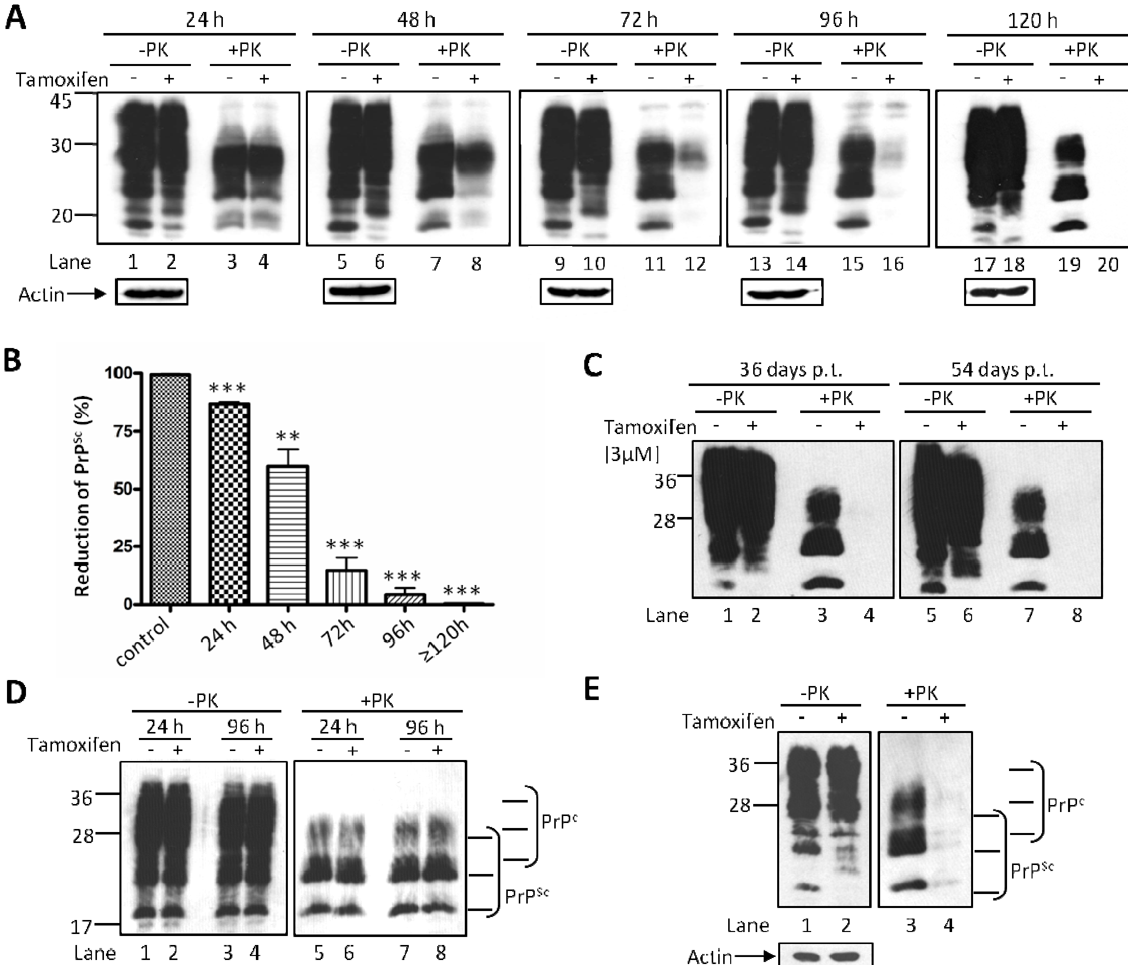

Figure 33. Tamoxifen is a potent anti-prion drug in ScN2a cells: time-dependent and robust clearance of PrP^{Sc}. (A) Monitoring time-dependent reduction of PrP^{Sc} by tamoxifen. ScN2a cells were either mock-treated (control) or treated with 3 µM tamoxifen for 24, 48, 72, 96 and ≥120 hours (h). Upon PK digestion, PrP was visualized by immunoblotting using anti-PrP mAb 4H11. **(B)** PrP^{Sc} signals in cells treated with 3 µM tamoxifen (for 24, 48, 72, 96 and ≥120 h are expressed as percentage of control and represent the mean ± S.E. of three independent experiments (**$p < 0.01$; ***$p < 0.001$). Reduction of cellular PrP^{Sc} upon tamoxifen treatment is time-dependent. **(C)** *Tamoxifen treatment leads to robust clearance of PrP^{Sc}*. ScN2a cells were either mock-treated or treated with 3 µM tamoxifen for 30 days, followed by cultivation without treatment for further 54 days. PrP^{Sc}-level in cells was monitored meanwhile by proteinase K digestion and immunblotting with anti-PrP mAb 4H11. Representative images are shown for 36 days and 54 days post treatment end (p.t.) (Adapted from T.Schäffler) **(D)** *Tamoxifen treatment of cell lysates.* Post-nuclear lysates from untreated cells were incubated either with control- treatment or with tamoxifen at room temperature for 1 or 4 days, respectively. Samples were analyzed by immunoblotting using anti-PrP mAb 4H11. (Adapted from T.Schäffler) **(E)** *Solubility analysis of PrP^{Sc} upon treatment with tamoxifen.* ScN2a cells were either mock-treated or treated with 3 µM tamoxifen for 72 h. Post-nuclear lysates were subjected to the detergent solubility assay. PrP in the supernatant (S) and in the pellet (P) were analyzed by Western blotting using anti-PrP mAb 4H11. (Adapted from T.Schäffler)

directly mediate the reduction of PrP^{Sc} (by e.g. destabilizing the PrP^{Sc} oligomer via chemical interactions), rather cellular mechanisms are necessary for tamoxifen to exert its effect on PrP^{Sc}.

In order to ensure that the observed reduction of PK resistant PrP derives from an actual decrease in the level of aggregated protein, and not from affected PK resistance, the effect of tamoxifen on the level of insoluble PrP was investigated as a second characteristic feature of PrP^{Sc}. This was accomplished by the detergent solubility assay where non-ionic detergent is added to post-nuclear lysates of ScN2a cells treated with tamoxifen for 72h prior to lysis and followed by ultracentrifugation (→Figure 33, E). Non-soluble PrP^{Sc} sediments in the pellet, soluble PrP^{c} remains in the supernatant. The level of insoluble PrP was strongly diminished in tamoxifen treated cells (lane 4) in contrast to control cells (lane 3), while levels of soluble PrP in the supernatant were comparable (lanes 1-2). Also the effect of tamoxifen on PrP^{Sc} in nonneuronal mouse fibroblast cells (L929) and in mouse embryonic fibroblasts (MEFs) persistently infected with 22L prion was investigated and clearance upon same treatment was observed (*data not shown*).

These results lead to the conclusion that tamoxifen provokes a reduction of PrP^{Sc} levels in persistent prion infections, as confirmed by analysis of two distinct characteristics of PrP^{Sc} upon tamoxifen treatment, namely PK resistance and insolubility in non-ionic detergents. The effect is not restricted to neuronal cells and clearance of PrP^{Sc} is robust; cellular prion infection does not recur.

3.2.4.2 Treatment does not alter cellular levels of PrP^{c}

In order to investigate, whether tamoxifen affects the cellular level of PrP^{c} and might thereby be limiting the substrate for PrP^{Sc}-conversion which would be an explanation of its reduction, PrP^{c}-level was analyzed via FACS analysis. Neither a short-term tamoxifen treatment of N2a cells for 72h (→Figure 34 A) nor a long-term treatment for 30 days (→Figure 34 **A**) led to an alteration of levels of cellular PrP^{c} when tamoxifen-treated cells where compared to untreated cells. To further analyze, whether PrP^{c} might be withdrawn from conversion to PrP^{Sc} by aggregation, the solubility of PrP^{c} was investigated upon tamoxifen treatment (→Figure 34 C). PrP^{c}-signals with strong, comparable intensities were obtained for the supernatant fraction from samples either from control cells (lane 1) or from tamoxifen-treated cells (lane 2). In the pellet fraction, PrP signals were barely detectable for both, cells treated with tamoxifen (lane 4) or

untreated (lane 5). Thus, both, levels of expression and solubility of PrP^c do not seem to be altered by treatment with tamoxifen.

To sum up; availability of PrP^c for conversion to PrP^{Sc} does not appear to be modified upon tamoxifen treatment and the effect of tamoxifen is not general to PrP (PrP^c + PrP^{Sc}), but selective to PrP^{Sc}.

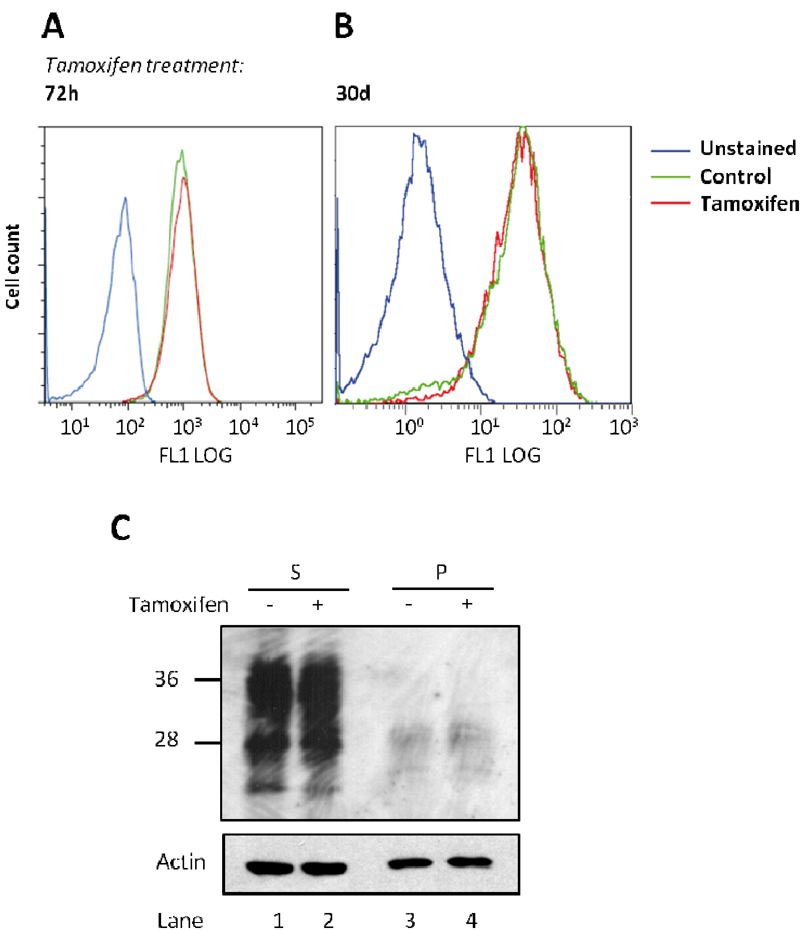

Figure 34. Tamoxifen does not alter cellular levels of PrP^c. (A) Level of cellular prion protein upon short term tamoxifen-treatment determined by flow cytometry analysis. N2a cells were either mock- treated with (green) or 3 µM tamoxifen (red) for 72 h upon analysis. Immunofluorescence staining of PrP^c was performed using anti-PrP mAb 4H11 and a Cy2™-conjugated secondary antibody. Negative controls without primary antibody were used for adjusting measurement gates (blue). X-axis indicates fluorescence intensity in a logarithmic scale. Y-axis shows cell count. For each experiment 15 000 events were measured. **(B)** Investigation of cellular prion protein level after long term cellular prion protein. ScN2a cells were either mock-treated (green) or 3 µM tamoxifen (red) for 30 days followed by cultivation without treatment for 34 days before analysis. Immunofluorescence staining of PrP was performed using anti-PrP mAb 4H11 and a Cy2™-conjugated secondary antibody. Negative controls without primary antibody were used for adjusting measurement gates (blue). X-axes indicate fluorescence intensity in a logarithmic scale. Y-axes show cell counts. For each experiment 15 000 events were measured. **(C)** Solubility analysis of PrP^c upon tamoxifen treatment. N2a cells were treated either mock-treated (-) or treated with 3 µM tamoxifen (+) for 72 h. Upon solubility assay of post-nuclear lysates, PrP contents of supernatant (S) and pellet fraction (P) were analyzed by immunoblotting using anti-PrP mAb 4H11. Actin served as a loading control. (Adapted from T.Schäffler)

3.2.4.3 Tamoxifen-induced autophagy in neuronal cells

Next, the effect of tamoxifen treatment on the autophagic pathway in neuronal N2a cells was assessed. Cells were treated for 24 h either with or without 3 µM tamoxifen and levels of LC3-II were monitored (→Figure 35 A). LC3-II-levels were significantly elevated (~ 2.5 fold) upon treatment with tamoxifen in comparison to control cells, indicating enhanced lipidation of LC3-I to LC3-II and autophagosome formation (→Figure 35 B). Investigation of LC3-levels in samples subjected to solubility assay also revealed that concomitant with a slight increase in the supernatant fraction, signal intensities of samples from tamoxifen-treated cells were considerably stronger than those from control cells in the pellet fraction (*data not shown*). To confirm these results and visualize enhanced autophagosome formation in tamoxifen treated cells, GFP-tagged LC3 in ScN2a cells was monitored by confocal microscopy (→Figure 35 C). For this purpose, ScN2a cells were transfected with an expression construct for rat-LC3 fused to green fluorescent protein (GFP-LC3). In control cells, GFP-LC3 was mostly evenly distributed in the entire cytoplasm, with only few and small EGFP-positive puncta (upper panel). In contrast, tamoxifen-treated cells showed significant increase in number and size of GFP-positive puncta, indicating increased association of EGFP-LC3 with autophagosomal membranes (lower panel). This observation correlates with data obtained in immunoblot analysis (→Figure 35 A and B).

As increased amounts of LC3-II or autophagosomes do not necessarily indicate total autophagic flux but can also result from impaired autophagosome-lysosome fusion, amounts of LC3-II in the presence and absence of bafilomycin A1, which is supposed to block autophagosome-lysosome fusion (Yamamoto el al., 1998), were measured and compaired in one experimental set (→3.1.2). The used dose of bafilomycin A1 is saturating for LC3-II levels in this assay (→Figure 22 B). ScN2a cells were either mock-treated, treated with tamoxifen or bafilomycin A1 alone or treated with 3 µM tamoxifen for 48 h with simultaneous bafilomycin A1 treatment and analyzed by immunoblotting (→Figure 35 D). Co-treated cells show a significantly increased amount of LC3-II compared to cells treated with bafilomycin A1 or tamoxifen alone (→Figure 35 E), confirming that tamoxifen activates autophagic flux in this cell culture model beyond autophagosome-lysosome fusion.

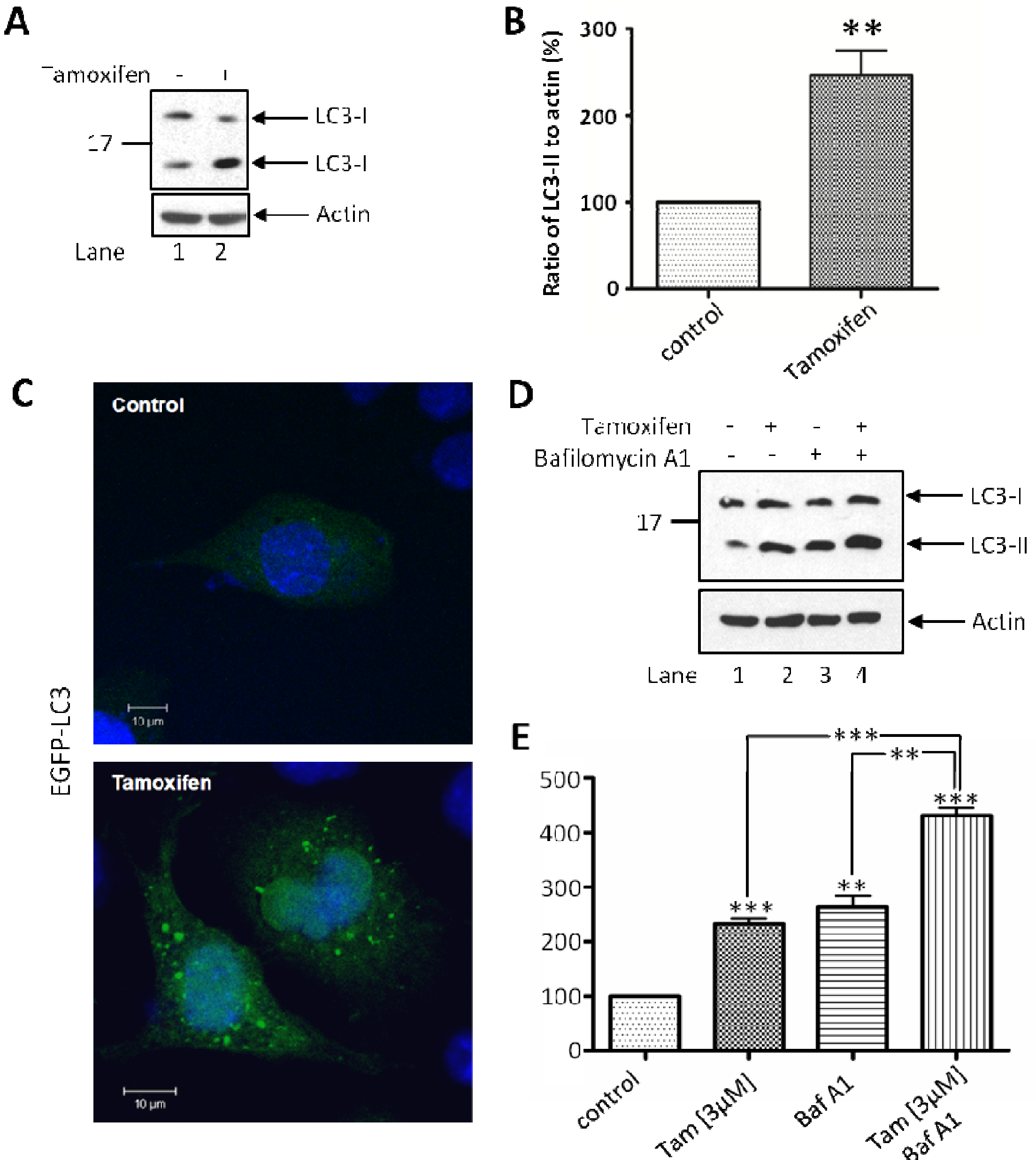

Figure 35. Assessment of tamoxifen-induced autophagy in prion-infected N2a cells. (A) To assess induction of autophagy at ascertained concentration of tamoxifen, namely 3 µM, ScN2a cells were either untreated or treated with for 24 h and analysed by immunoblotting using anti-LC3 monoclonal antibody (mAb) (Adapted from T.Schäffler). **(B)** An increased amount of LC3-II was observed upon 3 µM tamoxifen treatment. Values represent the mean ± S.E. of three independent experiments. Statistical analysis was performed using the unpaired two-tailed t-test **$p < 0.01$). (Adapted from T. Schäffler) **(C)** Autophagosome formation upon tamoxifen treatment. ScN2a cells were transiently transfected with pEGFP-LC3. 24h post transfection, cells were either mock-treated with vehicle (-Tam) or 3 µM tamoxifen for 24 h. Nuclear staining was performed with Hoechst 33342. Confocal microscopy images show cells containing EGFP-positive autophagic vesicles in tamoxifen treated cells. (Adapted from T. Schäffler) **(D)** ScN2a cells were either mock-treated (control, lane 1), treated with 3 µM tamoxifen for 24 h (lane 2), with bafilomycin A1 (200 nM) for 4 h prior to lysis of cells (lane 3), or treated with 3 µM tamoxifen for 48 h with simultaneous bafilomycin A1 (200 nM) treatment for 4 h prior to lysis of cells (lane 4). Cells were analyzed by immunoblotting using anti-LC3 mAb. **(E)** Levels of endogenous LC3-II in compound-treated cells are expressed as percentage of control and represent the mean ± S.E. of three independent experiments (**$p < 0.01$; ***$p < 0.001$). Tamoxifen activates autophagy in ScN2a cells beyond autophagosome-lysosome fusion.

3.2.5 Beclin 1 or Atg5 knock-down and the effect of tamoxifen

The previous studies and the results above strongly indicate that clearance of PrP^{Sc} seems to be the consequence of enhanced autophagic activation upon tamoxifen treatment. However, it is always relevant to gain evidence on the interconnection between

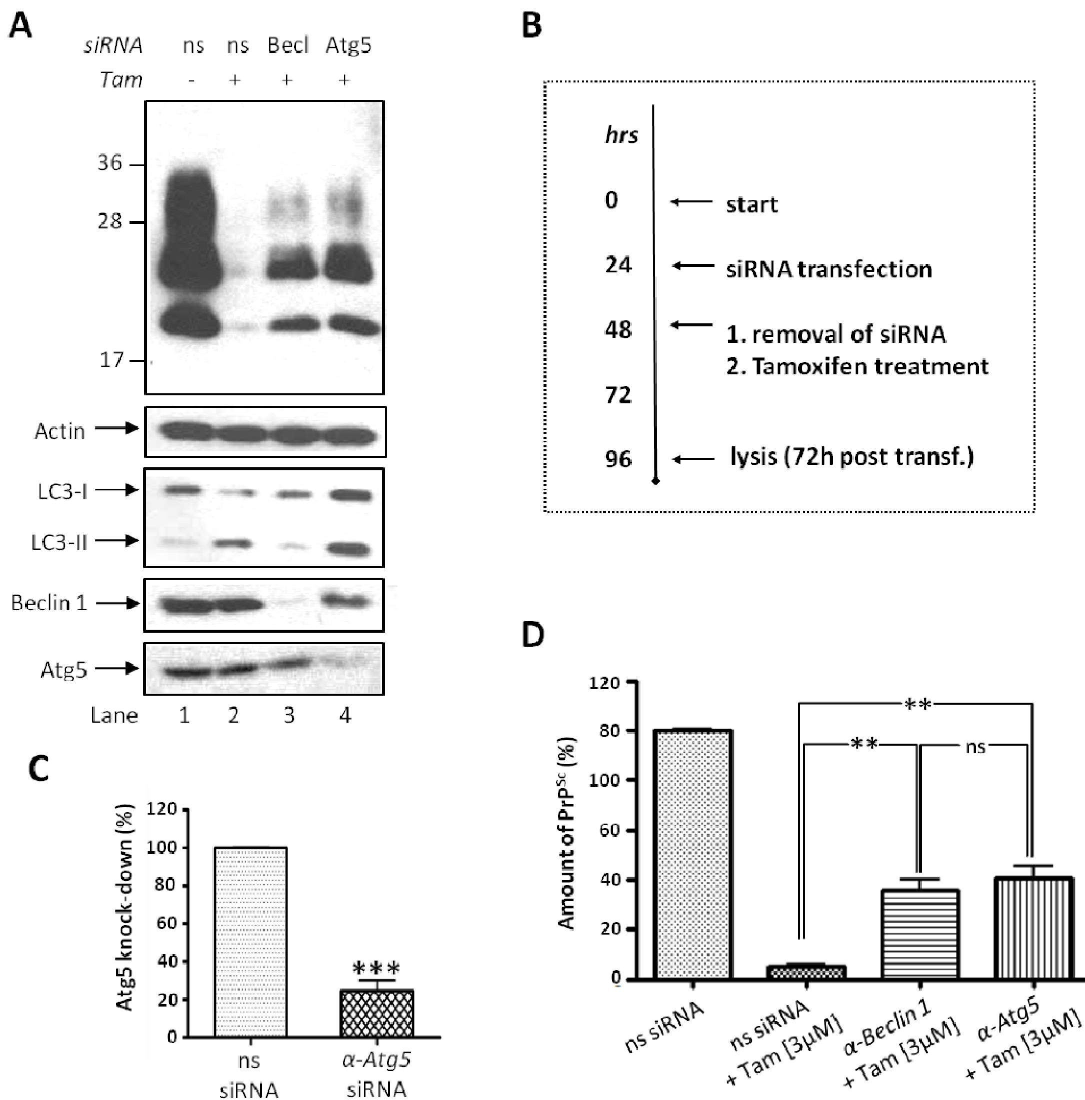

Figure 36. Attenuation of the autophagic pathway, via Beclin-1 or Atg5 knock-down, antagonizes the effect of tamoxifen on cellular prion infection. (A) ScN2a cells were either transfected with nonsilencing (ns) siRNA (lane1 and 2), with siRNA targeting *Beclin 1* (lane 3) or with siRNA targeting *Atg5* (lane 4). Cells were then treated with 3 µM tamoxifen (lane 2 to 4). Cells transfected with ns siRNA and left un-treated (lane 1) serve as a control. PrP^{Sc} was visualized by immunoblotting using anti-PrP mAb 4H11. **(B)** Chronological course of the experiment. **(C)** To confirm successful knockdown of Atg5, lysates were probed with anti-*Atg5* mAb by immunoblotting ATG51 signals in cells either transfected with anti- *Atg5* siRNA are expressed as percentage of cells transfected with ns siRNA and represent the mean ± S.E. of three independent experiments (***p < 0.001). Cells show a significant reduction of ATG5expression. **(D)** PrP^{Sc} signals in tamoxifen treated cells either transfected with ns siRNA, *Beclin 1* or *Atg5* siRNA are expressed as percentage of mock-treated cells transfected with ns siRNA and represent the mean ± S.E. of three independent experiments (ns: not significant; **p < 0.01). Transfection of tamoxifen treated cells with *Beclin 1* or *Atg5* siRNA reproducibly counteracts the antiprion effect when compared to tamoxifen-treated cells transfected with ns siRNA.

tamoxifen-induced upregulation of autophagy and effect on PrP^{Sc}. It had to be examined, whether decelerating or attenuating tamoxifen-induced autophagy would also influence the observed effect on PrP^{Sc}. For this purpose use was made of the straight-forward approach of knocking-down essential genes implicated in the autophagic pathway. SiRNA that target *Atg5* and *Beclin 1* were implied (→Figure 36). The siRNA was reproducibly able to significantly impair Atg5 expression, as shown in →Figure 36 A and C and Beclin 1 expression, as shown in →Figure 36 A and →Figure 39 B. ScN2a cells were either treated with non-silencing siRNA, treated with non-silencing siRNA and 3 µM tamoxifen, co-treated with anti-*Beclin 1* siRNA and 3 µM tamoxifen or co-treated with anti-*Atg5* siRNA and 3 µM tamoxifen and analyzed by immunoblotting (→Figure 36 A). Chronological sequence of the experiment is displayed in →Figure 36 B. Tamoxifen treatment led to the expected reduction of PrP^{Sc} (compare lanes 1 and 2). Compared to cells treated with tamoxifen alone (lane 2), reduction of PrP^{Sc} was strongly impaired by co-treatment with anti-*Beclin 1* siRNA (lane 3) or anti-*Atg5* siRNA (lane 4). Densitometric quantification was performed and analysed in →Figure 36 D. Same results were obtained when inhibition of autophagy was obtained pharmacologically (e.g. 3-MA). ScN2a cells co-treated with 3 µM tamoxifen and 10 mM 3-MA showed no reduction of PrP^{Sc} (*data not shown*).

These data show that PrP^{Sc} reduction upon tamoxifen treatment is antagonized by knock-down of *Beclin1* or *Atg5* and decelerated autophagic activity in the cell.

3.2.6 Tamoxifen-treatment of prion-infected mice

To explore whether tamoxifen has a therapeutic anti-prion potential in *in vivo*, prion infections of mice followed by tamoxifen treatment were performed. Mice were intracerebrally infected with prions and tamoxifen was orally given to mice starting at day 50 post-infection. Treatment of prion-infected mice with tamoxifen showed a small, however significant therapeutic effect ($^*p < 0.01$) (→Figure 37 A). At terminal time-points, mice were tested for the amount of PrP^{Sc} in the spleen (→Figure 37 B). PrP^{Sc} deposition was strongly reduced in spleens obtained from tamoxifen-treated mice (lanes 2 and 4) compared to control mice (lanes 1 and 3). Imunhistochemical analysis of disease associated microgliosis via detection of the ionized calzium binding adapter molecule 1 (Iba1) in brain sections (hippocampus and cortex) of tamoxifen-treated and non-treated mice at 125dpi showed reduced microgliosis in tissue of tamoxifen-treated mice (→Figure 37 C).

All the previous studies revealed, that drug-induced autophagy can accelerate clearance of PrP^{Sc} in persistently prion-infected cells. These results here confirm that another autophagy enhancer exerting an efficient clearance of PrP^{Sc} was identified.

To conclude, tamoxifen has an anti-prion potential in primary, persistent and *in vivo* prion infection and the data display that the efficient mode of action of the drug is directly connected to the autophagic pathway.

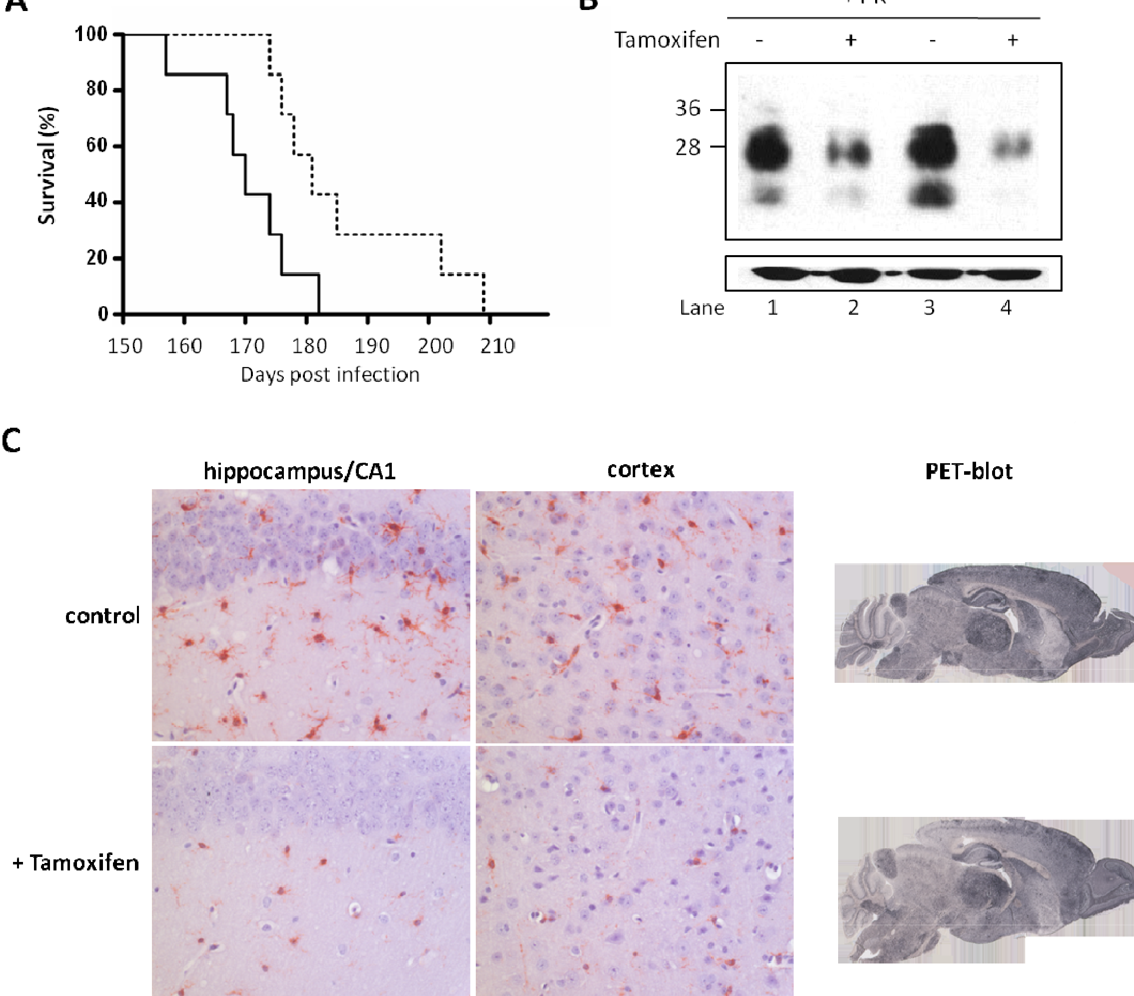

Figure 37. Effect of tamoxifen in *in vivo* prion infection. **(A)** *Prolongend survival times tamoxifen–treated mice.* Oral treatment with tamoxifen was initiated at day 50 post-intracerebral infection with prion strain 139A. Solid line depicts control mice (mean 170.6 ± 7.9 days); broken line depicts tamoxifen-treated mice (mean 186.4 ± 13.6 days, *p > 0.01); Group sizes were n = 7. Survival times in the tamoxifen show increased survival rates. **(B)** *Reduced PrP^{Sc} level in spleens of tamoxifen-treated mice compared to the untreated control.* Immunoblot detection of PrP^{Sc} in spleens of mock-treated (lanes 1 and 3) and tamoxifen-treated (lanes 2 and 4) mice at terminal time-points. **(C)** *Reduced microgliosis in brain tissue of tamoxifen-treated mice.* Immunhistochemical analysis of disease associated microgliosis via detection of the ionized calzium binding adapter molecule 1 (Iba1) in hippocampus (*left row*) and cortex (*right row*) tamoxifen-treated (*lower row*) and non-treated (*upper row*) mice at 125dpi. Representative sections are shown for indicated areas in a magnification of x400. PET blot analyses of PrP^{Sc} are shown on the right.

3.3 Autophagy impairment and persistent prion infections

In the past studies compound-induced (trehalose- and tamoxifen-induced) autophagy in degrading PrP^{Sc} in persistent- and primary prion infection was described and analysed. An interesting point that remained to be looked at was the role of basal constitutive autophagy and the consequence of its deceleration. This aspect was studied here in the scenario of persistent prion infections.

3.3.1 Pharmacological impairment of autophagy in prion-infected N2a cells

Pharmacological impairment of autophagy via 3MA was the first approach used when alteration of PrP^{Sc} was observed upon impairment of basal autophay levels in ScN2a cells. ScN2a cells were treated with 10 mM 3-MA for 24 h. Interestingly, a moderate though significant increase in the amount of PrP^{Sc} in 3MA treated cells was observed when compared to control cells (→Figure 38 A, compare lanes 1 and 2; (→Figure 38 B). This confirmed a previous data where immunoprecipitation assays of radioactively labelled PrP^{Sc} showed an increase of PrP^{Sc} upon treatment with 3MA. Increased levels of PrP^{Sc} upon pharmacological autophagic inhibition suggest an involvement of autophagic machinery in its degradation.

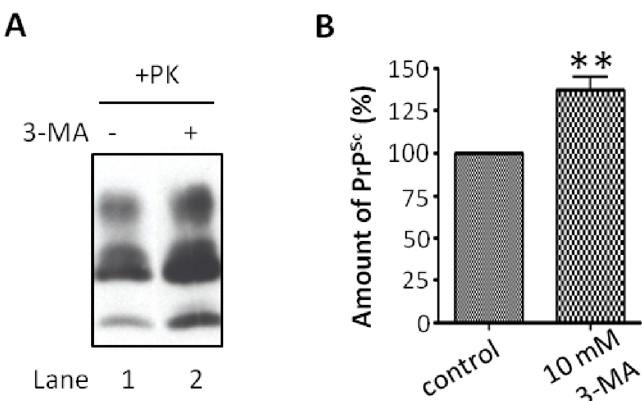

Figure 38. Inhibiton of autophagy via 3MA in prion-infected N2a cells. **(A)** ScN2a cells were either mock-treated or treated with10 mM for 48 h. Upon PK digestion, PrP was visualised by immunoblotting using anti-PrP mAb 4H11. Increase of PrP^{Sc} is observed when cells are treated with 3-MA. Blot adapted from Fig. 27 **(B)** PrP^{Sc} signal in immunoblot analysis in cells treated with 3-MA alone is expressed as percentage of mock-treated cells (control) and represents the mean ± S.E. of three independent experiments. Inhibition of autophagy increases cellular PrP^{Sc} levels.

3.3.2 Beclin 1 knock-down in prion-infected N2a cells

Based on the previous observation, it was important to confirm the involvement of basal autophagy in persisting cellular prion infections. For this purpose, genetic impairment of autophagy was thought to provide clear-cutting information about it.

Beclin 1 knock-down was first analysed to be able to better explain further results. When Beclin 1 and PrP were assayed 24h, 48h and 72h after transfection the following was observed: Beclin 1 was successfully down-regulated to approximately 20% already after 24 hours and this effect lasts up to 72h (further time-points were not monitored) (→Figure 39).

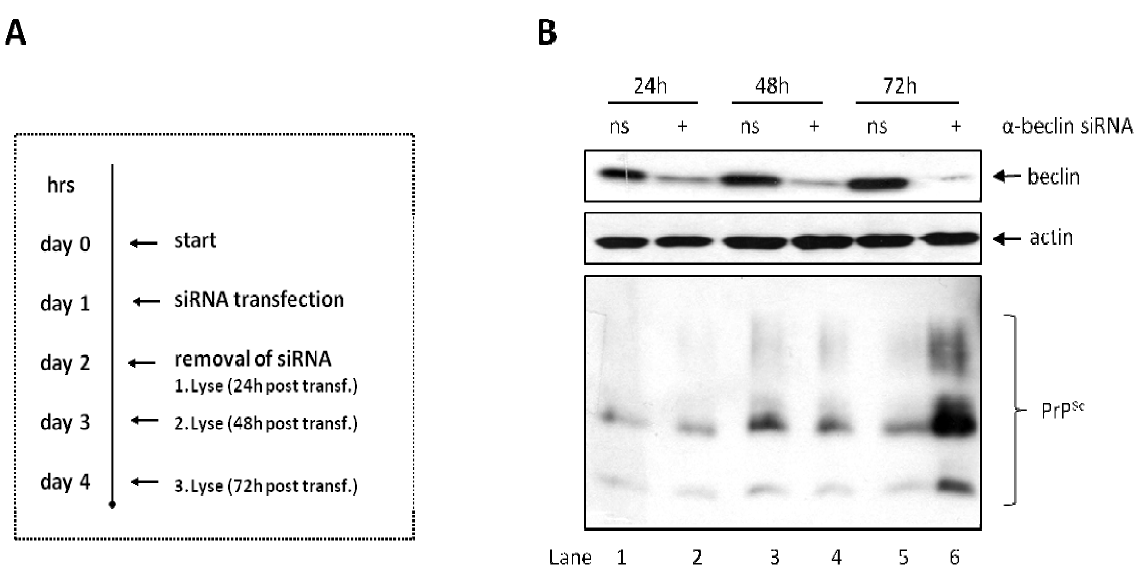

Figure 39. Establishment of Beclin-1 knock-down in prion-infected N2a cells. Persistently prion-infected ScN2a cells were transfected with non-silencing siRNA or anti-*Beclin 1* siRNA as shown in the time scheme in **(A)**. **(B)** Beclin 1- and PrP^{Sc}-levels were monitored 24h, 48h and 72h later. Beclin1 and PrP^{Sc} were visualised by immunoblotting using anti-Beclin 1 pAb and anti-PrP mAb 4H11. A strong increase of PrP^{Sc} upon down- regulation of Beclin 1 for 72h is observed.

When impact of Beclin 1 downregulation in persistently infected ScN2a cells was monitored, no effect was observed after 24h and 48h, however, a significant increase (> 3-fold) in the amount of PrP^{Sc} was observed at 72h. Analysis at time-point 72h (→Figure 40 A) was validated by detecting Beclin 1, PrP^{c} and PrP^{Sc} via immunoblotting (→Figure 40 B) and densitometric quantification (→Figure 40 C and D). A strong increase of PrP^{Sc} upon down- regulation of Beclin 1 for 72h was confirmed.

In preliminary experiments a first insight was gained about the effect of genetic attenuation of autophagy in the context of primary prion infections. Again, the possibility

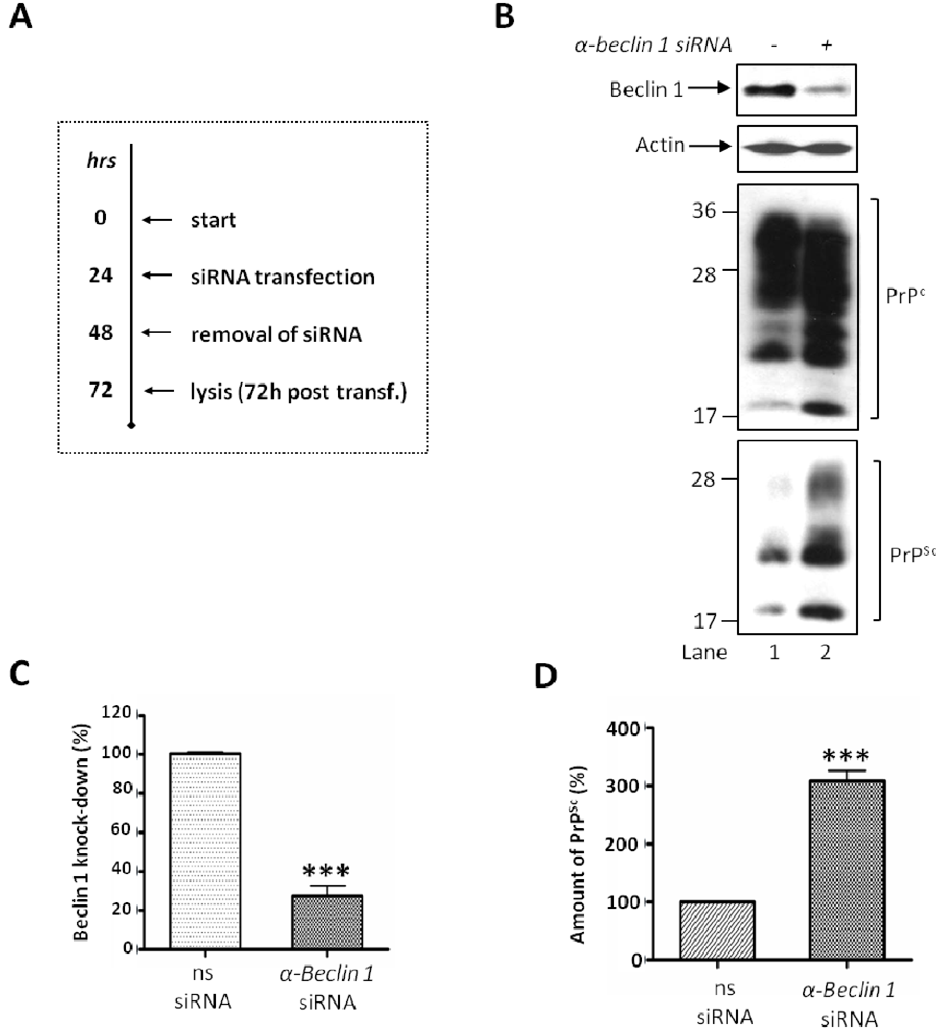

Figure 40. Effect of Beclin-1 knock-down for 72h in prion-infected N2a cells. Persistently prion-infected ScN2a cells were either transfected with non-silencing (ns) siRNA or siRNA targeting *Beclin 1*. Chronological course of the experiment is schematically in **(A)**. **(B)** Lysates were probed with anti-Beclin 1 pAb by immunoblotting. PrP^c and PrP^{Sc} were visualized by immunoblotting using anti-PrP mAb 4H11 in undigested and PK-digested probes. **(C)** Beclin 1 signals in cells either transfected with anti-*Beclin 1* siRNA are expressed as percentage of cells transfected with ns siRNA and represent the mean ± S.E. of three independent experiments (***$p < 0.001$). Cells show a significant reduction of Beclin 1 expression. **(D)** PrP^{Sc} signal in immunoblot analysis on cells transfected with anti-*Beclin 1* siRNA is expressed as percentage of cells transfected with ns siRNA (control) and represents the mean ± S.E. of three independent experiments. Attenuation of the Beclin 1 dependent autophagic pathway increases level of PrP^{Sc} in persistent prion infections.

to overexpress 3F4-tagged PrP^c was used to monitor specifically *de novo* formation of PrP^{Sc} (Vorberg *et al.*, 2004a, b) and how it is influenced upon genetic attenuation of autophagy. 3F4-PrP expressing N2a cells (3F4-N2a) were transfected with siRNA targeting *Atg5* or *Beclin 1*. When cells were analysed in Western blot one day, 3, 5 and 7 passages after infection with 22L prion-infected prion homogenate (→Figure 41 A), an increasing reduction of PrP^{Sc} in cells treated with *Atg5* or *Beclin 1*- targeting siRNA

compared to cells treated with non-silencing siRNA was observed (→Figure 41 B). These are interesting results that need to be confirmed in future studies.

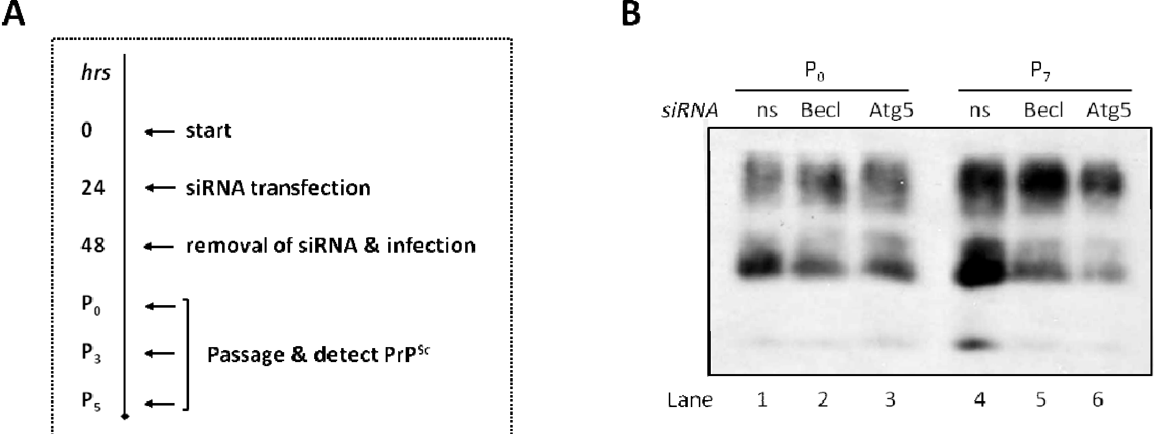

Figure 41. Attenuation of the Beclin 1 or Atg5 -dependent autophagic pathway seems to attenuate primary prion infections. **(A)** Chronological course of the experiment is schematically shown. **(B)** 3F4-PrP expressing N2a cells (3F4-N2a) were either transfected with non-silencing (ns) siRNA (lane 1 and 4), siRNA targeting *Beclin 1* (lanes 2 and 5) or siRNA targeting *Atg5* (lanes 3 and 6). 24h hours later transfection was terminated and cells were incubated with brain homogenate (1 %) derived from mice infected with prion strain 22L for 24h. After removal of brain homogenate cells were analysed at the given time-points. Postnuclear lysates of the cells were prepared (+/- PK-digestion) and subsequently analyzed by SDS-PAGE and immunoblot (here: passages 0 and 7) using anti-PrP monoclonal 3F4, which detects primary formed PrP^{Sc} and not the inocculated PrP^{Sc}. Levels of primary converted PrP^{Sc} are reduced in cells treated with *Beclin 1- or Atg5-* targeting siRNA.

4 Discussion

4.1 Modulation of autophagy and addressing its role in prion disease scenarios

Neurological abnormalities and neuronal death can be associated with impairment of autophagy in the CNS. Mice with defects in autophagy develop neurodegenerative symptoms in the CNS without the expression of disease-causing, aggregate prone proteins, suggesting a neuro-protective role of autophagy (Komatsu *et al*., 2006; Hara *et al*., 2006). Previous studies also implicated macroautophagy as a key pathway for removal of aggregated intracellular proteins, including mutant huntingtin, alpha-synuclein and ataxin-3 (Williams *et al*., 2006).

However, data on autophagy in prion diseases are still very limited (Boellaard *et al*., 1989, 1991; Schätzl *et al*., 1997; Sirkosa *et al*., 2004; Liberski *et al*., 1992, 1997, 2004; Liberski 2004, 2008; Mok *et al*., 2007). Electron microscopy studies pointed towards a possible contribution of autophagy in prion disease-associated neurodegeneration; a recent study suggests that spongiform vacuoles in prion diseases, may in reality originate from autophagic vacuoles (Liberski, 2004; Liberski *et al*., 2011). In addition a slight reduction in expression of genes mediating autophagy was observed in prion-infected brain tissue (Mok *et al*., 2007).

The beneficial effect of up-regulated autophagy was shown for several neurodegenerative diseases associated with aggregate-prone proteins (Berger *et al*., 2006; Fortun *et al*., 2003; Kabuta *et al*., 2006; Ravikumar *et al*., 2002; Webb *et al*., 2003). It was previously shown by our group that treatment of prion-infected cells with imatinib activates lysosomal degradation of PrP^{Sc} (Ertmer *et al*., 2004). In another separate study, it was demonstrated that imatinib induces autophagosome formation, leading to activation of the lysosomal degradation machinery (Ertmer *et al*., 2007). These two studies, however, did not determine whether the activation of the autophagic pathway per se has an anti-prion effect as a consequence, which became the aim of this study.

In this work, the role of autophagy in TSEs is analyzed via diverse approaches and scenarios. Autophagy modulators, activators and inhibitors were used for research purposes. Autophagic activators were also analyzed for potential therapeutic purposes. In line with studies on mutant forms of huntingtin or α-synuclein (Sarkar *et al*., 2007a;

Sarkar *et al*., 2005) it was now possible to show that trehalose, lithium (Aguib *et al*., 2009; Heiseke *et al*., 2009; Heiseke *et al*., 2010) and tamoxifen enhance the clearance of PrP^{Sc} in prion-infected cells by induction of autophagy. As mentioned, the most prominent method to monitor autophagosome formation is to analyse the level of LC3-II which is associated with autophagosome membranes (Kabeya *et al*., 2000). Autophagy inducers used in these studies all increased the amount of LC3-II in prion-infected cells in immunoblot analysis (→Figure 22, →Figure 35). LC3 fused to green fluorescent protein (GFP) was another tool to measure induction of autophagosome formation. Prion-infected neuroblastoma (ScN2a) cells treated with autophagy inducing drugs exhibited punctuate GFP staining (→Figure 23 and Figure 35), indicating the association of GFP-LC3 with autophagosomal membranes as a result of induction of autophagy. As monitoring autophagosome accumulation needs to be combined with methods that monitor the autophagic flux, assays with the lysosomal inhibitors Bafilomycin A1 were performed, to confirm the enhanced autophagic flux (Yamamoto *et al*. 1998, Mizushima *et al*., 2010) (→Figure 22 and Figure 35). In these studies the first direct evidence that induction of autophagy results in degradation of cellular PrP^{Sc} is provided. Inline with this, inhibition of autophagy by pharmacological interference and siRNA gene-silencing of essential members of the autophagy machinery impaired the capacity of compound-induced autophagy in reducing cellular levels of PrP^{Sc}. Of note, as induced autophagy was able to degrade aggregate-prone proteins accumulating within endosomal/lysosomal vesicles, as is the case for PrP^{Sc}, it is now shown that autophagy is not only an important clearance route for cytosolic aggregate-prone proteins lithium (Aguib *et al*., 2009; Heiseke *et al*., 2009; Heiseke *et al*., 2010).

4.1.1 A ‘trehalose study’ provides the proof of principle

In this study trehalose was utilized as an autophagic inducer that in fact has been seen to accelerate the clearance of other aggregate-prone proteins of mutant huntingtin and α-synuclein by activating autophagy in an mTOR independent manner (Sarkar *et al*., 2007a).

Trehalose is an alpha-linked disaccharide synthesised by fungi, plants and invertebrates. It protects cells against miscellaneous types of environmental stress like heat, cold, desiccation, dehydration and oxidation by preventing protein denaturation (Chen and Haddad, 2004). Many of its protective effects are apparently caused by its ability to act as a chemical chaperone (Welch and Brown, 1996). In order to exclude the option of

trehalose influencing levels of PrP^{Sc} as a chemical chaperone experiments with postnuclear cell lysates in an extracellular setting were performed, proving that no effects could be observed there (Figure 24).

Well-established prion infection cell culture models were then used to investigate the impact of autophagic activation via trehalose on cellular PrP^{Sc}. Obtained data presented in this study demonstrate that the degradation of cellular PrP^{Sc} induced by trehalose is directly mediated by activation of autophagy. The observed effect was time- and dosis-dependent (→Figure 21). As soon as trehalose-induced autophagy was constrained pharmacologically by 3-MA or wortmannin (*data not shown*), the effect on PrP^{Sc} was diminished or abolished (→Figure 27). As pharmacological inhibition of autophagy might be affected by non-warranted side effects of used drug, siRNA was additionally used to knock down the *Atg5* gene, an essential gene implicated in the autophagic pathway. Similar results were obtained when the *Atg5* gene, an essential member of the autophagic machinery, was genetically silenced prior to trehalose treatment (→Figure 28). These observations provide experimental evidence that the effect of trehalose, a mTOR-independent inducer of autophagy, on PrP^{Sc} depends on the autophagy machinery.

Even though trehalose treatment *in vivo* reduced PrP^{Sc} in spleens of intraperitoneally infected mice at early time points post infection (30 and 60 days), this effect was not evident at later time points (e.g. 90 days p.i.), and prion incubation times of trehalose- and sucrose-treated animals were not different from that of mock-treated mice (→Figure 29). Previously, similar results were obtained in mice treated with imatinib (Yun *et al*., 2007). Imatinib treatment at an early phase of peripheral prion-infection delayed both appearance of PrP^{Sc} in the CNS and onset of clinical disease in mice, but neither intraperitoneal nor intracerebroventricular delivery of the drug exerted significant PrP^{Sc} clearance effects in the brain. In terms of trehalose this finding was not unexpected as the anti-prion effect in cultured cells was highly dose-dependent and the effective anti-prion concentration of 100 mM is probably not achievable in tissues. Interestingly, Beranger and colleagues recently found that much lower doses of trehalose, which have no anti-prion effect in cultured cells, can protect prion-infected cells from oxidative damage, which suggests that this compound may have additional beneficial therapeutic effects (Beranger *et al*., 2008).

It is important to clearly mention the fact that the anti-prion effect is not restricted to mTOR-independent activators of autophagy, such as trehalose. Treatment with

rapamycin, an inhibitor of mTOR, also led to a detectable, although weak, PrP^{Sc} reduction (*data not shown*), which strongly confirms the relationship between autophagy and anti-prion effects, whether it was activated in an mTOR-depedent or independent manner.

These obtained data expand the impact of autophagy, its inducers (such as trehalose, imatinib and rapamycin) and inhibitors (such as 3-MA and wortmannin) on accumulated aggregate-prone proteins to the scenario of prion infections. It is shown here that autophagy, as a cytosolic, non-specific bulk degradation system, is not only an important clearance route for several cytosolic, toxic aggregate-prone proteins (like mutant forms of huntingtin and α-synuclein) but also for aggregate-prone proteins that mainly, if not exclusively, accumulate within intracellular vesicles, as is the case for PrP^{Sc} (Prusiner and DeArmond, 1994; Prusiner 1998).

To sum up, this study provides for the first time experimental evidence that induction of autophagy and concomitant reduction of PrP^{Sc} are obtained when persistently prion-infected cultured cells are treated with the autophagy inducer trehalose. The anti-prion effect was prevented by simultaneous application of potent autophagy inhibitors like 3-MA and by silencing the *Atg5* gene with siRNA. Now it is confirmed that the observed increased cellular degradation of prions is a result of induced autophagy. Proof of evidence was also obtained in the *in vivo* study, as trehalose treatment of intraperitoneally prion-infected mice delayed PrP^{Sc} accumulation in the spleen, although trehalose had not the potential to prolong prion incubation times in this situation.

After this study it was clear, that further *in vivo* experiments are required to elucidate the impact of autophagy on prion propagation and to validate whether autophagy plays a general role in different prion disease scenarios. It was also important to investigate whether other autophagy inducers could show better *in vivo* effects and provide a novel avenue for therapy against prion diseases.

4.1.2 The 'tamoxifen study' introduces an interesting therapeutic candidate

The study described above opened the door to interesting questions that need to be answered, such as: Does drug-induced autophagy also have a role in acute prion infection? Which autophagic proteins are involved in prion infection scenarios? How far can autophagy take us towards therapeutic applications? In fact, only little is known about mechanisms involved in autophagy-dependent clearance of the pathological prion protein.

In order to investigate a candidate with high potential, in respect to its ability to induce autophagy as well to its suitability for future therapies, the widely used anti-cancer drug tamoxifen , which was already reported to induce autophagy in breast cancer cells, was chosen (Bursch *et al*., 1996; Bilir *et al*., 2001; Pattingre *et al*., 2009; de Medina *et al*., 2009b). Originally classified as a nonsteroidal antiestrogen (Jordan, 1984), but now reclassified as a selective estrogen receptor modulator (SERM) (Jordan, 2001), tamoxifen is already widely used for hormonal therapy of estrogen receptor positive breast cancer. More than 30 years ago, tamoxifen was a pioneering intervention in this field (Ward, 1973).

Tamoxifen binds with comparable affinity to the microsomal antiestrogen binding site (AEBS), a secondary binding site distinct from the estrogen receptor (Kedjouar *et al*., 2004), and inhibits with a micromolar efficiency, protein kinase C (PKC) (Gundimeda *et al*., 1996), calmodulin-dependent enzymes and Acyl CoenzymeA: Cholesterol Acyl Transferase (de Medina *et al*., 2004).

In the study performed by Bursch *et al.* , tamoxifen appeared to induce autophagic cell death in breast cancer cells via the ER as this effect could be inhibited by estradiol (Bursch *et al*., 1996). More studies on the pathways involved in tamoxifen-induced autophagy followed and tamoxifen appears to interact with cellular signaling in a diverse manner. Studies on the proto-oncogene PKB, which is under the control of a tamoxifen-responsive ER, suggested involvement of the PKB/Akt family in tamoxifen-mediated autophagy (Jin and Woodgett, 2005). More recently, upregulation of cellular ceramide levels by tamoxifen have been reported, thereby inducing autophagy (Pattingre *et al*., 2009; Scarlatti *et al*., 2004). Tamoxifen appears to stimulate de novo synthesis of ceramide (Pattingre *et al*., 2009), which is a bioactive sphingolipid associated with a large range of cellular processes. Ceramide is suggested to interfere with the autophagic process by different mechanisms, including inhibition of the activation of PKB (Scarlatti *et al*., 2004), and increasing the levels of free Beclin 1. Beclin 1-levels are up-regulated either by increasing mRNA levels of Beclin 1 (Scarlatti *et al*., 2004) or by promoting the dissociation of the Beclin 1-Bcl-2 complex (Pattingre *et al*., 2009; Daido *et al*., 2004). The dissociation of this complex can be stimulated by phosphorylation of Bcl-2 dependent on c-Jun N-terminal protein kinase 1 (JNK1) (Pattingre *et al*., 2009) or via induction of the BH3-only protein BNIP3 which competes with Beclin 1 for binding Bcl2 (Daido *et al*., 2004). Moreover, tamoxifen-mediated induction of autophagy was recently reported to occur via modulation of the cholesterol metabolism. Binding of tamoxifen to

the AEBS was suggested to impair cholesterol metabolism and lead to accumulation of sterols (de Medina *et al*., 2009a, 2009b), thereby inducing autophagy (Cheng *et al*., 2006; de Medina *et al*., 2009a).

While the previous study provides the evidence of a role of autophagy in persistent prion infection, the data presented here take us a step further to confirm a general role of autophagy in prion disease scenarios based on a study with well characterized and established drug, namely Tamoxifen.

4.1.2.1 A novel effect of of the SERM Tamoxifen: PrP^{Sc} reduction

In this study tamoxifen had a double role: i) It was examined for its potential as an anti-prion drug and ii) was a tool that helped dissecting further aspects in the interconnection between the autophagic pathway and cellular prion infections.

From what was found in the previous study there is good experimental evidence that induced autophagy, e.g. by chemical compounds, can clearly have beneficial effects on persistent prion infection. In the present study, several experimental approaches were used addressing cellular prion infections to examine the effect of the drug tamoxifen on PrP^{Sc} accumulation. Compared to the previous work, the studied prion infection scenario was extended: besides monitoring the effects in cells, where prion infections are already established (→Figure 33), the initial prion infection process was analyzed (→Figure 30 and →Figure 31). For this purpose, the possibility to express 3F4-tagged PrP^{c} and to monitor specifically its de novo conversion (Vorberg *et al*., 2004a, b) was used. It was observed, that tamoxifen treatment leads to robust cellular clearance of PrP^{Sc}, which is antagonized when the autophagic pathway was attenuated, and strongly hinders *de novo* accumulation of intracellular PrP^{Sc}.

In screenings of compounds reported to induce autophagy, tamoxifen appeared to considerably interfere with prion infection (*data not shown*). The effect of tamoxifen on prion infection was investigated in a well-established neuronal cell culture model of prion infection. Upon treatment with tamoxifen at a non-toxic dose, the level of proteinase K resistant prion protein as well as the level of insoluble prion protein significantly decreased, while PrP^{c} remained unaltered. Prion infection levels decreased already upon treatment with tamoxifen for 24 h. Treatment time-kinetics revealed that tamoxifen reduces PrP^{Sc} levels below the detection level of Western blotting within 120 h (→Figure 33). For comparison, treatment with trehalose (or lithium chloride, Heiseke *et al*., 2009)

for 96 h resulted in reduced, but still detectable PrP^{Sc}-levels, pointing out the high efficiency of tamoxifen *in vitro*.

Interestingly, several studies suggest tamoxifen and other SERMs to be neuroprotective. It was shown, that Tamoxifen significantly reduced infarct size in permanent MCAO and transient occlusion/reperfusion models of cerebral ischemia (Kimelberg *et al.*, 2000, Mehta *et al.*, 2001, Osuka *et al.*, 2001). Tamoxifen was also recently shown to protect the striatum against 1-methyl-4-phenylpyridine- induced toxicity, suggesting that its protective abilities may extend to regions of the brain that are known to be affected in Parkinson disease (Obata *et al.*, 2001). In another study, selective estrogen receptor modulators, including Tamoxifen, were shown to exert neuroprotective effects and stimulate the expression of *Selective Alzheimer's Disease Indicator-1 (Seladin-1)*, a anti-apoptotic gene, in human neuroblast long-term cell cultures (Benvenutti *et al.*, 2005). These studies and others (Lee *et al.*, 2009; Tian at al., 2009; Liu *et al.*, 2010) suggest that clinically relevant SERMs such as tamoxifen may have potentially clinically important neuroprotective effects on the CNS and brain. Furthermore they point to a potential in studying the effect of tamoxifen on further neurodegenerative and protein aggregation diseases.

4.1.2.2 Tamoxifen treatment enhances clearance of already internalized PrP^{Sc} in de novo infection

Both, persistent and acute prion infections were hindered by tamoxifen treatment. Further dissection of the initial steps of prion infection showed that according to confocal microscopy and Western blot data, PrP^{Sc} taken up by the cells during *de novo* prion infection and was detectable in the absence and the presence of the drug (→Figure 32). As under both conditions cells were able to internalize PrP^{Sc}, it is assumed that the effect of tamoxifen treatment on prion infection is not due to an inhibition of an initial uptake of PrP^{Sc} by the cells. However, its intracellular accumulation and the progress of prion infection were hindered. It is known that trafficking and quality control events are crucial for the establishment of a persistent infection and chronic PrP^{Sc} formation (Nunziante *et al.*, 2003b). In neuroblastoma, cells, PrP^{c} is known to reside in the Golgi apparatus, on the plasma membrane, and in early and recycling endosomes (Borchelt *et al.*, 1990; Shyung *et al.*, 1993; Nunziante *et al.*, 2003a; 2003b; Prado *et al.*, 2004). In scrapie-infected cells, PrP^{c} is converted to PrP^{Sc} after transport to the cell surface before degradation in lysosomes (Caughey and Raymond, 1992; Caughey *et al.*, 1992, Borchelt *et al.*, 1992).

Also other cellular factors can have an influence. Rab proteins are involved with different aspects of vesicular trafficking in cells (Zerial and McBride, 2001) and can influence PrP^{Sc} formation (Beranger, 2002). A high cellular metabolism and a high rate of cell divisions or a 'suramin-induced like' (Gilch *et al.*, 2001) rerouting can also hamper PrP^{Sc} accumulation. Analysis of the subsequent time-points after *de novo* prion infection showed increased clearance celerity of PrP^{Sc}. The establishment of persistent cellular prion infections has requirements beyond simple exposure of cells to exogenous PrP^{Sc}. The kinetics of PrP^{Sc} formation and the balance between PrP^{Sc} formation and its degradation are a crucial aspect for a persistent infection, which seem to be altered upon tamoxifen treatment. In contrast to other anti-prion drugs that aim blocking PrP^{Sc} uptake by the cell impairing mechanisms of cellular prion invasion (reviewed in Krammer *et al.*, 2009b) the responsible mechanism upon tamoxifen treatment must take place after PrP^{Sc} resides intracellularly.

For characterization of different steps in the autophagic process upon tamoxifen-treatment in neuroblastoma cells, the autophagic process was monitored by Western blotting, immunofluorescence and confocal microscopy which confirmed functional autophagic flux upon tamoxifen treatment. 3µM tamoxifen were sufficient to induce autophagy and reduce prion load in the cells after 24h. The effect of tamoxifen on PrP^{Sc} depends on the autophagy machinery; as soon as tamoxifen-mediated autophagy was constrained genetically by siRNA knock down of autophagy essential genes, Beclin 1 or Atg5, the effect on PrP^{Sc} was diminished (→Figure 36).

These results uncover a novel function for the SERM, Tamoxifen and demonstrate a further connection between drug-induced autophagy and prion infections.

4.1.2.3 Tamoxifen as a candidate for further *in vivo* studies

In recent years, especially with the potential threat of a large outbreak of variant Creutzfeldt-Jakob disease, a proliferation of research into the understanding and identification of therapeutics against prion diseases was initiated. *In vitro* and animal model studies have suggested a number of categories of drugs as candidates for treatment (MacLeod *et al.*, 2003, Krammer *et al.*, 2009b).

A huge number of compounds was tested in assays, cell culture-based systems, and animal studies, with some drugs also being tested in humans (Stewart *et al.*, 2008). However, formal clinical studies are still at an early stage, as numerous challenges are encountered including toxicity and non-tolerable side effects, suboptimal

pharmacokinetics and limited bioavailability and, the ultralarge obstacle, ineffective passing of the blood-brain-barrier.

As mentioned above, imatinib treatment in prion-infected mice at an early phase of peripheral infection delayed both the neuroinvasion of PrP^{Sc} and the onset of clinical disease (Yun *et al.*, 2007). Unfortunately, drug application at time points when neuroinvasion was already accomplished provoked no clear PrP^{Sc} clearance effects in CNS, probably due to ineffective blood-brain barrier crossing of the drug. Trehalose treatment did not prolong incubation times, but showed effects on the appearance of PrP^{Sc} in spleens (→3.1.7). Depending on when trehalose treatment was started, peripheral accumulation of PrP^{Sc} was delayed. As was the case with imatinib treatment, this probably also reflects that the process of neuroinvasion was decelerated.

The conditions under which the *in vivo* experiments in this work were performed, resemble a pre-clinical therapeutic intervention situation (Mok *et al.* 2006; Riemer *et al.* 2008). Mice showed a small but significant prolonged survival and a strong decrease in PrP^{Sc} in the spleen. This effect is stronger than the effects observed previously upon Lithium and rapamycin treatment. Moreover, Tamoxifen treatment seems to reduce disease-associated microgliosis, as indicated by iba-1 staining of brain tissue (→Figure 37). Microgliosis has been reported as characteristic of trauma and stroke as well as inflammatory and chronic neurodegenerative disease (Ladeby *et al.*, 2005) and in cellular pathogenesis in prion diseases (Gilch *et al.*, 2007b, Crozet *et al.*, 2008). Activation of microglia is a physiological response from neural tissue to cope with infections and neurodegeneration and its aim is to protect neural tissue. However, exaggerated and chronic activation of microglia may lead to neurotoxicity and may be detrimental for neural tissue (Thomas 1992, Streit *et al.* 1999, Polazzi & Contestabile 2002, Depino *et al.* 2003, Kim & de Vellis 2005, Marchetti & Abbracchio 2005, Minghetti 2005, Suzumura *et al.* 2006, Block *et al.* 2007). There is the hypothesis that regulative experimental approaches of the response of microglial cells to inflammation may represent a therapeutic approach to control neurodegeneration.

Effects of tamoxifen in the CNS are likely to be further facilitated by its known ability to readily cross the blood–brain barrier and accumulate in the CNS (Biegon *et al.* 1996), which is an important prerequisite for neuroprotection. There are reports about treatment of brain tumors and metastases with tamoxifen (Lien *et al.*, 1991; Pors *et al.*, 1991; Salvati *et al.*, 1993; Desai *et al.*, 2006). Tamoxifen has been used in humans for potential

glioma therapy (Perez *et al*. 2003) and for pubertal gynecomastia (Derman *et al*. 2003). Thus, it is of interest to monitor the potential of tamoxifen application as an approach for post-exposure prophylaxis in accordingly designed experimental settings (Aguzzi & Collinge, 1997). Furthermore, there is an enormous number of derivatives available, allowing further refinement and excluding unspecific side-effects by selection (McCague *et al*., 1989; Pathak *et al*., 1996; Top *et al*., 2003).

Obviously, the next important step is to perform further detailed *in vivo* studies, including different infection scenarios which are also of relevance for vCJD infection cases. It should not be underestimated that there is a high amount of expertise in clinical usage of tamoxifen and a lot of clinical data available for tamoxifen treatment of humans, as it is one of the most frequently prescribed drugs for the treatment of breast-cancer. On one hand , autophagy has been reported to play a role in many other neurodegenerative disorders, including neurodegenerative diseases like Alzheimer's disease (Nixon *et al*., 2005), Huntington's disease (Ravikumar *et al*., 2004) or Parkinson's disease (Webb *et al*., 2003). On the other hand, a number of studies report neuroprotective effects of Tamoxifen (Kimelberg *et al*. 2000, 2003; Tian *et al*., 2009; Liu *et al*., 2010). Therefore, correlations between tamoxifen treatment and development of diseases associated with autophagy might be attractive for retrospective studies. All the above makes this compound a potentially neuroprotective drug which might be beneficial in prion disease scenarios and therefore it would be useful to include tamoxifen in further studies.

To conclude, activation of the autophagy pathway by potent pharmacological means such as the SERM tamoxifen might reduce the pathology associated with the neuronal accumulation of PrP^{Sc}. This study bridges previous knowledge about the interplay between prion infection and autophagy with a promising novel type of candidate for therapeutic anti-prion strategy.

4.2 Attenuation of basal autophagy enhances persistent prion infection and indicates a possible role of Beclin 1

As activation of Autophagy via different inducers was able to provide interesting alteration on levels of PrP^{Sc}, it was of interest to study the other scenario, of attenuated or diminished autophagy and its influence on cellular prion infections.

Cell culture models in which prion infections were persistent were used. When autophagy was compromised alone by 3-MA or wortmannin (*data not shown*) treatment in prion-

infected cells, an increase of cellular PrP^{Sc} was observed (→Figure 38), indicating that repression of endogenous autophagy seems to compromise the physiological degradation of cellular PrP^{Sc} which would result in more PrP^{Sc} available for the conversion process, and leading subsequently to an increase in cellular PrP^{Sc}. These results also suggest the possibility that autophagy is involved in the physiological degradation process of PrP^{Sc} during cellular prion infection.

Given the lack of specificity of these currently available autophagy inhibitors, it is recommended (as also mentioned above) to avoid drawing conclusions about the functions of autophagy based upon studies that rely uniquely upon the pharmacological inhibition of autophagy. Rather, pharmacological studies should be combined with genetic approaches to more specifically inhibit the autophagy pathway. More specific inhibition of the autophagy pathway can be achieved by knockout or knockdown of different *Atg* genes. To date, autophagy deficiency/reduction has been confirmed in cells lacking Atg3 (Sou *et al.*, 2008), Atg5 (Mizushima *et al.*, 2001), Beclin 1 (Qu *et al.*, 2003; Yue *et al.*, 2003), Atg7 (Komatsu *et al.*, 2005), Atg9a (Saitoh *et al.*, 2009), Atg16L1 (Cadwell *et al.*, 2008; Saitoh *et al.*, 2008), FIP200 (Hara *et al.*, 2008) and Ambra1 (Fimia *et al.*, 2007). The knockout of Atg4C (Mariño *et al.*, 2007), LC3B (Cann *et al.*, 2007), and ULK1 (Kundu *et al.*, 2008) results in mild phenotypes *in vivo*, probably because related isoforms compensate for the gene deficiency. Therefore, these genes (Atg4C, LC3B and ULK1) should not be used as first choice RNAi targets for autophagy knockdown experiments.

Moreover, recent evidence suggests that autophagosomes can form from late endosomes and trans-Golgi in cells that are deficient for Atg5 or Atg7 (Nishida at al, 2009), and that this appears to be partly involved in eliminating mitochondria from reticulocytes (Nishida at al, 2009; Zhang *et al.*, 2009). However, Atg5/Atg7-independent autophagy requires Beclin1, suggesting that knockdown of Beclin1 may be a more generally robust approach to complete inhibition of macroautophagy, and to determining the importance of autophagy in an experimental system, than knockdown of either Atg5 or Atg7.

In untreated cells with basal autophagy levels, knocking-down autophagy using *Beclin 1* siRNA led to drastic increase of accumulated PrP^{Sc} (> 3-fold), confirming that repression of endogenous autophagy compromises the physiological degradation of cellular PrP^{Sc} in cells were prion infection is already persistent. This goes in line and confirms previous results that suggested that also basal autophagy *per se* might have PrP^{Sc}-reducing

potential in prion-infected cultured cells, as pharmacological inhibition of basal autophagy resulted in increased cellular PrP^{Sc} load (~ 1.3 fold).

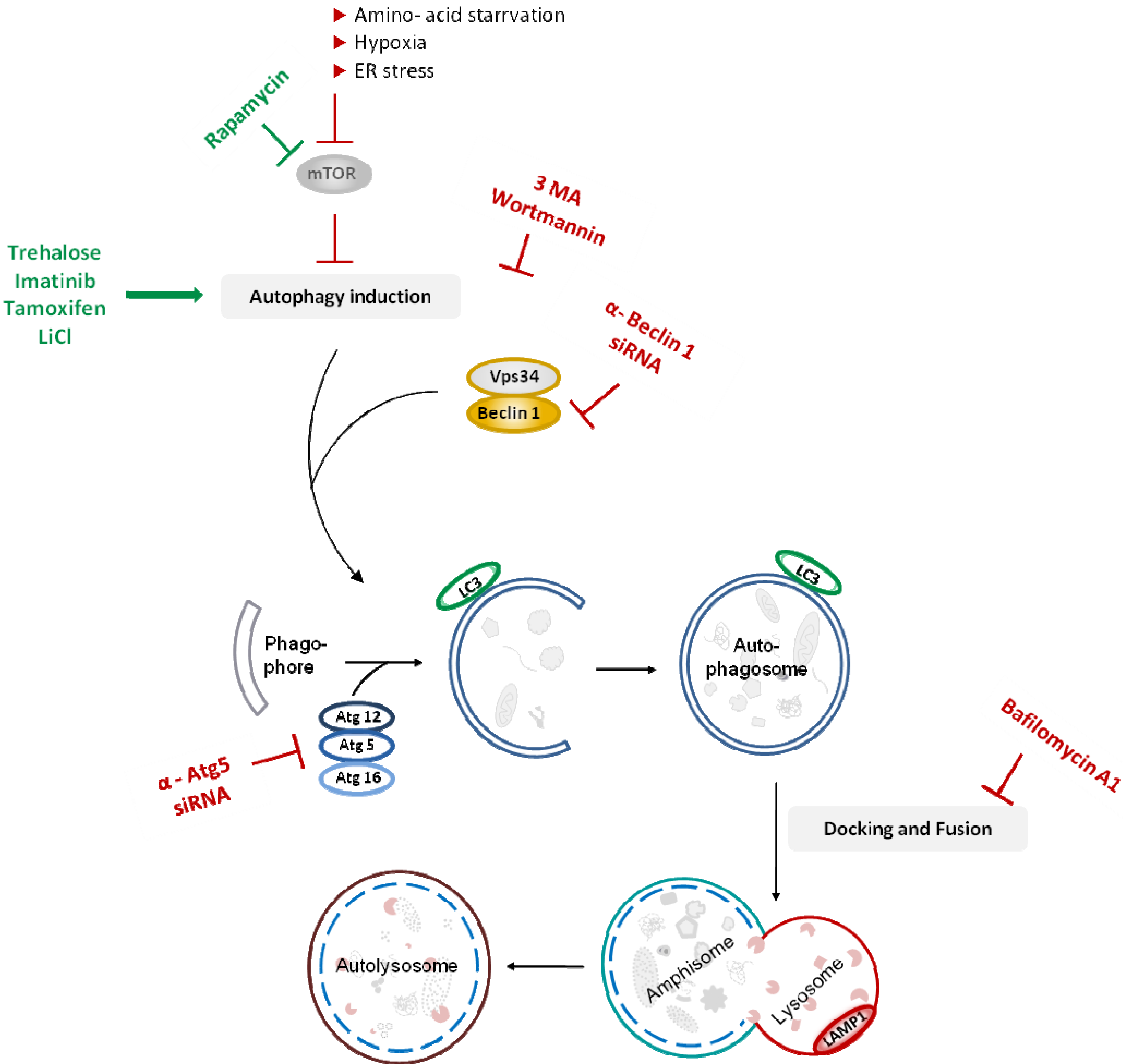

Figure 42. Autophagic modulation as analysed in this work

Beclin 1 is a coiled-coil, 60kDa protein that is analogous to the yeast autophagy protein Apg6 (Liang *et al.*, 1998; Liang *et al.*, 1999). Overexpression of Beclin 1 reduces apoptosis and increases autophagy (Erlich *et al.*, 2006; Hamacher-Brady *et al.*, 2006). Beclin 1 has been localized in some instances to the Golgi apparatus of the ER and may play a role in sorting proteins for degradation via the autophagy pathway (Furuya *et al.*, 2005; Takacs-Vellai *et al.*, 2005). Thus, Beclin 1 might be playing a role in neurons in PD and LBD in targeting excess α-syn to the autophagy pathway thereby reducing the potential for accumulation. For example, in the brains of patients with AD, levels of the

protein Beclin 1 are severely down modulated (Pickford *et al*., 2008). In neuronal cell cultures (Xilouri *et al*. 2009) and in transgenic mice, α-syn overexpression is associated with impaired autophagy and it was shown that *Beclin 1* gene transfer is also capable of reducing the accumulation of α-syn aggregates and associated neuronal deficits (Spencer *et al*., 2009).

It is interesting that data gained from this work suggest a molecular connection between Beclin 1 and accumulation of PrP^{Sc}. These results here confirm previous studies that implicate macroautophagy as a key pathway for removal of aggregated intracellular proteins, including mutant huntingtin,-synuclein, ataxin-3 and prions (Williams *et al*., 2006).

This is consistent with recent studies showing that α-syn aggregates are cleared via autophagy and that alterations in the lysosomal pathway might participate in the mechanisms of α-syn mediated neurodegeneration (Stefanis *et al*., 2001; Meredith *et al*., 2002; Webb *et al*., 2003; Cuervo *et al*., 2004; Rideout *et al*., 2004; Rockenstein *et al*., 2005). Further recent studies supporting a role for autophagic dysfunction in these disorders, have shown increased susceptibility to develop parkinsonism and α-syn accumulation in lysosomal storage diseases such as Gaucher's disease (Tayebi *et al*., 2001; Varkonyi *et al*., 2003) and Niemann-Pick disease (Saito *et al*., 2004).

To sum up, Beclin 1 is an important player in the intracellular degradation of PrP^{Sc} and might present another interesting therapeutic target.

4.3 Conclusions: About the role of autophagy in prion infection

Autophagic vacuoles were described in neurons in experimental models of prion disease in mice and hamsters (Boellaard *et al*., 1991; Boellaard *et al*., 1989). In addition, the appearance of multi-vesicular bodies and autophagic vacuoles was observed in prion-infected cultured neuronal cells (Schatzl *et al*., 1997). More recently, it was found that autophagic vacuoles are formed in neuronal perikarya, neurites and synapses in experimentally induced scrapie, Creutzfeldt-Jakob disease (CJD) and Gerstmann-Sträussler- Scheinker (GSS) syndrome (Liberski *et al*., 2004; Liberski *et al*., 2011) and autophagic vacuoles were identified in synapses in various forms of human prion disease (Sikorska *et al*., 2004). Another interesting correlation between prion diseases and autophagy was observed in studies on *scrg1* (scrapie responsive gene 1). This gene was

up-regulated in brains of scrapie prion- and BSE-infected mice and in brains of patients with sporadic CJD (Dandoy-Dron *et al*., 1998; 2000; Dron *et al*., 1998). In the CNS of prion-infected mice up-regulated Scrg1 was associated with autophagic vacuoles which were observed at the terminal stage of disease (Dron *et al*., 2005). Consequently, it was suggested that Scrg1 might be useful as a marker for neuronal autophagy in prion diseases (Dron *et al*., 2006). With regard to the PrP homologue doppel (Dpl), it was shown that ectopic expression of Dpl in CNS neurons of prion protein knockout-mice (*Ngsk*; NP0/0) results in late onset ataxia due to extensive Purkinje cell (PC) death (Moore *et al*., 1999; Rossi *et al*., 2001; Sakaguchi *et al*., 1996). In line with this, it was demonstrated that preceding and during such PC loss the protein levels of both Scrg1 and the well established autophagic markers LC3-II and p62 were increased, whereas mRNA expression levels were stable (Heitz *et al*., 2008). It was suggested that CNS expression of Dpl might trigger autophagy and that the apoptotic cascade might be triggered by a progressive dysfunction of autophagy.

Besides such descriptions of autophagy in prion disease models, the putative involvement of PrP^c in autophagic pathways was described in several studies. An increased expression of LC3-II was observed in hippocampal neurons of Zürich I *Prnp-/-* mice as compared to wild-type control neurons under serum deprivation and this up-regulation was counter-acted by reintroduction of PrP^c into *Prnp-/-* cells (Oh *et al*., 2008). As such counter-regulation was not detectable for PrP^c lacking the octapeptide region, it was suggested that the octapeptide region of PrP^c may play a crucial role in control of autophagy in neuronal cells as mediated by PrP^c. Concerning again the role of autophagy in prion disease, it was proposed that autophagy may contribute to formation of spongiform changes, a pathological hallmark in prion-affected brains, and may be activated by apoptosis (Liberski *et al*., 2002; 2004; 2008; 2011). In contrast to this assumption that autophagy plays a disease-promoting role, it is also quite conceivable that the observed increase in autophagic vacuoles in prion disease models is due to activation of the autophagic machinery as a defense mechanism, leading even to degradation of prions, which is also confirmed by this study and discussed in → 4.1 and 4.2. Support for such a protective role of autophagy in prion disease was described in studies addressing a member of the galactin family of proteins, namely galactin-3. Reduced levels of the lysosomal activation marker LAMP-2 were observed in prion-infected galactin-3-/--mice and, interestingly, in brain tissue of prion-infected wild-type and galactin-3-/--mice, lower mRNA levels of autophagy markers Beclin 1 and Atg5 were detected as compared to

mock-infected control brains (Mok *et al.*, 2007). Therefore, the authors suggested that endosomal/lysosomal dysfunction in combination with reduced autophagy may contribute to development of prion disease.

4.3.1 Autophagy and prions: Different scenarios, different roles?

In this work the interconnection between drug-induced autophagy and prion propagation is validated and confirmed, by implying different approaches and methods for modulation of autophagy (→Figure 42). Upon treatment with autophagy activating drugs, the cellular load of PrP^{Sc} and prion infectivity is reduced, most probably by an increase in lysosomal degradation, shifting the equilibrium between prion propagation and clearance towards the latter. This was evidenced by the fact that attenuating Atg5- and Beclin 1-dependent autophagy antagonized the observed effect in trehalose and tamoxifen-treated cells. This effect of PrP^{Sc} reduction due to pharmacologically induced autophagy was observed in different prion infection scenrarios: persistent, primary *in vitro* and *in vivo* prion infections (→Figure 43).

In line with these data that state that induced autophagy has beneficial effects in prion infections sceneraios, further data presented in this work suggest a beneficial role of basal autophagy in prion infected cells. The basic process of autophagy seems to have a physiological role in persistent prion infection and might be used by cells for controlling or counter-acting cellular prion infection, as increased amounts of PrP^{Sc} were observed in cells with pharmacologically or genetically attenuated autophagy.

While these results state, that autophagy is involved in the physiological degradation process of PrP^{Sc} in a persisting cellular prion infection, where accumulation and propagation in prions is already established, the scenario of impaired autophagy in primary infection, where prions are still about to occupy their usual trafficking tracks and how the autophagic pathway is involved in this phase of infection remains a different point. Preeliminary experiments showed that Beclin 1 and Atg5 knock down during primary infection attenuated the infection process (→Figure 41), which is the opposite of what is observed in persistent prion infection. Interesting experiments performed by A. Heiseke, in wich autophagy-incompetent MEFs (MEF*Atg*$5^{-/-}$) and their wild-type counterparts (MEF^{wt}) were freshly prion-infected, also shed a light into the role of basal constitutive autophagy in primary prion infection, much less efficient susceptibility to prions was observed in (MEF*Atg*$5^{-/-}$) when compared to MEF^{wt} cells. In turn, reintroduction *of Atg*5 into (MEFAtg$5^{-/-}$) rendered cells more susceptible to prions,

indicating that basal autophagy enhances primary prion infection (Heiseke, 2010). In PrP^{Sc}-susceptible N2a clones, autophagosome formation was increased when newly converted PrP^{Sc} was detected upon primary prion infection. In contrast, increased autophagosome formation was not observed in a PrP^{Sc} -unsusceptible N2a clone (cells that do not convert endogenous PrP^{c} into PrP^{Sc}) upon primary prion infection, indicating that autophagosome formation accompanies and supports primary prion infection.

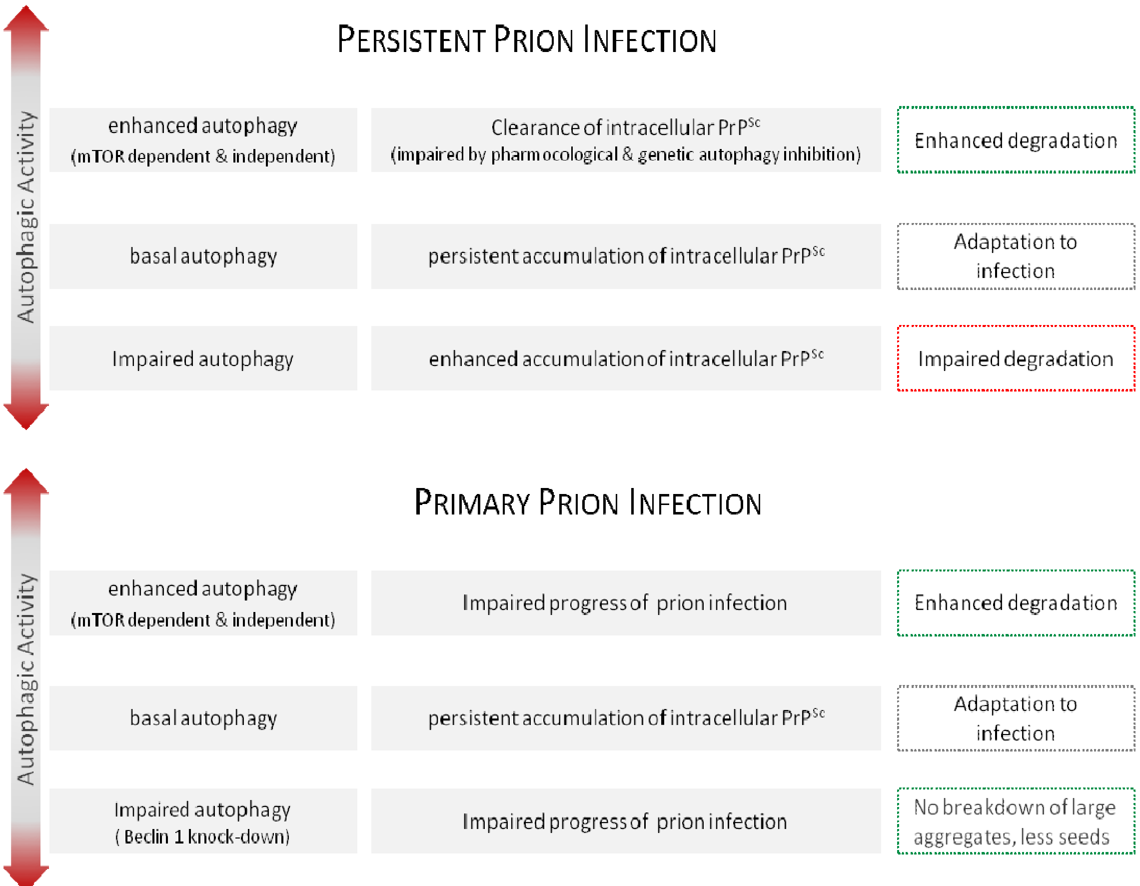

Figure 43. Prion infection scenarios and observed effects of pharmacological and genetic manipulation of autophagy and resulting theories.

It seems that the impact of basal autophagy in cellular events strongly depends on the steps of the autophagic process that are relevant for the according scenarios. Thus it is important to differentiate between effects in *de novo* or persistent infections, as the location of PrP^{Sc} while activating or inhibiting the autophagic process seems to decide about the resulting effect on prion infection. However, identifying the subcellular sites of PrP^{Sc} formation and accumulation proved difficult and cellular compartments involved in prion biogenesis seems to differ during the initial stages of prion infection. It is also unknown if PrP^{Sc} formation by different prion strains might involve alternative

compartments for conversion or if cellular mechanisms of PrP^{Sc} formation differ depending on the cell type. Early studies revealed that PrP^{c} first traverses the cell surface before it acquires protease resistance, suggesting that the secretory pathway including endoplasmic reticulum (ER) and Golgi is not involved in PrP^{Sc} biogenesis (Caughey and Raymond, 1991; Borchelt *et al.*, 1992). Studies with viruses or other GPI-anchored proteins now demonstrate that plasma membrane bound proteins and cargo can be retrogradely sorted to the early secretory pathway, arguing that required PrP^{c} cell surface expression for conversion does not necessarily exclude that these cellular compartments are involved in PrP^{Sc} formation. In N2a cells, only very small amounts of PrP^{Sc} are located at the plasma membrane (Caughey and Raymond, 1991; Borchelt *et al.*, 1990; Vey *et al.*, 1996), the majority appears to accumulate in late endosomes and lysosomes (McKinely *et al.*, 1991; Arnold *et al.*, 1995; Mironov *et al.*, 2003). The lysosome is thought to be the main compartment in the cell in regard to degradation and clearance of PrP^{Sc} (Taraboulos *et al.* 1992; Ertmer *et al.* 2004). In this compartment the amino terminus of nascent PrP^{Sc} is truncated by acidic proteases (Caughey and Raymond 1991; Taraboulos *et al.* 1992). Furthermore, it seems possible that leakage of PrP^{Sc}-containing late endosomes or lysosomes cause PrP^{Sc} to accumulate in the cytosol forming aggresomes (Kristiansen *et al.* 2005) which have been shown to impair proteasomal function (Kristiansen *et al.* 2007). Clearing aggresomes, and thereby PrP^{Sc}, might also be accomplished by autophagy.

On the other hand, the low pH-value of late endosomes or lysosomes may enhance denaturation and refolding of the prion protein and therefore can also represent a compartment for prion replication (Taraboulos *et al.* 1992; Arnold *et al.* 1995; Marijanovic *et al.* 2009).

Although there is now strong evidence from the experiments performed in different prion infection scenarios that induction of autophagy is beneficial in prion-infected cells and animals, it is not clear whether increased or decreased autophagy also can have deleterious effects. One scenario would be that cells impaired in autophagy might be more susceptible to prion infection as they are lacking a putative defence mechanism. In primary infection models experiments addressing whether increase or decrease of basal autophagy is a modifier of prion infection and susceptibility to prion infection were initiated. Another possibility worth to envision is that autophagy also might be a positive factor for prion propagation. A moderate basal level of cellular autophagy, that is likely to be present even in treatment situations with autophagy inhibitors, might be beneficial for

generating smaller PrP^{Sc} seeds, which are known to be more efficient templates for conversion of PrP^{c} into PrP^{Sc} than are larger aggregates (Silveira *et al.*, 2005). Hence, although induced autophagy can reduce or clear prions and PrP^{Sc} seeds, a moderate level of autophagy might support PrP^{Sc} seed production at certain stages of prion infection, thereby promoting prion disease, which would explain reduced PrP^{Sc} levels in cells with genetically attenuated basal autophagy.

Based on exisiting and newly gained data it is now suggested that a certain level of autophagy can be both a positive modifier of prion infection and an enhancing factor, which in certain scenarios is even crucial for prion propagation. As outlined in →Figure 43, a combination of anti-prion and prion-promoting effects of autophagy cannot be excluded as well. This states a complex role of autophagy in prion diseases and presents a scenario reminding of the seeming paradox of the role of autophagy in cell death and cancer biology (Mizushima *et al.*, 2008).

To sum up, the type of interconnection between autophagy and prion infection strongly depends on the existing conditions and settings and the autophagy-prion infection interaction may manifest itself in various ways.

4.3.2 Future directions

From the data obtained so far there is good experimental evidence from *in vitro* and partly also *in vivo* studies that drug-induced autophagy can clearly have beneficial effects on prion infection. The cellular load of PrP^{Sc} and prion infectivity is reduced, most probably by an increase in lysosomal degradation, shifting the equilibrium between prion propagation and clearance towards the latter. On the one hand this clearly shows that not only cytosolic materials are prone to autophagic degradation, on the other hand the systematic analysis of autophagy in prion infection scenarios and the exact molecular mechanisms are still incompletely understood. All the steps where prion infection meets late endosomes and lysosomes are candidates for how and why autophagy is interconnected with prion infection. As the very vast majority of PrP^{Sc}/prions reside within endosomal and lysosomal vesicles, it remains interesting to investigate, whether the aggregates are reached there by the autophagic machinery or whether prion aggregates also reside in autophagosomes. In studies analyzing the role of the autophagy in Alzheimer's disease, purified autophagic vesicles (AVs) are shown to contain APP and β-cleaved APP and are highly enriched in PS1, nicastrin, and PS-dependent γ-secretase

activity (Yu *et al.*, 2004; 2005; Nixon, 2007). It would be interesting to perform similar studies regarding PrP^{Sc} and to characterize autophagosome in different prion infection scenarios.

Furthermore this work demonstrates how the use of antiprion compounds as biochemicals can uncover mechanisms of prion propagation and prion clearance. In future work it has to be studied how prion propagation, prion trafficking and recycling, and finally prion clearance are interconnected with the autophagic pathway. For example combination of well-characterized autophagy manipulators with systematic analysis of the autophagic machinery and involved compartments can address questions auch as: The biological function of autophagy *per se* in prion infection: Is autophagy a cause or a consequence of disease progression? How is the autophagy pathway regulated in prion disease? Is there a crucial molecular key?

Another benefit is that these details can help identifying the intracellular target(s) and molecular mechanism of action of the drugs. Once those are known, the biological activity of the compounds can be optimized on a rational basis, their potential side effects understood and minimized. This work here suggests that the drug tamoxifen and its analogues provide a tool that can be used incorporated in such studies.

Questions like this are readily accessible in *in vitro* studies. As mentioned, autophagy seems to have a dual function and there might be situations in which autophagy is needed for prion propagation. Work from yeast prions and nowadays from mammalian cell culture systems for studying prion-like properties (Krammer *et al.*, 2009a) indicates that the kinetics of aggregate formation needs breaking-up of aggregates and fibrils, probably involving disaggregase activities. It will be interesting to study whether autophagy is a cellular mechanism involved in this scenario.

Another challenge will be to establish reliable *in vivo* models for studying prion infection and autophagy side by side. One possibility is using mice expressing GFP-LC3, allowing direct correlation analysis of hallmarks of prion infection and autophagy *in vivo* (Baier, Aguib, Heiseke and Schatzl, personal communication). As presently available mice with a neuron-specific conditional knockout of *Atg5* or *Atg7* die soon after birth at time points which make them not accessible for classical prion infection studies, alternative Cre deleter mice have to be crossed in, in order to generate a neuron-specific and postnatal knockout of these genes. This would enable to directly assess the impact of autophagy in

prion incubation time and prion disease in *in vivo* models, due to increased life-span of neuron-specific *Atg* knockout mice.

Finally, bringing this knowledge to translational research and bridging therapeutic anti-prion concepts will be a huge challenge. However developing a therapeutic concept in which a) a well characterized (pharmacokinetics, side-effects, …) drug with the ability of passing the blood-brain-barrier, such as tamoxifen and b) a cellular target (the autophagic pathway) studied in detail and c) a sophisticated treatment concept customized to the mode of action of the first and the specific interaction of the latter is still a reasonable goal that could lead to an efficient anti-prion strategy..

In summary, there seems to be a fascinating interplay between prion infections and autophagy. Although extensive future studies will be necessary, there is a high probability that it is both worth and feasible to decipher their mutual interaction at molecular, cellular and whole organisms' level.

5 Abbreviations

aa	Amino acid
Aβ	Amyloid β
AD	Alzheimer's disease
AEBS	Antiestrogen binding site
ALS	Amyotrophic lateral sclerosis
APS	Ammoniumpersulfate
APP	Amyloid precursor protein
ATCC	American Type Culture Collection
Atg	Autophagy related protein
Bp	Base pairs
Baf A1	Bafilomycin A1
BBB	Blood-brain-barrier
BSE	Bovine spongiform encephalopathy
CDC	Centre for disease control and prevention
CJD	Creutzfeldt-Jakob disease
CLD	Caveolae-like domains
CLSM	Confocal laser-scanning microscope
CMA	Chaperone-mediated autophagy
CNS	Central nervous system
CWD	Chronic wasting disease
Dest.	Destilled
DNA	Deoxyribonucleic acid
DOC	Sodium deoxycholate
dNTP	Deoxynucleotide
dpi	Days post infection
EGFP	Enhanced green fluorescent protein
EEG	Electroencephalographic
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
ENS	Enteric nervous system
EUE	Exotic ungulate encephalopathy
FACS	Fluorescence-activated cell-sorting
FAE	Follicle-associated epithelium

FCM	Flow cytometry
FCS	Fetal calf serum
FDC	Follicular dendritic cells
FFI	Fatal familial insomnia
FTD	Frontotemporal dementia
FSE	Feline spongiform encephalopathy
GAG	Glycosaminoglycan
Gdn HCl	Guanidinium hydrochloride
GFP	Green fluorescent protein
GPI	Glycosyl-phosphatidyl-inositol
GSS	Gerstmann-Sträußler-Scheinker disease
HRP	Horseradish peroxidase
Hsp	Heat shock protein
IgG	Immunoglobulin G
IF	Immunofluorescence
Kb	Kilo base pairs
kDa	Kilodalton
LAMP	Lysosomal-associated membrane protein
LC3	Microtubule-associated protein 1 light chain 3
LR	Laminin receptor
LRP	Laminin receptor precursor
mAb	Monoclonal antibody
MBM	Meat and bone meal
mRNA	Messenger ribonucleic acid
MEF	Mouse embryonic fibroblasts
mTOR	Mammalian target of rapamycin
MVB	Multi vesicular body
NCAM	Neural cell adhesion molecules
NMR	Nuclear magnetic resonance
nvCJD	New variant Creutzfeldt-Jakob disease
N2a	Mouse neuroblastoma cell line
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PD	Parkinson’s disease
PE	Phosphatidylethanolamine
PK	Proteinase K
PKC	Protein kinase C
Prnp	Prion protein gene (human)
prnp	Prion protein gene (animal)
PrP	Prion protein
$PrP^{0/0}$	PrP knock-out
PrP^{c}	Cellular non-pathogenic form of the prion protein
PrP^{Sc}	Pathogenic form of the prion protein
PK	Proteinase K
PVDV	Polyvinyl-difluoride membrane
RML	Rocky Mountain Laboratories
RNA	Ribonucleic acid
rpm	Rounds per minute
RT	Room temperature
SAF	Scrapie-associated-fibrils
ScN2a	Scrapie-infected neuroblastoma cells
SERM	Selective estrogen receptor modulator
SDS	Sodium dodecyl sulfate
siRNA	Small interfering RNA
S. E.	Standard error of the mean
TBST	Tris buffered saline tween-20
TEMED	N,N,N,N-tetramethylethylendiamin
TGN	Trans-Golgi network
TME	Transmissible mink encephalopathy
TSE	Transmissible spongiform encephalopathy
UK	United Kingdom
vCJD	Variant CJD
WB	Western blot
wt/vol	Weight by volume
3-MA	Methyladenine

6 References

Aguib Y., Gilch S., Krammer C., Ertmer A., Groschup M.H. and Schatzl H.M. (2008) Neuroendocrine cultured cells counteract persistent prion infection by downregulation of PrP^c. *Molecular and cellular neurosciences* 38, 98-109.

Aguib Y., Heiseke A., Gilch S., Riemer C., Baier M., Schatzl H.M. and Ertmer A. (2009) Autophagy induction by trehalose counteracts cellular prion infection. *Autophagy* 5, 361-369.

Aguzzi A. and Collinge J. (1997) Post-exposure prophylaxis after accidental prion inoculation. *Lancet* 350, 1519-20.

Aguzzi A. (2002) Absence of the prion protein homologue Doppel causes male sterility. *EMBO J.* 21, 3652-8.

Aguzzi A., Montrasio F., Kaeser P.S. (2001a) Prions: health scare and biological challenge. *Nat Rev Mol Cell Biol.*2, 118-26. Review.

Aguzzi A., Glatzel M., Montrasio F., Prinz M., Heppner F. L. (2001b) Interventional strategies against prion diseases. *Nat Rev Neurosci.* 2, 745-9.

Aguzzi A. and Haass C. (2003) Games played by rogue proteins in prion disorders and Alzheimer's disease. *Science NY* 302, 814-818.

Aguzzi A. and Polymenidou M. (2004) Mammalian prion biology: one century of evolving concepts. *Cell* 116, 313-327.

Alexandrenne C., Hanoux V., Dkhissi F., Boquet D., Couraud J.Y. and Wijkhuisen A. (2009) Curative properties of antibodies against prion protein: a comparative in vitro study of monovalent fragments and divalent antibodies. *J Neuroimmunol* 209, 50-6.

Alper T., Haig D. A. and Clarke M. C. (1966) The exceptionally small size of the scrapie agent. *Biochemical and biophysical research communications* **22,** 278-284.

Alper T., Cramp W.A., Haig D.A. and Clarke MC. (1967) Does the agent of scrapie replicate without nucleic acid? *Nature* 214, 764-6.

Alpers D.H. (1987) Another piece to the celiac puzzle. *J Pediatr Gastroenterol Nutr.* 6, 5-7.

Arnold J.E., Tipler C., Laszlo L., Hope J., Landon M. and Mayer R. J. (1995) The abnormal isoform of the prion protein accumulates in late-endosome-like organelles in scrapie-infected mouse brain. *J. Pathol.,* 176, 403-11.

Asante E.A., Linehan J.M., Desbruslais M., Joiner S., Gowland I., Wood A.L., Welch J., Hill A.F., Lloyd S.E., Wadsworth J.D. and Collinge J.(2002) BSE prions propagate as either variant CJD-like or sporadic CJD-like prion strains in transgenic mice expressing human prion protein. *EMBO* J. 21, 6358-66.

Bach S., Talarek N., Andrieu T., Vierfond J.M., Mettey Y., Galons H., Dormont D., Meijer L., Cullin C. and Blondel M. (2003) Isolation of drugs active against mammalian prions using a yeast-based screening assay. *Nat. Biotechnol.* 21, 1075-81.

Bar-On P., Crews L., Koob AO., Mizuno H., Adame A., Spencer B. and Masliah E. (2008) Statins reduce neuronal alpha-synuclein aggregation in in vitro models of Parkinson's disease. *J Neurochem*. 105:1656-67.

Barth S., Glick D. and Macleod K.F. (2010) Autophagy: assays and artifacts. *J. Pathol*. 221, 117-24. Review.

Bartolini M. and Andrisano V. (2010) Strategies for the inhibition of protein aggregation in human diseases. *Chembiochem.* 11, 1018-35. Review.

Basler K., Oesch B., Scott M., Westaway D., Walchli M., Groth D.F., McKinley M. P., Prusiner S.B. and Weissmann C. (1986) Scrapie and cellular PrP isoforms are encoded by the same chromosomal gene. *Cell.* 46**,** 417-428.

Beekes M. and McBride P.A. (2000) Early accumulation of pathological PrP in the enteric nervous system and gut-associated lymphoid tissue of hamsters orally infected with scrapie. *Neurosci Lett.* 278, 181-184.

Belay E.D., (1999) Transmissible spongiform encephalopathies in humans. *Annu. Rev. Microbiol.* 53, 283-314. Review.

Bellingham S.A., Coleman L.A., Masters C.L., Camakaris J. and Hill A.F. (2009) Regulation of prion gene expression by transcription factors SP1 and metal transcription factor-1. *J. Biol. Chem.* 284, 1291-301.

Benvenuti S., Luciani P., Vannelli G.B., Gelmini S., Franceschi E., Serio M. and Peri A. (2005) Estrogen and selective estrogen receptor modulators exert neuroprotective effects and stimulate the expression of selective Alzheimer's disease indicator-1, a recently discovered antiapoptotic gene, in human neuroblast long-term cell cultures. *J. Clin. Endocrinol Metab.* 90:1775-82.

Béranger F., Mangé A., Goud B. and Lehmann S. (2002) Stimulation of PrP(C) retrograde transport toward the endoplasmic reticulum increases accumulation of PrP(Sc) in prion-infected cells. *J. Biol. Chem. 277*, 38972-7.

Béranger F., Crozet C., Goldsborough A. and Lehmann S. (2008) Trehalose impairs aggregation of PrP^{Sc} molecules and protects prion-infected cells against oxidative damage. *Biochem. Biophys. Res. Commun.* 374, 44-8.

Berger Z., Ravikumar B., Menzies F.M., Oroz L.G., Underwood B.R., Pangalos M.N., Schmitt I., Wullner U., Evert B.O., O'Kane C.J. and Rubinsztein D.C. (2006) Rapamycin alleviates toxicity of different aggregate-prone proteins. *Human molecular genetics 15*, 433-442.

Beringue V., Vilette D., Mallinson G., Archer F., Kaisar M., Tayebi M., Jackson G.S., Clarke A.R., Laude H., Collinge J. and Hawke S. (2004) PrP^{Sc} binding antibodies are potent inhibitors of prion replication in cell lines. *J. Biol. Chem.* 279, 39671 -6.

Bernales S., Schuck S. and Walter P. (2007) ER-phagy: selective autophagy of the endoplasmic reticulum. *Autophagy* 3, 285-287.

Bertsch U., Winklhofer K.F., Hirschberger T., Bieschke J., Weber P., Hartl F.U., Tavan P., Tatzelt J., Kretzschmar H.A. and Giese A. (2005) Systematic identification of antiprion drugs by highthroughput screening based on scanning for intensely fluorescent targets. *J Virol.* 79, 7785- 91.

Bessen R.A. and Marsh R.F. (1992) Biochemical and physical properties of the prion protein from two strains of the transmissible mink encephalopathy agent. *Journal of virology* 66, 2096-2101.

Bessen R. A. and Marsh R. F. (1994) Distinct PrP properties suggest the molecular basis of strain variation in transmissible mink encephalopathy. *Journal of virology* 68, 7859-7868.

Bessen R.A., Raymond G.J. and Caughey B. (1997) In situ formation of protease-resistant prion protein in transmissible spongiform encephalopathy-infected brain slices. *The Journal of biological chemistry* 272, 15227-15231.

Bhutani N., Venkatraman P. and Goldberg A.L. (2007) Puromycin-sensitive aminopeptidase is the major peptidase responsible for digesting polyglutamine sequences released by proteasomes during protein degradation. *EMBO J.* 26, 1385-96.

Biegon A., Mathis C.A., Hanrahan S.M. and Jagust W.J. (1993) [125I]5-iodo-6-nitroquipazine: a potent and selective ligand for the 5-hydroxytryptamine uptake complex. II. In vivo studies in rats. *Brain Res.* 619, 236-46.

Bilir A., Altinoz M.A., Erkan M., Ozmen V. and Aydiner A. (2001) Autophagy and nuclear changes in FM3A breast tumor cells after epirubicin, medroxyprogesterone and tamoxifen treatment in vitro. *Pathobiology* 69,120-6.

Bird, S.M. (2004) Attributable testing for abnormal prion protein, database linkage, and blood-borne vCJD risks. *Lancet*. 364, 1362-4.

Bjorkoy G., Lamark T., Brech A., Outzen H., Perander M., Overvatn A., Stenmark H. and Johansen T. (2005) p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtininduced cell death. *The Journal of cell biology* 171, 603-614.

Block M.L. and Hong J.S. (2007) Chronic microglial activation and progressive dopaminergic neurotoxicity. *Biochem. Soc Trans.* 35, 1127-32. Review.

Blommaart E.F., Krause U., Schellens J.P., Vreeling- Sindelarova H., and Meijer A.J. (1997) The phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 inhibit autophagy in isolated rat hepatocytes. *European journal of biochemistry / FEBS 243*, 240-246.

Blommaart E.F., Luiken J.J., Meijer A.J. (1997) Autophagic proteolysis: control and specificity. *Histochem J.* 29, 365-85.

Boellaard J.W., Schlote W. and Tateishi J. (1989) Neuronal autophagy in experimental Creutzfeldt-Jakob's disease. *Acta neuropathologica* 78, 410-418.

Boellaard J.W., Kao M., Schlote W. and Diringer H. (1991) Neuronal autophagy in experimental scrapie. *Acta neuropathologica 82*, 225-228.

Bolton D.C., McKinley M.P. and Prusiner S.B. (1982) Identification of a protein that purifies with the scrapie prion. *Science.* 218, 1309-11.

Borchelt D.R., Taraboulos A. and Prusiner S. B. (1992) Evidence for synthesis of scrapie prion proteins in the endocytic pathway. *The Journal of biological chemistry* 267, 16188-16199.

Borchelt, D.R., Scott, M., Taraboulos, A., Stahl, N. and Prusiner, S.B. (1990) Scrapie and cellular prion proteins differ in their kinetics of synthesis and topology in cultured cells. *J. Cell Biol.* 110, 743–752.

Borchelt D.R., Koliatsos V.E., Guarnieri M., Pardo C.A., Sisodia S.S. and Price D.L. (1994) Rapid anterograde axonal transport of the cellular prion glycoprotein in the peripheral and central nervous systems. *The Journal of biological chemistry* 269, 14711-14714.

Botti J., Djavaheri-Mergny M., Pilatte Y. and Codogno P. (2006) Autophagy signaling and the cogwheels of cancer. *Autophagy* 2, 67-73.

Bounhar Y., Zhang Y., Goodyer C.G. and LeBlanc A. (2001) Prion protein protects human neurons against Bax-mediated apoptosis. *J Biol. Chem.* 19, 39145-9.

Brandner S., Isenmann S., Raeber A., Fischer M., Sailer A., Kobayashi Y., Marino S., Weissmann C. and Aguzzi A. (1996) Normal host prion protein necessary for scrapie-induced neurotoxicity. *Nature.* 379, 339-43.

Breydo L., Bocharova O.V. and Baskakov I.V. (2005) Semiautomated cell-free conversion of prion protein: applications for high-throughput screening of potential antiprioin drugs. *Anal Biochem.* 339, 165-73

Brown F. (1987) Unconventional viruses and the central nervous system. *Br. Med. J. (Clin. Res. Ed.).* 8, 347-8.

Brown P., Preece M.A. and Will R.G. (1992) "Friendly fire" in medicine: hormones, homografts, and Creutzfeldt-Jakob disease. *Lancet* 340**,** 24-27.

Brown D.R., Qin K., Herms J.W., Madlung A., Manson J., Strome R., Fraser P.E., Kruck T., von Bohlen A., Schulz-Schaeffer W., Giese A., Westaway D. and Kretzschmar H. (1997) The cellular prion protein binds copper in vivo. *Nature.* 390, 684-7.

Brown D.R. and Besinger A. (1998) Prion protein expression and superoxide dismutase activity. *Biochem. J.* 334, 423-9.

Brown P., Preece M., Brandel J.P., Sato T., McShane L., Zerr I., Fletcher A., Will R.G., Pocchiari M., Cashman N.R., d'Aignaux J.H., Cervenáková L., Fradkin J., Schonberger L.B. and Collins S.J. (2000) Iatrogenic Creutzfeldt-Jakob disease at the millennium. *Neurology.* 55, 1075-81.

Brown D.R. (2001) Microglia and prion disease. *Microsc Res Tech.* 54, 71-80.

Bruce M.E. and Fraser H. (1991) Scrapie strain variation and its implications. *Curr. Top Microbiol. Immunol.* 172, 125-38.

Bruce M., Chree A., McConnell I., Foster J., Pearson G. and Fraser H. (1994) Transmission of bovine spongiform encephalopathy and scrapie to mice: strain variation and the species barrier. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 343, 405-11.

Bueler H., Fischer M., Lang Y., Bluethmann H., Lipp H.P., DeArmond S.J., Prusiner S.B., Aguet M. and Weissmann C. (1992) Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. *Nature* 356, 577-582.

Bueler H., Aguzzi A., Sailer A., Greiner R.A., Autenried P., Aguet M. and Weissmann C. (1993) Mice devoid of PrP are resistant to scrapie. *Cell* 73, 1339-1347.

Bursch W., Ellinger A., Kienzl H., Török L., Pandey S., Sikorska M., Walker R. and Hermann R.S. (1996) Active cell death induced by the anti-estrogens tamoxifen and ICI 164 384 in human mammary carcinoma cells (MCF-7) in culture: the role of autophagy. *Carcinogenesis* 17, 1595-607.

Bursch W. and Ellinger A. (2005) Autophagy--a basic mechanism and a potential role for neurodegeneration. *Folia neuropathologica / Association of Polish Neuropathologists and Medical Research Centre, Polish Academy of Sciences* 43, 297-310.

Cadwell K., Liu J.Y., Brown S.L., Miyoshi H., Loh J., Lennerz J.K., Kishi C., Kc W., Carrero J.A., Hunt S., Stone C.D., Brunt E.M., Xavier R.J., Sleckman B.P., Li E., Mizushima N., Stappenbeck T.S. and Virgin H.W. 4th. (2008). A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. *Nature 456*, 259–263.

Cann G.M., Guignabert C., Ying L., Deshpande N., Bekker J.M., Wang L., Zhou B., and Rabinovitch M. (2007) Developmental expression of LC3alpha and beta: absence of fibronectin or autophagy phenotype in LC3beta knockout mice. *Dev. Dyn. 237*, 187–195.

Cao Y., Espinola J.A., Fossale E., Massey A.C., Cuervo A.M., MacDonald M.E. and Cotman S. L. (2006) Autophagy is disrupted in a knock-in mouse model of juvenile neuronal ceroid lipofuscinosis. *J. Biol. Chem*. 281, 20483- 20493.

Castilla J, Saa P, Hetz C. and Soto C. (2005) In vitro generation of infectious scrapie prions. *Cell* 121, 195-206

Caspi S., Halimi M., Yanai A., Sasson S.B., Taraboulos A. and Gabizon R. (1998) The anti-prion activity of Congo red. Putative mechanism. *J. Biol. Chem.* 273, 3484-9.

Caughey B. (1991) In vitro expression and biosynthesis of prion protein. *Curr. Top. Microbiol. Immunol.* 172, 93-107.

Caughey B. and Raymond G.J. (1991) The scrapie-associated form of PrP is made from a cell surface precursor that is both protease- and phospholipase-sensitive. *J. Biol. Chem.* 266, 18217-23.

Caughey B., Raymond G.J., Ernst D. and Race R.E. (1991) N-terminal truncation of the scrapie-associated form of PrP by lysosomal protease(s): implications regarding the site of conversion of PrP to the protease-resistant state. *J. Virol*. 65, 6597-603.

Caughey B. and Race R.E. (1992) Potent inhibition of scrapie-associated PrP accumulation by Congo red. *J Neurochem*; 59, 768 -71.

Caughey B., Ernst D. and Race R.E. (1993) Congo red inhibition of scrapie agent replication. *J Virol.* 67, 6270 -2.

Caughey B. and Race R.E. (1994) Scrapie-associated PrP accumulation and its inhibition: revisiting the amyloid-glycosaminoglycan connection. *Ann. N Y Acad. Sci.* 724, 290-5.

Caughey B., Kocisko D.A., Raymond G.J. and Lansbury P.T. Jr. (1995) Aggregates of scrapieassociated prion protein induce the cell-free conversion of protease-sensitive prion protein to the protease-resistant state. *Chem. Biol*. 2, 807-17.

Caughey W.S., Raymond L.D., Horiuchi M. and Caughey B. (1998) Inhibition of protease-resistant prion protein formation by porphyrins and phthalocyanines. *Proc. Natl. Acad. Sci. U S A*. 95, 12117- 12122.

Caughey B. (2001) Prion protein interconversions. *Philos. Trans. R. Soc. B*, 356, 197–200; discussion 200–202. Review

Chen Q. and Haddad G.G. (2004) Role of trehalose phosphate synthase and trehalose during hypoxia: from flies to mammals. *J. Exp. Biol.* 207, 3125-9.

Cheng J., Ohsaki Y., Tauchi-Sato K., Fujita A. and Fujimoto T. (2006) Cholesterol depletion induces autophagy. *Biochem. Biophys. Res. Commun.* 351, 246-52.

Chesebro B., Race R., Wehrly K., Nishio J., Bloom M., Lechner D., Bergstrom S., Robbins K., Mayer L., Keith J.M., Garon C. and Haase A. (1985) Identification of scrapie prion protein-specific mRNA in scrapie-infected and uninfected brain. *Nature 315*, 331-333.

Chini M.G., Scrima M., D'Ursi A.M. and Bifulco G. (2009) Fibril aggregation inhibitory activity of the beta-sheet breaker peptides: a molecular docking approach. *J. Pept. Sci.* 15, 229-34.

Chiti F. and Dobson C.M. (2006) Protein misfolding, functional amyloid, and human disease. *Annual review of biochemistry* 75, 333-366.

Clarke M.C. and Haig D.A. (1970) Evidence for the multiplication of scrapie agent in cell culture. *Nature* 225, 100-1.

Clarke M.C. and Millson G.C. (1976) Infection of a cell line of mouse L fibroblasts with scrapie agent. *Nature* 261, 144-5.

Clarke P.G. (1990) Developmental cell death: morphological diversity and multiple mechanisms. *Anatomy and embryology* 181, 195-213.

Clarke A.R., Jackson G.S. and Collinge J. (2001) The molecular biology of prion propagation. *Philos. Trans. R. Soc. Lond. B. Biol. Sci*. 356, 185-95.

Clinton J., Royston M.C., Gentleman S.M. and Roberts G.W. (1992) Amyloid plaque: morphology, evolution, and etiology. *Mod Pathol* 5, 439-443.

Cohen F.E., Pan K.M., Huang Z., Baldwin M., Fletterick R.J. and Prusiner S.B. (1994) Structural clues to prion replication. *Science NY* 264, 530-531.

Colling S.B., Collinge J. and Jefferys J.G. (1996) Hippocampal slices from prion protein null mice: disrupted Ca(2+)-activated K+ currents. *Neurosci Lett*. 209, 49-52.

Collinge J., Beck J., Campbell T., Estibeiro K. and Will R.G. (1996a) Prion protein gene analysis in new variant cases of Creutzfeldt-Jakob disease. *Lancet.* 348, 56.

Collinge J., Sidle K.C., Meads J., Ironside J. and Hill A.F. (1996b) Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. *Nature.* 383, 685-90.

Collinge J. and Rossor M. (1996) A new variant of prion disease. *Lancet*. 347, 916-7.

Collinge J. (1997) Human prion diseases and bovine spongiform encephalopathy (BSE). *Hum. Mol. Genet*. 6, 1699-705.

Collinge J.(1999) Variant Creutzfeldt-Jakob disease. *Lancet*. 354, 317-23. Review.

Collinge J. (2001) Prion diseases of humans and animals: their causes and molecular basis. *Annu. Rev. Neurosci*. 24: 519-50.

Collinge J. (2005) Molecular neurology of prion disease. *Journal of neurology, neurosurgery and psychiatry* 76, 906-919.

Collins S.J., Sanchez-Juan P., Masters C.L., Klug G.M., van Duijn C., Poleggi A., Pocchiari M., Almonti S., Cuadrado-Corrales N., de Pedro-Cuesta J., Budka H., Gelpi E., Glatzel M., Tolnay M., Hewer E., Zerr I., Heinemann U., Kretszchmar H. A., Jansen G.H., Olsen E., Mitrova E., Alperovitch A., Brandel J.P., Mackenzie J., Murray K. and Will R.G. (2006) Determinants of diagnostic investigation sensitivities across the clinical spectrum of sporadic Creutzfeldt-Jakob disease. *Brain* 129, 2278-2287.

Come J.H., Fraser P.E. and Lansbury P.T. Jr. (1993) A kinetic model for amyloid formation in the prion diseases: importance of seeding. *Proc. Natl. Acad. Sci. U S A.* 90, 5959-63.

Creutzfeldt H.G. (1920) Über eine eigenartige herdförmige Erkrankung des zentralen Nervensystems. *Ges. Neuro. Psychiatr.* 57, 1-18.

Criollo A., Maiuri M.C., Tasdemir E., Vitale I., Fiebig A.A., Andrews D., Molgó J., Díaz J., Lavandero S., Harper F., Pierron G., di Stefano D., Rizzuto R., Szabadkai G. and Kroemer G. (2007) Regulation of autophagy by the inositol trisphosphate receptor. *Cell death and differentiation* 14, 1029-1039.

Cronier S., Beringue V., Bellon A., Peyrin J.M. and Laude H. (2007) Prion strain- and species-dependent effects of antiprion molecules in primary neuronal cultures. *J. Virol.* 81, 13794 -800.

Crozet C., Beranger F. and Lehmann S. (2008) Cellular pathogenesis in prion diseases. *Vet. Res.* 39,44. Review.

Cuervo A.M. (2010) Chaperone-mediated autophagy: selectivity pays off. *Trends Endocrinol. Metab.* 21, 142–150.

Cuervo A.M., Stefanis L., Fredenburg R., Lansbury P.T. and Sulzer D. (2004) Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy. *Science* 305, 1292-5.

Cuille J., and Chelle P.L. (1939) Experimental transmission of trembling to the goat. *Comptes Rendus des Seances de l'Academie des Sciences* 208, 1058-1160.

Daido S., Kanzawa T., Yamamoto A., Takeuchi H., Kondo Y. and Kondo S. (2004) Pivotal role of the cell death factor BNIP3 in ceramide-induced autophagic cell death in malignant glioma cells. *Cancer Res.* 64, 4286-93.

Dandoy-Dron F., Guillo F., Benboudjema L., Deslys J.P., Lasmezas C., Dormont D., Tovey M.G. and Dron M. (1998) Gene expression in scrapie. Cloning of a new scrapie-responsive gene and the identification of increased levels of seven other mRNA transcripts. *The Journal of biological chemistry* 273, 7691-7697.

Dandoy-Dron F., Benboudjema L., Guillo F., Jaegly A., Jasmin C., Dormont D., Tovey M.G. and Dron M. (2000) Enhanced levels of scrapie responsive gene mRNA in BSE-infected mouse brain. *Brain research* 76, 173-179.

Daude N., Marella M. and Chabry J. (2003) Specific inhibition of pathological prion protein accumulation by small interfering RNAs. *J. Cell Sci.* 116, 2775 -9.

De Médina P., Favre G. and Poirot M. (2004) Multiple targeting by the antitumor drug tamoxifen: a structure-activity study. *Curr. Med. Chem. Anticancer Agents* 4, 491-508.

De Medina P., Payré B., Boubekeur N., Bertrand-Michel J., Tercé F., Silvente-Poirot S. and Poirot M. (2009a) Ligands of the antiestrogen-binding site induce active cell death and autophagy in human breast cancer cells through the modulation of cholesterol metabolism. *Cell Death Differ.* 16, 1372-84.

De Medina P., Silvente-Poirot S. and Poirot M. (2009b) Tamoxifen and AEBS ligands induced apoptosis and autophagy in breast cancer cells through the stimulation of sterol accumulation. *Autophagy* 5, 1066-7.

DeArmond S.J., Mobley W.C., DeMott D.L., Barry R.A., Beckstead J. H. and Prusiner S.B. (1987) Changes in the localization of brain prion proteins during scrapie infection. *Neurology* 37, 1271-1280

DeArmond S.J. and Prusiner S.B. (1995) Etiology and pathogenesis of prion diseases. *The American journal of pathology* 146, 785-811.

Depino A.M., Earl C., Kaczmarczyk E., Ferrari C., Besedovsky H., del Rey A., Pitossi F.J. and Oertel W.H. (2003) Microglial activation with atypical proinflammatory cytokine expression in a rat model of Parkinson's disease. *Eur. J. Neurosci.* 18, 2731-42.

Derman O., Kanbur N.O. and Kutluk T. (2003) Tamoxifen treatment for pubertal gynecomastia. *Int. J. Adolesc. Med. Health* 15, 359-63.

Desai P.B., Duan J.Z., Zhu Y.W. and Kouzi S. (1998) Human liver microsomal metabolism of paclitaxel and drug interactions. *Eur. J. Drug Metab. Pharmacokinet.* 23, 417-24.

Diarra-Mehrpour M., Arrabal S., Jalil A., Pinson X., Gaudin C., Piétu G., Pitaval A., Ripoche H., Eloit M., Dormont D. and Chouaib S. (2004) Prion protein prevents human breast carcinoma cell line from tumor necrosis factor alpha-induced cell death. *Cancer Res.* 15, 719-27.

Dice J.F. (2007) Chaperone-mediated autophagy. *Autophagy* 3, 295–299.

Dickinson A.G., Meikle V.M. and Fraser H. (1968) Identification of a gene which controls the incubation period of some strains of scrapie agent in mice. *J. Comp. Pathol.* 78, 293-9.

Dickinson A.G., Stamp J.T. and Renwick C.C. (1974) Maternal and lateral transmission of scrapie in sheep. *J. Comp. Pathol.* 84, 19-25.

Diringer H. and Ehlers B. (1991) Chemoprophylaxis of scrapie in mice. J. Gen. Virol. 72, 457 -60.

Dodelet V.C. and Cashman N.R. (1998) Prion protein expression in human leukocyte differentiation. *Blood* 91, 1556-1561.

Donnelly, C.A., Ferguson, N.M., Ghani, A.C. and Anderson, R.M. (2002) Implications of BSE infection screening data for the scale of the British BSE epidemic and current European infection levels. *Proc. Biol. Sci.* 269, 2179-90.

Dron M., Dandoy-Dron F., Guillo F., Benboudjema L., Hauw J.J., Lebon P., Dormont D. and Tovey M.G. (1998) Characterization of the human analogue of a Scrapie-responsive gene. *The Journal of biological chemistry* 273, 18015-18018.

Dron M., Bailly Y., Beringue V., Haeberle A.M., Griffond B., Risold P.Y., Tovey M.G., Laude H. and Dandoy-Dron F. (2005) Scrg1 is induced in TSE and brain injuries, and associated with autophagy. *The European journal of neuroscience* 22, 133-146.

Dron M., Bailly Y., Beringue V., Haeberle A.M., Griffond B., Risold P.Y., Tovey M.G., Laude H. and Dandoy-Dron F. (2006) SCRG1, a potential marker of autophagy in transmissible spongiform encephalopathies. *Autophagy* 2, 58-60.

Dunn W.A. Jr., Cregg J. M., Kiel J. A., van der Klei I. J., Oku M., Sakai Y., Sibirny A. A., Stasyk O. V. and Veenhuis M. (2005) Pexophagy: the selective autophagy of peroxisomes. *Autophagy* 1, 75-83.

Dupiereux I., Falisse-Poirrier N., Zorzi W., Watt N.T., Thellin O., Zorzi D., Pierard O., Hooper N.M., Heinen E. and Elmoualij B. (2007) Protective effect of prion protein via the N-terminal region in mediating a protective effect on paraquat-induced oxidative injury in neuronal cells. *J. Neurosci. Res*. 86, 653-659.

Edenhofer F., Rieger R., Famulok M., Wendler W., Weiss S. and Winnacker EL. (1996) Prion protein PrP^c interacts with molecular chaperones of the Hsp60 family. *J. Virol*. 70, 4724-8.

Enari M., Flechsig E. and Weissmann C. (2001) Scrapie prion protein accumulation by scrapie-infected neuroblastoma cells abrogated by exposure to a prion protein antibody. *Proc. Natl. Acad. Sci. USA*. 98, 9295 -9.

Endo T., Groth D., Prusiner S.B. and Kobata A. (1989) Diversity of oligosaccharide structures linked to asparagines of the scrapie prion protein. *Biochemistry* 28, 8380-8.

Erlich S., Shohami E. and Pinkas-Kramarski R. (2006) Neurodegeneration induces upregulation of Beclin 1. *Autophagy.* 2, 49-51.

Ertmer A., Gilch S., Yun S.W., Flechsig E., Klebl B., Stein-Gerlach M., Klein M.A. and Schätzl H.M. (2004) The tyrosine kinase inhibitor STI571 induces cellular clearance of PrP^{Sc} in prion-infected cells. *J. Biol. Chem*. 279, 41918 -27.

Ertmer A., Huber V., Gilch S., Yoshimori T., Erfle V., Duyster J., Elsässer H.P. and Schätzl H.M. (2007) The anticancer drug imatinib induces cellular autophagy. *Leukemia* 21, 936 -42.

Eskelinen E.L. (2008) New insights into the mechanisms of macroautophagy in mammalian cells. *Int. Rev. Cell Mol. Biol.* 266, 207-247.

Farquhar C.F. and Dickinson A.G. (1986) Prolongation of scrapie incubation period by an injection of dextran sulphate 500 within the month before or after infection. J. Gen.Virol. 67, 463 -73

Felbor U., Kessler B., Mothes W., Goebel H. H., Ploegh H. L., Bronson R. T. and Olsen B. R. (2002) Neuronal loss and brain atrophy in mice lacking cathepsins B and L. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 7883-7888.

Féraudet C., Morel N., Simon S., Volland H., Frobert Y., Créminon C., Vilette D., Lehmann S. and Grassi J. (2005) Screening of 145 anti-PrP monoclonal antibodies for their capacity to inhibit PrP^{Sc} replication in infected cells. *J. Biol. Chem.* 280, 11247-58.

Fevrier B., Vilette D., Archer F., Loew D., Faigle W., Vidal M., Laude H. and Raposo G. (2004) Cells release prions in association with exosomes. *Proc. Natl. Acad. Sci. U S A* 101, 9683-8.

Filimonenko M., Stuffers S., Raiborg C., Yamamoto A., Malerod L., Fisher E. M., Isaacs A., Brech A., Stenmark H. and Simonsen A. (2007) Functional multivesicular bodies are required for autophagic clearance of protein aggregates associated with neurodegenerative disease. *J. Cell Biol.* 179, 485-500.

Fimia G. M., Stoykova A., Romagnoli A., Giunta L., Di Bartolomeo S., Nardacci R., Corazzari M., Fuoco C., Ucar A., Schwartz P., Gruss P., Piacentini M., Chowdhury K. and Cecconi F. (2007) Ambra1 regulates autophagy and development of the nervous system. *Nature* 447, 1121-1125.

Flöck D., Colacino S., Colombo G. and Di Nola A. (2006) Misfolding of the amyloid beta-protein: a molecular dynamics study. *Proteins* 62, 183-92.

Ford M.J., Burton L.J., Li H., Graham C.H., Frobert Y., Grassi J., Hall S. M. and Morris R.J. (2002) A marked disparity between the expression of prion protein and its message by neurones of the CNS. *Neuroscience* 111, 533-551.

Fortun J., Dunn W.A. Jr., Joy S., Li J. and Notterpek L. (2003) Emerging role for autophagy in the removal of aggresomes in Schwann cells. *J. Neurosci. 23*, 10672-10680.

Fournier J.G., Escaig-Haye F., Billette de Villemeur T. and Robain O. (1995) Ultrastructural localization of cellular prion protein (PrP^c) in synaptic boutons of normal hamster hippocampus. *Comptes rendus de l'Academie des sciences* 318, 339-344.

Fournier J.G., Escaig-Haye F. and Grigoriev V. (2000) Ultrastructural localization of prion proteins: physiological and pathological implications. *Microsc. Res. Tech.* 50, 76-88.

Frosh A., Smith L.C., Jackson C.J., Linehan J.M., Brandner S., Wadsworth J.D. and Collinge J. (2004) Analysis of 2000 consecutive UK tonsillectomy specimens for disease-related prion protein. *Lancet*. 364, 1260-2.

Furuya N., Yu J., Byfield M., Pattingre S. and Levine B. (2005) The evolutionarily conserved domain of Beclin 1 is required for Vps34 binding, autophagy and tumor suppressor function. *Autophagy* 1, 46-52.

Gabriel J.M., Oesch B., Kretzschmar H., Scott M. and Prusiner S.B. (1992) Molecular cloning of a candidate chicken prion protein. *Proceedings of the National Academy of Sciences of the United States of America* 89, 9097-9101.

Gajdusek D.C, Gibbs C.J. and Alpers M. (1966) Experimental transmission of a Kuru-like syndrome to chimpanzees. *Nature* 209, 794-6.

Gajdusek D.C. (1994) Nucleation of amyloidogenesis in infectious and noninfectious amyloidoses of brain. *Ann N Y Acad Sci*. 6,173-90.

Gambetti P., Parchi P., Petersen R.B., Chen S.G. and Lugaresi E. (1995) Fatal familial insomnia and familial Creutzfeldt-Jakob disease: clinical, pathological and molecular features. *Brain Pathol.* 5, 43-51. Review.

Gasset M., Baldwin M.A., Fletterick R.J. and Prusiner S.B. (1993) Perturbation of the secondary structure of the scrapie prion protein under conditions that alter infectivity. *Proceedings of the National Academy of Sciences of the United States of America* 90**,** 1-5.

Gebauer M., Skerra A. (2009) Engineered protein scaffolds as next-generation antibody therapeutics. *Curr Opin Chem Biol.* 13,245-55. Review.

Gendron T.F. and Petrucelli L. (2009) The role of tau in neurodegeneration. *Mol Neurodegener.* 11, 4-13.

Ghosh A.K., Gemma S. and Tang J.(2008) beta-Secretase as a therapeutic target for Alzheimer's disease. *Neurotherapeutics* 5, 399-408.

Gilch S., Spielhaupter C. and Schatzl H.M. (2000) Shortest known prion protein allele in highly BSE susceptible lemurs. *Biol Chem.* 381, 521-3.

Gilch S., Winklhofer K.F., Groschup M.H., Nunziante M., Lucassen R., Spielhaupter C., Muranyi W., Riesner D., Tatzelt J. and Schatzl H.M. (2001) Intracellular re-routing of prion protein prevents propagation of PrP(Sc) and delays onset of prion disease. *EMBO J.* 20, 3957-66.

Gilch S., Wopfner F., Renner-Muller I., Kremmer E., Bauer C., Wolf E., Brem G., Groschup M. H. and Schatzl H.M. (2003) Polyclonal anti-PrP auto-antibodies induced with dimeric PrP interfere efficiently with PrP^{Sc} propagation in prion-infected cells. *J. Biol. Chem.* 278, 18524-31.

Gilch S. and Schatzl H.M. (2003) Promising developments bringing prion diseases closer to therapy and prophylaxis. *Trends Mol. Med.* 9, 367-9. Review.

Gilch S., Nunziante M. Ertmer A., Wopfner F., Laszlo L. and Schatzl H.M. (2004) Recognition of lumenal prion protein aggregates by post-ER quality control mechanisms is mediated by the preoctarepeat region of PrP. *Traffic* 5, 300–313.

Gilch S., Kehler C. and Schatzl H.M. (2006) The prion protein requires cholesterol for cell surface localization. *Mol. Cell Neurosci.* 31, 346-53.

Gilch S., Kehler C. and Schätzl H.M. (2007a) Peptide aptamers expressed in the secretory pathway interfere with cellular PrP^{Sc} formation. *J Mol. Biol.* 371, 362-73.

Gilch S., Schmitz F., Aguib Y., Kehler C., Bülow S., Bauer S., Kremmer E. and Schätzl H.M. (2007b) CpG and LPS can interfere negatively with prion clearance in macrophage and microglial cells. *FEBS J.* 274, 5834-44.

Gilch S., Krammer C., Schätzl H. M. (2008) Targeting prion proteins in neurodegenerative disease. *Expert Opin Biol Ther.* 8, 923-40. Review.

Gilch S. and Schätzl H.M. (2009) Aptamers against prion proteins and prions. *Cell Mol. Life Sci.* 66, 2445-55.

Glabe C.C. (2005) Amyloid accumulation and pathogensis of Alzheimer's disease: significance of monomeric, oligomeric and fibrillar Abeta. *Subcell Biochem.* 38, 167-77. Review

Glabe C.G. (2006) Common mechanisms of amyloid oligomer pathogenesis in degenerative disease. *Neurobiol Aging* 27, 570-5. Review.

Gorodinsky A. and Harris D.A. (1995) Glycolipid-anchored proteins in neuroblastoma cells form detergent-resistant complexes without caveolin. *J. Cell. Biol.* 129, 619-27.

Ghani A.C., Donnelly C.A., Ferguson N. M. and Anderson R.M. (2002) The transmission dynamics of BSE and vCJD. *C. R. Biol*. 325, 37-47. Review.

Golde T.E., Petrucelli L. and Lewis J.(2010) Targeting Abeta and tau in Alzheimer's disease, an early interim report. *Exp. Neurol.* 223, 252-66.

Ghosh S. (2002). Intestinal entry of prions. *Z. Gastroenterol* 40, 37-39.

Govaerts C., Wille H., Prusiner S.B. and Cohen F.E. (2004) Evidence for assembly of prions with left-handed beta-helices into trimers. *Proc. Natl. Acad. Sci. USA*. 101, 8342-7.

Gozuacik D. and Kimchi A. (2007) Autophagy and cell death. *Current topics in developmental biology* 78, 217-245.

Gundimeda U., Chen Z.H. and Gopalakrishna R. (1996) Tamoxifen modulates protein kinase C via oxidative stress in estrogen receptor-negative breast cancer cells. *J. Biol. Chem.* 7, 13504-14.

Hadlow W.J. (1959) Scrapie and kuru. *Lancet* 2, 289-290.

Haeberlé A.M., Ribaut-Barassin C., Bombarde G., Mariani J., Hunsmann G., Grassi J. and Bailly Y. (2000) Synaptic prion protein immuno-reactivity in the rodent cerebellum.Microsc *Res. Tech.,* 50(1), 66-75.

Haigh C.L. and Brown D.R. (2006) Prion protein reduces both oxidative and non-oxidative copper toxicity. *J. Neurochem*. 98, 677-89.

Hamacher-Brady A, Brady N.R. and Gottlieb R.A. (2006) Enhancing macroautophagy protects against ischemia/reperfusion injury in cardiac myocytes. *J. Biol. Chem.* 281, 29776-87.

Hanger D.P., Anderton B.H. and Noble W. (2009) Tau phosphorylation: the therapeutic challenge for neurodegenerative disease. *Trends Mol. Med*. 15, 112-9. Review.

Hara T., Nakamura K., Matsui M., Yamamoto A., Nakahara Y., Suzuki-Migishima R., Yokoyama M., Mishima K., Saito I., Okano H. and Mizushima N. (2006) Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature* 441, 885-889.

Harding T.M., Morano K.A., Scott S.V., and Klionsky, D.J. (1995). Isolation and characterization of yeast mutants in the cytoplasm to vacuole protein targeting pathway. *The Journal of cell biology* 131, 591-602.

Haybaeck J., Heikenwalder M., Klevenz B., Schwarz P., Margalith I., Bridel C., Mertz K., Zirdum E., Petsch B., Fuchs T.J., Stitz L. and Aguzzi A. (2011) Aerosols transmit prions to immunocompetent and immunodeficient mice. *PLoS Pathog.* 7, e1001257.

He C. and Klionsky D.J. (2009) Regulation mechanisms and signaling pathways of autophagy. *Annu. Rev. Genet.* 43, 67–93.

Head M.W., Northcott V., Rennison K., Ritchie D., McCardle L., Bunn T.J.R., McLennan N.F., Ironside J.W., Tullo A.B. and Bonshek R.E. (2002) Prion protein accumulation in eyes ofpatients with sporadic and variant Creutzfeldt-Jakob disease. *Invest. Ophthalmol. Vis. Sci.* 44, 342–6.

Hegde R.S., Mastrianni J.A., Scott M.R., Defea K.A., Tremblay P., Torchia M., DeArmond S. J., Prusiner S.B., and Lingappa V.R. (1998) A transmembrane Form of the prion protein in Neurodegenerative Disease. *Science* 279, 827–834.

Henley D.B., May P.C., Dean R.A. and Siemers E.R.(2009) Development of semagacestat (LY450139), a functional gamma-secretase inhibitor, for the treatment of Alzheimer's disease. *Expert Opin. Pharmacother.* 10, 1657-64.

Heiseke A., Schobel S., Lichtenthaler S.F., Vorberg I., Groschup M.H., Kretzschmar H., Schatzl H.M., and Nunziante M. (2008). The novel sorting nexin SNX33 interferes with cellular PrP formation by modulation of PrP shedding. *Traffic 9*, 1116-1129.

Heiseke A., Aguib Y., Riemer C., Baier M., and Schatzl H.M. (2009). Lithium induces clearance of protease resistant prion protein in prion-infected cells by induction of autophagy. *Journal of neurochemistry* 109, 25-34.

Heiseke, A., Aguib, Y. and Hermann M. Schätzl. Autophagy, Prion Infection and their Mutual Interactions. (2010) *Curr Issues Mol Biol*. 12(2):87-98.

Heiseke A. (2010) Prions and autophagy: Effect of lithium on prion infection and role of basal autophagy in primary prion infection. *Dissertation.* TU München.

Heitz S., Grant N.J., Leschiera R., Haeberle A.M., Demais V., Bombarde G., and Bailly Y. (2008). Autophagy and Cell Death of Purkinje Cells Overexpressing Doppel in Ngsk Prnp-deficient Mice. *Brain pathology*.

Herms J., Tings T., Gall S., Madlung A., Giese A., Siebert H., Schurmann P., Windl O., Brose N. and Kretzschmar H. (1999) Evidence of presynaptic location and function of the prion protein. *J. Neurosci.* 19, 8866-8875.

Hijazi N., Kariv-Inbal Z., Gasset M. and Gabizon R. (2005) PrP^{Sc} incorporation to cells requires endogenous glycosaminoglycan expression. *J. Biol. Chem*. 280, 17057 -61.

Hill A.F., Desbruslais M., Joiner S., Sidle K.C., Gowland I., Collinge J., Doey L.J. and Lantos P. (1997) The same prion strain causes vCJD and BSE. *Nature* 389,448-50.

Hill A.F., Butterworth R.J., Joiner S., Jackson G., Rossor M.N., Thomas D.J. and Frosh A. (1999a) Investigation of variant Creutzfeldt-Jakob disease and other human prion diseases with tonsil biopsy samples. *Lancet* 353, 183–189.

Hill A.F., Antoniou M. and Collinge J. (1999b) Protease-resistant prion protein produced in vitro lacks detectable infectivity. J. Gen. Virol. 80, 11-4.

Hill A.F., Joiner S., Linehan J., Desbruslais M., Lantos P.L. and Collinge J. (2000) Species-barrierindependent prion replication in apparently resistant species.*Proc. Natl. Acad. Sci. USA* 97, 10248-53.

Hill A.F., Joiner S., Wadsworth J.D., Sidle K.C., Bell J.E., Budka H., Ironside J.W. and Collinge J. (2003) Molecular classification of sporadic Creutzfeldt-Jakob disease. *Brain*. 126, 1333-46.

Hilmert H. and Diringer H. (1984) A rapid and efficient method to enrich SAF-protein from scrapie brains of hamsters. *Biosci. Rep.* 4, 165-70.

Hilton D.A.(2006) Pathogenesis and prevalence of variant Creutzfeldt-Jakob disease.*J. Pathol.* 208, 134-41. Review.

Holme A., Daniels M., Sassoon J. and Brown D.R. (2003) A novel method of generating neuronal cell lines from gene-knockout mice to study prion protein membrane orientation. *Eur. J. Neurosci.* 18, 571-9.

Horiuchi M. and Caughey B. (1999) Specific binding of normal prion protein to the scrapie form via a localized domain initiates its conversion to the protease-resistant state. *EMBO J.* 18, 3193-203.

Horonchik L., Tzaban S., Ben-Zaken O., Yedidia Y., Rouvinski A., Papy-Garcia D., Barritault D., Vlodavsky I. and Taraboulos A. (2005) Heparan sulfate is a cellular receptor for purifi ed infectious prions. *J. Biol. Chem.* 280, 17062 -7.

Hörnlimann B., Riesner D., und Kretzschmar H. (2001) Prionen und Prionkrankheiten. *De Gruyter, Berlin.*

Hornshaw M.P., McDermott J.R. and Candy J.M. (1995) Copper binding to the N-terminal tandem repeat regions of mammalian and avian prion protein. *Biochem. Biophys. Res. Commun.* 207, 621-9.

Horwich A.L. and Weissman J.S. (1997) Deadly conformations--protein misfolding in prion disease. *Cell.* 89, 499-510. Review.

Hosokawa N., Hara Y. and Mizushima N. (2006) Generation of cell lines with tetracycline-regulated autophagy and a role for autophagy in controlling cell size. *FEBS Lett. 580*, 2623–2629.

Hosokawa N., Hara T., Kaizuka T., Kishi C., Takamura A., Miura Y., Iemura S., Natsume T., Takehana K., Yamada N., Guan J.L., Oshiro N. and Mizushima N. (2009). Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. *Mol. Biol. Cell 20*, 1981–1991.

Hsiao K., Baker H.F., Crow T.J., Poulter M., Owen F., Terwilliger J.D., Westaway D., Ott J. and Prusiner S.B. (1989) Linkage of a prion protein missense variant to Gerstmann-Straussler syndrome. *Nature* 338, 342-345.

Hundt C., Peyrin J.M., Haïk S., Gauczynski S., Leucht C., Rieger R., Riley M.L., Deslys J.P., Dormont D., Lasmézas C.I. and Weiss S. (2001) Identification of interaction domains of the prion protein with its 37-kDa/67-kDa laminin receptor. *EMBO J.* 20, 5876-86.

Ingrosso L., Ladogana A. and Pocchiari M. (1995) Congo red prolongs the incubation period in scrapie-infected hamsters. *J. Virol.* 69, 506 -8.

Iwata A., Christianson J.C., Bucci M., Ellerby L.M., Nukina N., Forno L.S. and Kopito R.R. (2005a) Increased susceptibility of cytoplasmic over nuclear polyglutamine aggregates to autophagic degradation. *Proc. Natl. Acad. Sci. USA* 102, 13135–13140.

Iwata A., Riley B.E., Johnston J.A., and Kopito R.R. (2005b). HDAC6 and microtubules are required for autophagic degradation of aggregated huntingtin. *The Journal of biological chemistry 280*, 40282-40292.

Jacobsen J.S., Comery T.A., Martone R.L., Elokdah H., Crandall D.L., Oganesian A., Aschmies S., Kirksey Y., Gonzales C., Xu J., Zhou H., Atchison K., Wagner E., Zaleska M.M., Das I., Arias R.L., Bard J., Riddell D., Gardell S.J., Abou-Gharbia M., Robichaud A., Magolda R., Vlasuk G.P., Bjornsson T., Reinhart P.H. and Pangalos M.N. (2008) Enhanced clearance of Abeta in brain by sustaining the plasmin proteolysis cascade.*Proc. Natl. Acad. Sci. U S A.* 105, 8754-9.

Jakob A.M. (1921) Über eigenartige Erkrankungen des Zentralnervensystems mit bemerkenswerten anatomischen Befunden (Spastische Pseudosklerose-Encephalomyelopathie mit disseminierten Degererationsherden). *Dtsch. Z. Nervenheilk.* 70, 132-146.

Jarrett J.T. and Lansbury P.T. Jr. (1993) Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie? *Cell.* 18, 1055-8. Review.

Jeffrey M. and Wells G.A. (1988) Spongiform encephalopathy in a nyala (Tragelaphus angasi).*Vet. Pathol.* 25, 398-9.

Jin J. and Woodgett J.R. (2005) Chronic activation of protein kinase Bbeta/Akt2 leads to multinucleation and cell fusion in human epithelial kidney cells: events associated with tumorigenesis. *Oncogene* 24, 5459-70

Jordan V.C. (1984) Biochemical pharmacology of antiestrogen action. *Pharmacol. Rev.* 36, 245-76. Review.

Jordan VC. (2001) Selective estrogen receptor modulation: a personal perspective. *Cancer Res.* 61, 5683-7.

Kabeya Y., Mizushima N., Ueno T., Yamamoto A., Kirisako T., Noda T., Kominami E., Ohsumi Y., and Yoshimori T. (2000) LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *The EMBO journal 19*, 5720-5728.

Kabuta T., Suzuki Y., and Wada K. (2006). Degradation of amyotrophic lateral sclerosis-linked mutant Cu, Znsuperoxide dismutase proteins by macroautophagy and the proteasome. *The Journal of biological chemistry 281*, 30524-30533.

Kamada Y., Funakoshi T., Shintani T., Nagano K., Ohsumi M., and Ohsumi Y. (2000) Tor-mediated induction of autophagy via an Apg1 protein kinase complex. *The Journal of cell biology 150*, 1507-1513.

Kaneko K., Vey M., Scott M., Pilkuhn S., Cohen F.E. and Prusiner S.B. (1997a) COOH terminal sequence of the cellular prion protein directs subcellular trafficking and controls conversion into the scrapie isoform. *Proceedings of the National Academy of Sciences of the United States of America* 94**,** 2333-2338.

Kaneko K., Zulianello L., Scott M., Cooper C.M., Wallace A.C., James T.L., Cohen F.E. and Prusiner S.B. (1997b) Evidence for protein X binding to a discontinuous epitope on the cellular prion protein during scrapie prion propagation. *Proc. Natl. Acad. Sci. USA.* 94, 10069-10074.

Kanki T. and Klionsky D.J. (2008) Mitophagy in yeast occurs through a selective mechanism. *The Journal of biological chemistry* 283, 32386-32393.

Kanu N., Imokawa Y., Drechsel D.N., Williamson R.A., Birkett C.R., Bostock C.J. & Brockes J.P. (2002) Transfer of scrapie prion infectivity by cell contact in culture. *Curr.Biol.* 12, 523-530.

Kawai A., Takano S., Nakamura N., and Ohkuma S. (2006). Quantitative monitoring of autophagic degradation. *Biochemical and biophysical research communications 351*, 71-77.

Kedjouar B., de Médina P., Oulad-Abdelghani M., Payré B., Silvente-Poirot S., Favre G., Faye J.C. and Poirot M. (2004) Molecular characterization of the microsomal tamoxifen binding site. *J. Biol. Chem.* 279, 34048-61.

Kim S.U. and de Vellis J. (2005) Microglia in health and disease. J. Neurosci. Res. 81, 302-13.

Kimelberg H.K., Feustel P.J., Jin Y., Paquette J., Boulos A., Keller R.W. Jr. and Tranmer B.I. (2000) Acute treatment with tamoxifen reduces ischemic damage following middle cerebral artery occlusion. Neuroreport 11, 2675–2679.

Kimelberg H.K., Jin Y., Charniga C. and Feustel P.J. (2003) Neuroprotective activity of tamoxifen in permanent focal ischemia. *J. Neurosurg.* 99, 138-42.

Kirkwood J.K., Wells G.A., Wilesmith J.W., Cunningham A.A. and Jackson S.I. (1990) Spongiform encephalopathy in an arabian oryx (Oryx leucoryx) and a greater kudu (Tragelaphus strepsiceros) *Vet. Rec.* 127, 418-20.

Klionsky D.J., and Ohsumi Y. (1999). Vacuolar import of proteins and organelles from the cytoplasm. *Annual review of cell and developmental biology 15*, 1-32.

Klionsky D.J. (2007) Autophagy: from phenomenology to molecular understanding in less than a decade. *Nat. Rev. Mol. Cell Biol.* 8, 931–937.

Klionsky D.J., Abeliovich H., Agostinis P., Agrawal D.K., Aliev G., Askew D.S., Baba M., Baehrecke E.H., Bahr B.A., Ballabio A., Bamber B.A., Bassham D.C., Bergamini E., Bi X., Biard-Piechaczyk M., Blum J.S., Bredesen D.E., Brodsky J.L., Brumell J.H., Brunk U.T., Bursch W., Camougrand N., Cebollero E., Cecconi F., Chen Y., Chin L.S., Choi A., Chu C.T., Chung J., Clarke P.G., Clark R.S., Clarke S.G., Clavé C., Cleveland J.L., Codogno P., Colombo M.I., Coto-Montes A., Cregg J.M., Cuervo A.M., Debnath J., Demarchi F., Dennis P.B., Dennis P.A., Deretic V., Devenish R.J., Di Sano F., Dice J.F., Difiglia M., Dinesh-Kumar S., Distelhorst C.W., Djavaheri-Mergny M., Dorsey F.C., Dröge W., Dron M., Dunn W.A. Jr., Duszenko M., Eissa N.T., Elazar Z., Esclatine A., Eskelinen E.L., Fésüs L., Finley K.D., Fuentes J.M., Fueyo J., Fujisaki K., Galliot B., Gao F.B., Gewirtz D.A., Gibson S.B., Gohla A., Goldberg A.L., Gonzalez R., González-Estévez C., Gorski S., Gottlieb R.A., Häussinger D., He Y.W., Heidenreich K., Hill J.A., Høyer-Hansen M., Hu X., Huang W.P., Iwasaki A., Jäättelä M., Jackson W.T., Jiang X., Jin S., Johansen T., Jung J.U., Kadowaki M., Kang C., Kelekar A., Kessel D.H., Kiel J.A., Kim H.P., Kimchi A., Kinsella T.J., Kiselyov K., Kitamoto K., Knecht E., Komatsu M., Kominami E., Kondo S., Kovács A.L., Kroemer G., Kuan C.Y., Kumar R., Kundu M., Landry J., Laporte M., Le W., Lei H.Y., Lenardo M.J., Levine B., Lieberman A., Lim K.L., Lin F.C., Liou W., Liu L.F., Lopez-Berestein G., López-Otín C., Lu B., Macleod K.F., Malorni W., Martinet W., Matsuoka K., Mautner J., Meijer A.J., Meléndez A., Michels P., Miotto G., Mistiaen W.P., Mizushima N., Mograbi B., Monastyrska I., Moore M.N., Moreira P.I., Moriyasu Y., Motyl T., Münz C., Murphy L.O., Naqvi N.I., Neufeld T.P., Nishino I., Nixon R.A., Noda T., Nürnberg B., Ogawa M., Oleinick N.L., Olsen

L.J., Ozpolat B., Paglin S., Palmer G.E., Papassideri I., Parkes M., Perlmutter D.H., Perry G., Piacentini M., Pinkas-Kramarski R., Prescott M., Proikas-Cezanne T., Raben N., Rami A., Reggiori F., Rohrer B., Rubinsztein D.C., Ryan K.M., Sadoshima J., Sakagami H., Sakai Y., Sandri M., Sasakawa C., Sass M., Schneider C., Seglen P.O., Seleverstov O., Settleman J., Shacka J.J., Shapiro I.M., Sibirny A., Silva-Zacarin E.C., Simon H.U., Simone C., Simonsen A., Smith M.A., Spanel-Borowski K., Srinivas V., Steeves M., Stenmark H., Stromhaug P.E., Subauste C.S., Sugimoto S., Sulzer D., Suzuki T., Swanson M.S., Tabas I., Takeshita F., Talbot N.J., Tallóczy Z., Tanaka K., Tanaka K., Tanida I., Taylor G.S., Taylor J.P., Terman A., Tettamanti G., Thompson C.B., Thumm M., Tolkovsky A.M., Tooze S.A., Truant R., Tumanovska L.V., Uchiyama Y., Ueno T., Uzcátegui N.L., van der Klei I., Vaquero E.C., Vellai T., Vogel M.W., Wang H.G., Webster P., Wiley J.W., Xi Z., Xiao G., Yahalom J., Yang J.M., Yap G., Yin X.M., Yoshimori T., Yu L., Yue Z., Yuzaki M., Zabirnyk O., Zheng X., Zhu X. and Deter R.L. (2008). Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy 4*, 151-175.

Kocisko D.A., Baron G.S., Rubenstein R., Chen J., Kuizon S. and Caughey B. (2003) New inhibitors of scrapie-associated prion protein formation in a library of 2000 drugs and natural products. *J. Virol.* 88, 1062-1067.

Kocisko D.A. and Caughey B. (2006) Searching for anti-prion compounds: cell-based highthroughput in vitro assays and animal testing strategies. *Methods Enzymol*. 412, 223-34.

Koike M., Shibata M., Waguri S., Yoshimura K., Tanida I., Kominami E., Gotow T., Peters C., von Figura K., Mizushima N., Saftig P. and Uchiyama Y. (2005) Participation of autophagy in storage of lysosomes in neurons from mouse models of neuronal ceroid-lipofuscinoses (Batten disease). *Am. J. Pathol.* 167, 1713-1728.

Komatsu M., Waguri S., Ueno T., Iwata J., Murata S., Tanida I., Ezaki J., Mizushima N., Ohsumi Y., Uchiyama Y., Kominami E., Tanaka K. and Chiba T. (2005). Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. *The Journal of cell biology* 169, 425-434.

Komatsu M., Waguri S., Chiba T., Murata S., Iwata J., Tanida I., Ueno T., Koike M., Uchiyama Y., Kominami E. and Tanaka K. (2006) Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature* 441, 880-884.

Komatsu M., Ueno T., Waguri S., Uchiyama Y., Kominami E., and Tanaka K. (2007a). Constitutive autophagy: vital role in clearance of unfavorable proteins in neurons. Cell death and differentiation *14*, 887-894.

Komatsu M., Wang Q.J., Holstein G.R., Friedrich V.L. Jr, Iwata J., Kominami E., Chait B.T., Tanaka K. and Yue Z. (2007b) Essential role for autophagy protein Atg7 in the maintenance of axonal homeostasis and the prevention of axonal degeneration. *Proc. Natl. Acad. Sci. USA* 104, 14489–14494.

Kovacs A.L., Gordon P.B., Grotterod E.M. and Seglen P.O. (1998) Inhibition of hepatocytic autophagy by adenosine, adenosine analogs and AMP. *Biol. Chem.* 379, 1341-1347.

Kovacs G.G., Trabattoni G., Hainfellner J.A., Ironside J.W., Knight R.S. and Budka H. (2002) Mutations of the prion protein gene phenotypic spectrum. *Journal of neurology* 249, 1567-1582.

Krammer C., Kryndushkin D., Suhre M.H., Kremmer E., Hofmann A., Pfeifer A., Scheibel T., Wickner R.B., Schatzl H.M., and Vorberg I. (2009a). The yeast Sup35NM domain propagates as a prion in mammalian cells. PNAS *106*, 462-467.

Krammer C., Vorberg I., Schätzl H.M. and Gilch S. (2009b) Therapy in prion diseases: from molecular and cellular biology to therapeutic targets. Infect Disord Drug Targets. 9, 3-14.

Kretzschmar H.A., Prusiner S.B., Stowring L.E. and DeArmond S.J. (1986) Scrapie prion proteins are synthesized in neurons. *Am. J. Pathol.* 122, 1-5.

Kristiansen M., Messenger M.J., Klohn P.C., Brandner S., Wadsworth J.D., Collinge J. and Tabrizi S.J. (2005) Disease-related prion protein forms aggresomes in neuronal cells leading to caspase activation and apoptosis. *The Journal of biological chemistry* 280, 38851-38861.

Kristiansen M., Deriziotis P., Dimcheff D.E., Jackson G.S., Ovaa H., Naumann H., Clarke A.R., van Leeuwen F.W., Menendez-Benito V., Dantuma N.P., Portis J.L., Collinge J. and Tabrizi S.J. (2007) Disease-associated prion protein oligomers inhibit the 26S proteasome. *Molecular cell* 26, 175-188.

Kroemer G. and Levine B. (2008) Autophagic cell death: the story of a misnomer. *Nat. Rev. Mol. Cell Biol.* 9, 1004-1010.

Kuma A., Hatano M., Matsui M., Yamamoto A., Nakaya H., Yoshimori T., Ohsumi Y., Tokuhisa T., and Mizushima N. (2004). The role of autophagy during the early neonatal starvation period. *Nature* 432, 1032-1036.

Kundu M., Lindsten T., Yang C.Y., Wu J., Zhao F., Zhang J., Selak M.A., Ney P.A., and Thompson C.B. (2008). Ulk1 plays a critical role in the autophagic clearance of mitochondria and ribosomes during reticulocyte maturation. *Blood* 112, 1493–1502.

Kurschner C. and Morgan J.I. (1996) Analysis of interaction sites in homo- and heteromeric complexes containing Bcl-2 family members and the cellular prion protein. *Brain research* 37, 249-258.

Kuwahara C., Takeuchi A.M., Nishimura T., Haraguchi K., Kubosaki A., Matsumoto Y., Saeki K., Matsumoto Y., Yokoyama T., Itohara S. and Onodera T.(1999) Prions prevent neuronal cell-line death.15, 225-6.

Ladeby R., Wirenfeldt M., Garcia-Ovejero D., Fenger C., Dissing-Olesen L., Dalmau I. and Finsen B. (2005) Microglial cell population dynamics in the injured adult central nervous system. *Brain Res. Rev.* 48, 196-206. Review.

Ladogana A., Casaccia P., Ingrosso L., Cibati M., Salvatore M., Xi Y.G., Masullo C. and Pocchiari M. (1992) Sulphate polyanions prolong the incubation period of scrapie-infected hamsters. J. *Gen. Virol*. 73, 661-5.

Laine J., Marc M.E., Sy M.S. and Axelrad H. (2001) Cellular and subcellular morphological localization of normal prion protein in rodent cerebellum. *Eur. J. Neurosci*. 14, 47-56.

Larramendy-Gozalo C., Barret A., Daudigeos E., Mathieu E., Antonangeli L., Riffet C., Petit E., Papy-Garcia D., Barritault D., Brown P. and Deslys J.P. (2007) Comparison of CR36, a new heparan mimetic, and pentosan polysulfate in the treatment of prion diseases. *J. Gen. Virol*. 88, 1062 -7.

Lasmézas C.I., Deslys J.P., Demaimay R., Adjou K.T., Lamoury F., Dormont D., Robain O., Ironside J. and Hauw J.J. (1996) BSE transmission to macaques. *Nature.* 381, 743-4.

Lauren J., Gimbel D.A., Nygaard H.B., Gilbert J.W. and Strittmatter S.M. (2009) Cellular prion protein mediates impairment of synaptic plasticity by amyloid-beta oligomers. *Nature* 457, 1128-1132.

Lee J.A., Beigneux A., Ahmad S.T., Young S.G. and Gao F.B. (2007) ESCRT-III dysfunction causes autophagosome accumulation and neurodegeneration. *Curr. Biol.* 17, 1561-1567.

Lee J.A. (2009) Autophagy in neurodegeneration: two sides of the same coin. *BMB Rep.* 42, 324-30.

Lee E.S., Yin Z., Milatovic D., Jiang H. and Aschner M. (2009) Estrogen and tamoxifen protect against Mn-induced toxicity in rat cortical primary cultures of neurons and astrocytes. *Toxicol. Sci.* 110, 156-67.

Legname G., Baskakov I.V., Nguyen H.O., Riesner D., Cohen F.E., DeArmond S.J. and Prusiner S.B. (2004) Synthetic mammalian prions. *Science*. 305, 673-6.

Lehmann S. and Harris D.A. (1996) Two mutant prion proteins expressed in cultured cells acquire biochemical reminiscent of scrapie isoform. *Proc. Natl. Acad. Sci. USA* 93, 5610-5614.

Leucht C., Simoneau S., Rey C., Vana K., Rieger R., Lasmezas C.I. & Weiss S. (2003) The 37 kDa/67 kDa laminin receptor is required for PrP(Sc) propagation in scrapie-infected neuronal cells. *EMBO Rep.* 4, 290-295.

Levine B. and Klionsky D.J. (2004) Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev. Cell* 6, 463-477.

Levine B., and Yuan J. (2005). Autophagy in cell death: an innocent convict? *The Journal of clinical investigation* 115, 2679-2688.

Levine B. (2005) Eating oneself and uninvited guests: autophagy-related pathways in cellular defense. *Cell* 120, 159-162.

Levine B. and Deretic V. (2007) Unveiling the roles of autophagy in innate and adaptive immunity. *Nat Rev Immunol 7*, 767-777.

Levine B. and Kroemer G. (2008) Autophagy in the pathogenesis of disease. *Cell 132*, 27-42.

Lewis V., Hooper N. M. (2011) The role of lipid rafts in prion protein biology. *Front Biosci.* 16,151-68.

Li A., Harris D.A. (2005) Mammalian prion protein suppresses Bax-induced cell death in yeast. J. Biol. Chem. 29, 17430-4.

Liang X.H., Kleeman L.K., Jiang H.H., Gordon G., Goldman J.E., Berry G., Herman B. and Levine B. (1998) Protection against fatal Sindbis virus encephalitis by beclin, a novel Bcl-2-interacting protein. *J. Virol.* 72, 8586-96.

Liang X.H., Jackson S., Seaman M., Brown K., Kempkes B., Hibshoosh H. and Levine B. (1999) Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* 402, 672-6.

Liberski P.P., Yanagihara R., Gibbs C.J. Jr. and Gajdusek D.C. (1992) Neuronal autophagic vacuoles in experimental scrapie and Creutzfeldt-Jakob disease. *Acta Neuropathol* 83, 134-9.

Liberski P.P. and Mori S. (1997) The Echigo-1: a panencephalopathic strain of Creutzfeldt-Jakob disease: a passage to hamsters and ultrastructural studies. *Folia Neuropathol.* 35, 250–4.

Liberski P.P., Gajdusek D.C., and Brown P. (2002) How do neurons degenerate in prion diseases or transmissible spongiform encephalopathies (TSEs): neuronal autophagy revisited. *Acta neurobiologiae experimentalis 62*, 141-147.

Liberski PP. Spongiform change--an electron microscopic view. *Folia Neuropathol.* 2004; 42, 59-70.

Liberski P.P., Sikorska B., Bratosiewicz-Wasik J., Gajdusek D.C. and Brown P. (2004) Neuronal cell death in transmissible spongiform encephalopathies (prion diseases) revisited: from apoptosis to autophagy. *The international journal of biochemistry & cell biology* 36, 2473-2490.

Liberski P.P., Brown D.R., Sikorska B., Caughey B., and Brown P. (2008) Cell death and autophagy in prion diseases (transmissible spongiform encephalopathies). *Folia neuropathologica / Association of Polish Neuropathologists and Medical Research Centre, Polish Academy of Sciences* 46, 1-25.

Liberski P.P., Sikorska B, Gibson P, Brown P. (2011) Autophagy contributes to widespread neuronal degeneration in hamsters infected with the Echigo-1 strain of Creutzfeldt-Jakob disease and mice infected with the Fujisaki strain of Gerstmann-Sträussler-Scheinker (GSS) syndrome. *Ultrastruct Pathol.* 35, 31-6.

Lien E.A., Wester K., Lønning P.E., Solheim E. and Ueland P.M. (1991) Distribution of tamoxifen and metabolites into brain tissue and brain metastases in breast cancer patients. *Br. J. Cancer* 63, 641-5.

Liu F., Iqbal K., Grundke-Iqbal I., Hart G.W. and Gong C.X. (2004) O-GlcNAcylation regulates phosphorylation of tau: a mechanism involved in Alzheimer's disease. *Proc. Natl. Acad. Sci. USA* 101,10804-9.

Liu J.L., Tian D.S., Li Z.W., Qu W.S., Zhan Y., Xie M.J., Yu Z.Y., Wang W. and Wu G. (2010) Tamoxifen alleviates irradiation-induced brain injury by attenuating microglial inflammatory response in vitro and in vivo. *Brain Res.* 1316, 101-11.

Lledo P.M., Tremblay P., DeArmond S.J., Prusiner S.B. and Nicoll R.A. (1996) Mice deficient for prion protein exhibit normal neuronal excitability and synaptic transmission in the hippocampus. Proc. *Natl. Acad. Sci.* USA 19, 2403-7.

Llewelyn C.A., Hewitt P.E., Knight R.S., Amar K., Cousens S., Mackenzie J. and Will R.G. (2004) Possible transmission of variant Creutzfeldt- Jakob disease by blood transfusion. *Lancet* 363, 417–421.

Lueck C.J., McIlwaine G.G. and Zeidler M. (2000) Creutzfeldt-Jakob disease and the eye. I Background and patient management. *Eye* 14, 263–90

Lugaresi E., Medori R., Montagna P., Baruzzi A., Cortelli P., Lugaresi A., Tinuper P., Zucconi M. and Gambetti P. (1986) Fatal familial insomnia and dysautonomia with

selective degeneration of thalamic nuclei. *The New England journal of medicine* 315, 997-1003.

Lum J.J., DeBerardinis R.J. and Thompson C.B. (2005) Autophagy in metazoans: cell survival in the land of plenty. *Nat. Rev. Mol. Cell Biol. 6*, 439-448.

Ma J. and Lindquist S. (2002) Conversion of PrP to a self-perpetuating PrP^{Sc}-like conformation in the cytosol. *Science* 298, 1785-1788.

Ma J., Wollmann R. and Lindquist S. (2002) Neurotoxicity and neurodegeneration when PrP accumulates in the cytosol. *Science* 298, 1781-1785.

Macleod M.A. (2003) Potential treatments and treatment strategies in Creutzfeldt-Jakob disease. *IDrugs* 6, 345-50. Review.

Madeo F, Eisenberg T, Kroemer G. (2009) Autophagy for the avoidance of neurodegeneration. *Genes Dev*. 23, 2253-9.

Madore N., Smith K.L., Graham C.H., Jen A., Brady K., Hall S. and Morris R. (1999) Functionally different GPI proteins are organized in different domains on the neuronal surface. *The EMBO journal* 18, 6917-6926.

Maignien T., Lasmezas C. I., Beringue V., Dormont D. and Deslys J.P. (1999) Pathogenesis of the oral route of infection of mice with scrapie and bovine spongiform encephalopathy agents. *J. Gen.Virol.* 80, 3035-3042

Maiuri M.C., Zalckvar E., Kimchi A. and Kroemer G. (2007). Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nat. Rev. Mol. Cell Biol.* 8, 741-752.

Mallucci G., Dickinson A., Linehan J., Klöhn P.C., Brandner S. and Collinge J. (2003) Depleting neuronal PrP in prion infection prevents disease and reverses spongiosis. *Science* 302, 871- 4.

Mangé A., Nishida N., Milhavet O., McMahon H.E., Casanova D. and Lehmann S. (2000) Amphotericin B inhibits the generation of the scrapie isoform of the prion protein in infected cultures. *J. Virol*. 74, 3135-40.

Manson J.C., Clarke A.R., Hooper M.L., Aitchison L., McConnell I. and Hope J. (1994) 129/Ola mice carrying a null mutation in PrP that abolishes mRNA production are developmentally normal. *Mol. Neurobiol*. 8, 121-7.

Marchetti B. and Abbracchio M.P. (2005) To be or not to be (inflamed)--is that the question in anti-inflammatory drug therapy of neurodegenerative disorders? *Trends Pharmacol. Sci.* 26, 517-25.

Marella M., Lehmann S., Grassi J., and Chabry J. (2002) Filipin prevents pathological prion protein accumulation by reducing endocytosis and inducing cellular PrP release. *The Journal of biological chemistry 277*, 25457-25464.

Marijanovic Z., Caputo A., Campana V. and Zurzolo C. (2009) Identification of an intracellular site of prion conversion. *PLoS pathogens* **5,** e1000426.

Mariño G., Salvador-Montoliu N., Fueyo A., Knecht E., Mizushima N. and López-Otín C. (2007) Tissue-specific autophagy alterations and increased tumorigenesis in mice deficient in Atg4C/autophagin-3. *J. Biol. Chem. 282*, 18573–18583.

Martinez-Vicente M. and Cuervo A.M. (2007) Autophagy and neurodegeneration: when the cleaning crew goes on strike. *Lancet neurology 6*, 352-361.

Mathew R., Karantza-Wadsworth V. and White E. (2007) Role of autophagy in cancer. *Nat. Rev. Cancer 7*, 961-967.

McCague R., Leclercq G., Legros N., Goodman J., Blackburn G.M., Jarman M. and Foster A.B. (1989) Derivatives of tamoxifen. Dependence of antiestrogenicity on the 4-substituent. *J. Med. Chem.* 32, 2527-33.

McGoan J.P. (1914) Invesigation into the disease of sheep called "scrapie" with special reference to its association with sarcospoidiosis. *Rept.* 232.

McGoan J.P. (1922) Scrapie in sheep. *Scott. J. Agric.* 5, 365-375.

McIntire S.L., Garriga G., White J., Jacobson D. and Horvitz H.R. (1992) Genes necessary for directed axonal elongation or fasciculation in C. elegans. *Neuron* 8, 307-322.

McKinley M.P., Bolton D.C. and Prusiner S.B. (1983) A protease-resistant protein is a structural component of the scrapie prion. *Cell.* 35, 57-62.

McKinley M.P., Taraboulos A., Kenaga L., Serban D., Stieber A., Dearmond S. J., Prusiner S. B. and Gonatas N. (1991) *Lab. Invest. 65,* 622.

McKnight S. and Tjian R. (1986) Transcriptional selectivity of viral genes in mammalian cells. *Cell* 46, 795-805.

Mead S. (2006) Prion disease genetics. *Eur J Hum Genet* 14, 273-281.

Mehta S.H., Dhandapani K.M., Webb R.C., Mahesh V.B. and Brann D.W. (2001) Therapeutic doses of tamoxifen exert neuroprotective effects in acute ischemic stroke. *Soc Neurosci Abstr* 27, 437-11.

Meijer A.J. and Codogno P. (2006). Signalling and autophagy regulation in health, aging and disease. *Molecular aspects of medicine 27*, 411-425.

Meredith G.E., Totterdell S., Petroske E., Santa Cruz K., Callison R.C. Jr. and Lau Y.S. (2002) Lysosomal malfunction accompanies alpha-synuclein aggregation in a progressive mouse model of Parkinson's disease. *Brain Res.* 956, 156-65.

Meyer R.K., McKinley M.P., Bowman K.A., Braunfeld M.B., Barry R.A. and Prusiner S.B. (1986) Separation and properties of cellular and scrapie prion proteins. *Proc. Natl. Acad. Sci. USA.* 83, 2310–2314.

Meyer R.K., Lustig A., Oesch B., Fatzer R., Zurbriggen A. and Vandevelde M. (2000) A monomer-dimer equilibrium of a cellular prion protein (PrP^c) not observed with recombinant PrP. *J. Biol. Chem.* 275, 38081-7.

Mizushima N., Yamamoto A., Hatano M., Kobayashi Y., Kabeya Y., Suzuki K., Tokuhisa T., Ohsumi Y. and Yoshimori T. (2001) Dissection of autophagosomeformation using Apg5-deficient mouse embryonic stem cells. *J. Cell Biol. 152*, 657–668.

Minghetti L., Ajmone-Cat M.A., De Berardinis M.A. and De Simone R. (2005) Microglial activation in chronic neurodegenerative diseases: roles of apoptotic neurons and chronic stimulation. *Brain. Res. Rev.* 48, 251-6. Review.

Mironov A. Jr., Latawiec D., Wille H., Bouzamondo-Bernstein E., Legname G., Williamson R. A., Burton D., DeArmond S.J., Prusiner S. B. and Peters P. J. (2003) Cytosolic Prion Protein in Neurons. *J. Neurosci.* 23, 7183-7193

Mizushima N. (2004) Methods for monitoring autophagy. *The international journal of biochemistry & cell biology 36*, 2491-2502.

Mizushima N., Yamamoto A., Matsui M., Yoshimori T. and Ohsumi Y. (2004) *In vivo* analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. *Molecular biology of the cell 15*, 1101-1111.

Mizushima N. and Hara T. (2006) Intracellular quality control by autophagy: how does autophagy prevent neurodegeneration? *Autophagy 2*, 302-304.

Mizushima N. and Klionsky D.J. (2007) Protein turnover via autophagy: implications for metabolism. *Annual review of nutrition 27*, 19-40.

Mizushima N. and Yoshimori T. (2007) How to interpret LC3 immunoblotting. *Autophagy* 3, 542-545.

Mizushima N., Levine B., Cuervo A.M. and Klionsky D.J. (2008). Autophagy fights disease through cellular selfdigestion. *Nature* 451, 1069-1075.

Mizushima N., Yoshimori T. and Levine B. (2010) Methods in mammalian autophagy research. *Cell* 140, 313-26.

Mok S.W., Riemer C., Madela K., Hsu D.K., Liu F.T., Gultner S., Heise I. and Baier M. (2007) Role of galectin-3 in prion infections of the CNS. *Biochemical and biophysical research communications* 359, 672-678.

Moore R.C., Lee I.Y., Silverman G.L., Harrison P.M., Strome R., Heinrich C., Karunaratne A., Pasternak S.H., Chishti M.A., Liang Y., Mastrangelo P., Wang K., Smit A.F., Katamine S., Carlson G.A., Cohen F.E., Prusiner S.B., Melton D.W., Tremblay P., Hood L.E. and Westaway D. (1999) Ataxia in prion protein (PrP)-deficient mice is associated with upregulation of the novel PrP-like protein doppel. *J. Mol. Biol*. 292, 797-817.

Moore R.A., Taubner L.M. and Priola S.A.(2009) Prion protein misfolding and disease. *Curr. Opin. Struct. Biol.* 19, 14-22.

Morel E., Andrieu T., Casagrande F., Gauczynski S., Weiss S., Grassi J., Rousset M., Dormont D. and Chambaz J. (2005) Bovine prion is endocytosed by human enterocytes via the 37 kDa/67 kDa laminin receptor. *The American journal of pathology* 167, 1033-1042.

Mortimore G.E., Poso A.R. and Lardeux B.R. (1989) Mechanism and regulation of protein degradation in liver. *Diabetes Metab. Rev.* 5, 49–70.

Moser M., Colello R.J., Pott U. and Oesch B. (1995) Developmental expression of the prion protein gene in glial cells. *Neuron* 14, 509-517.

Mouillet-Richard S., Ermonval M., Chebassier C., Laplanche J.L., Lehmann S., Launay J.M. and Kellermann O. (2000) Signal transduction through prion protein. *Science*. 289, 1925-8.

Moya K.L., Sales N., Hassig R., Creminon C., Grassi J. and Di Giamberardino L. (2000) Immunolocalization of the cellular prion protein in normal brain. *Microsc. Res. Tech.* 50, 58-65.

Moore R.C., Lee I.Y., Silverman G.L., Harrison P.M., Strome R., Heinrich C., Karunaratne A., Pasternak S.H., Chishti M.A., Liang Y., Mastrangelo P., Wang K., Smit A.F., Katamine S., Carlson G.A., Cohen F.E., Prusiner S.B., Melton D.W., Tremblay P., Hood L.E. and Westaway D. (1999) Ataxia in prion protein (PrP)-deficient mice is associated with upregulation of the novel PrP-like protein doppel. *Journal of molecular biology 292*, 797-817.

Nagai Y. and Popiel H.A. (2008) Conformational changes and aggregation of expanded polyglutamine proteins as therapeutic targets of the polyglutamine diseases: exposed beta-sheet hypothesis. *Curr. Pharm. Des.*14, 3267-79. Review.

Nakai A., Yamaguchi O., Takeda T., Higuchi Y., Hikoso S., Taniike M., Omiya S., Mizote I., Matsumura Y., Asahi M., Nishida K., Hori M., Mizushima N. and Otsu K. (2007). The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress. *Nature medicine 13*, 619-624.

Nishida Y., Arakawa S., Fujitani K., Yamaguchi H., Mizuta T., Kanaseki T., Komatsu M., Otsu K., Tsujimoto Y. and Shimizu S. (2009) Discovery of Atg5/ Atg7-independent alternative macroautophagy. *Nature* 461, 654–659.

Nishimura T., Sakudo A., Hashiyama Y., Yachi A., Saeki K., Matsumoto Y., Ogawa M., Sakaguchi S., Itohara S. and Onodera T. (2007) Serum withdrawal-induced apoptosis in ZrchI prion protein (PrP) gene-deficient neuronal cell line is suppressed by PrP, independent of Doppel. *Microbiol Immunol*. 51: 457-66.

Nixon R.A., Wegiel J., Kumar A., Yu W.H., Peterhoff C., Cataldo A. and Cuervo A.M. (2005) Extensive involvement of autophagy in Alzheimer disease: an immuno-electron microscopy study. *J Neuropathol Exp Neurol* 64, 113-22.

Nixon R.A. (2006) Autophagy in neurodegenerative disease: friend, foe or turncoat? Trends Neurosci. 29, 528-35.

Nixon R.A. (2007) Autophagy, amyloidogenesis and Alzheimer disease. *J. Cell Sci.* 120, 4081-91. Review.

Nixon R.A., Yang D.S. and Lee J.H. (2008) Neurodegenerative lysosomal disorders: a continuum from development to late age. *Autophagy* 4, 590–599.

Novitskaya V., Bocharova O.V., Bronstein I. and Baskakov I.V. (2006) Amyloid fibrils of mammalian prion protein are highly toxic to cultured cells and primary neurons. *J. Biol. Chem.* 281, 13828- 36.

Nunziante M., Gilch S. and Schätzl H.M. (2003a) Essential role of the prion protein N terminus in subcellular trafficking and half-life of cellular prion protein. *J. Biol. Chem.* 278, 3726-34.

Nunziante M., Gilch S. and Schätzl H.M. (2003b) Prion diseases: from molecular biology to intervention strategies. *Chembiochem.* 4, 1268-84. Review.

Nunziante M., Kehler C., Maas E., Kassack M.U., Groschup M. and Schätzl H.M. (2005) Charged bipolar suramin derivatives induce aggregation of the prion protein at the cell surface and inhibit PrP^{Sc} replication. *J. Cell Sci.* 118, 4959-73.

Obata T. and Kubota S. (2001) Protective effect of tamoxifen on 1-methyl-4-phenylpyridine-induced hydroxyl radical generation in the rat striatum. *Neurosci. Lett.* 308, 87-90.

Oboznaya M.B., Gilch S., Titova M.A., Koroev D.O., Volkova T.D., Volpina O.M.and Schätzl H.M. (2007) Antibodies to a nonconjugated prion protein peptide 95-123 interfere with PrP Sc propagation in prion-infected cells. *Cell Mol. Neurobiol*. 27 3, 271-84.

Oesch B., Westaway D., Wälchli M., McKinley M.P., Kent S.B., Aebersold R., Barry R.A., Tempst P., Teplow D.B., Hood L.E., Prusiner S.B. and Weissmann C. (1985) A cellular gene encodes scrapie PrP 27-30 protein. *Cell* 40, 735–746.

Oh J.M., Shin H.Y., Park S.J., Kim B.H., Choi J.K., Choi E.K., Carp R.I. and Kim Y.S. (2008) The involvement of cellular prion protein in the autophagy pathway in neuronal cells. *Molecular and cellular neurosciences 39*, 238-247.

Osuka K., Feustel P.J., Mongin A.A., Tranmer B.I. and Kimelberg H.K. (2001) Tamoxifen inhibits nitrotyrosine formation after reversible middle cerebral artery occlusion in the rat. J Neurochem. 76, 1842-50.

Pacheco C.D. and Lieberman A.P. (2008) The pathogenesis of Niemann-Pick type C disease: a role for autophagy? *Expert Rev. Mol. Med*. 10:e26. Review.

Pan K.M., Baldwin M., Nguyen J., Gasset M., Serban A., Groth D., Mehlhorn I., Huang Z., Fletterick R.J. and Cohen F.E. (1993) Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. *Proc. Natl. Acad. Sci. USA*. 90, 10962-6.

Pan T., Wong B.S., Liu T., Li R., Petersen R.B. and Sy M.S. (2002) Cell-surface prion protein interacts with glycosaminoglycans. *Biochem. J.* 368, 81-90.

Pankiewicz J., Prelli F., Sy M.S., Kascsak R.J., Kascsak R.B., Spinner D.S., Carp R.I., Meeker H.C., Sadowski M. and Wisniewski T.T. (2006) Clearance and prevention of prion infection in cell culture by anti-PrP antibodies. *Eur. J. Neurosci*. 23, 2635 -47.

Pankiv S., Clausen T.H., Lamark T., Brech A., Bruun J.A., Outzen H., Overvatn A., Bjorkoy G., and Johansen T. (2007) p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *The Journal of biological chemistry 282*, 24131-24145.

Parchi P., Castellani R., Capellari S., Ghetti B., Young K., Chen S.G., Farlow M., Dickson D.W., Sima A.A., Trojanowski J.Q., Petersen R.B. and Gambetti P. (1996) Molecular basis of phenotypic variability in sporadic Creutzfeldt-Jakob disease. *Ann. Neurol.* 39, 767-78.

Parchi P., Capellari S., Chen S.G., Petersen R.B., Gambetti P., Kopp N., Brown P., Kitamoto T., Tateishi J., Giese A. and Kretzschmar H. (1997) Typing prion isoforms. *Nature*. 386, 232-4.

Parchi P., Giese A., Capellari S., Brown P., Schulz-Schaeffer W., Windl O., Zerr I., Budka H., Kopp N., Piccardo P., Poser S., Rojiani A., Streichemberger N., Julien J., Vital C., Ghetti B., Gambetti P. and Kretzschmar H. (1999) Classification of sporadic Creutzfeldt-Jakob disease based on molecular and phenotypic analysis of 300 subjects. *Ann. Neurol*. 46, 224-33.

Parchi P., Zou W., Wang W., Brown P., Capellari S., Ghetti B., Kopp N., Schulz-Schaeffer W.J., Kretzschmar H.A., Head M.W., Ironside J.W., Gambetti P. and Chen S.G. (2000) Genetic influence on the structural variations of the abnormal prion protein. *Proc. Natl. Acad. Sci. USA.* 97, 10168- 72.

Parkin E.T., Watt N.T., Turner A.J. and Hooper N.M. (2004). Dual mechanisms for shedding of the cellular prion protein. *The Journal of biological chemistry 279*, 11170-11178.

Parkyn C.J., Vermeulen E.G., Mootoosamy R.C., Sunyach C., Jacobsen C., Oxvig C., Moestrup S., Liu Q., Bu G., Jen A. and Morris R.J. (2008) LRP1 controls biosynthetic and endocytic trafficking of neuronal prion protein. *J Cell Sci.* 121, 773-83.

Pathak D.N., Pongracz K. and Bodell W.J. (1996) Activation of 4-hydroxytamoxifen and the tamoxifen derivative metabolite E by uterine peroxidase to form DNA adducts: comparison with DNA adducts formed in the uterus of Sprague-Dawley rats treated with tamoxifen. *Carcinogenesis* 17, 1785-90.

Pattingre S., Bauvy C., Carpentier S., Levade T., Levine B. and Codogno P. (2009) Role of JNK1-dependent Bcl-2 phosphorylation in ceramide-induced macroautophagy. *J. Biol. Chem.* 284, 2719-28.

Pattison I.H. (1965) Scrapie in the welsh mountain breed of sheep and its experimental transmission to goats. *Vet. Rec.* 77, 1388-1390.

Pauly P.C. and Harris D.A. (1998) Copper stimulates endocytosis of the prion protein. *J. Biol. Chem.* 273, 33107-10.

Petersen A., Larsen K.E., Behr G.G., Romero N., Przedborski S., Brundin P., and Sulzer D. (2001) Expanded CAG repeats in exon 1 of the Huntington's disease gene stimulate dopamine-mediated striatal neuron autophagy and degeneration. *Human molecular genetics 10*, 1243-1254.

Peden A.H., Head M.W., Ritchie D.L., Bell J.E. and Ironside J.W. (2004) Preclinical vCJD after blood transfusion in a PRNP codon 129 heterozygous patient. *Lancet* 364, 527–529.

Peretz D., Williamson R.A., Kaneko K., Vergara J., Leclerc E., Schmitt-Ulms G., Mehlhorn I.R., Legname G., Wormald M.R., Rudd P.M., Dwek R.A., Burton D.R. and Prusiner S.B. (2001) Antibodies inhibit prion propagation and clear cell cultures of prion infectivity. *Nature* 412, 739 -43

Perez E.A., Gandara D.R., Edelman M.J., O'Donnell R., Lauder I.J. and DeGregorio M. (2003) Phase I trial of high-dose tamoxifen in combination with cisplatin in patients with lung cancer and other advanced malignancies. *Cancer Invest.* 21,1-6.

Pergami P., Jaffe H. and Safar J. (1996) Semipreparative chromatographic method to purify the normal cellular isoform of the prion protein in nondenatured form. *Anal. Biochem. 236*, 63-73.

Perrier V., Solassol J., Crozet C., Frobert Y., Mourton-Gilles C., Grassi J. and Lehmann S. (2004) Anti-PrP antibodies block PrP^{Sc} replication in prion-infected cell cultures by accelerating PrPc degradation. *J. Neurochem.* 89, 454 -63.

Perry V.H. (2004) The influence of systemic inflammation on inflammation in the brain: implications for chronic neurodegenerative disease. *Brain Behav. Immun.* 18, 407-13.

Pfeifer A., Eigenbrod S., Al Khadra S., Hofmann A., Mitteregger G., Moser M., Bertsch U. and Kretzschmar H. (2006) Lentivector-mediated RNAi effi ciently suppresses prion protein and prolongs survival of scrapie-infected mice. *J. Clin. Invest*. 116, 3204 -10.

Phillips N., Bridgeman J., and Ferguson-Smith M. (2000) BSE inquiry report. *Science. (Sationary Office, London).* 6, 413–451.

Piccardo P., Safar J., Ceroni M., Gajdusek D.C. and Gibbs Jr. C.J. (1990) Immunohistochemical localization of prion protein in spongiform encephalopathies and normal brain tissue. *Neurology*, 40, 518-522.

Pickford F., Masliah E., Britschgi M., Lucin K., Narasimhan R., Jaeger P. A., Small S., Spencer B., Rockenstein E., Levine B. and Wyss-Coray T. (2008) The autophagyrelated protein beclin 1 shows reduced expression in early Alzheimer disease and regulates amyloid beta accumulation in mice. *J. Clin. Invest*. 118, 2190-2199.

Polazzi E. and Contestabile A. (2002) Reciprocal interactions between microglia and neurons: from survival to neuropathology. *Rev. Neurosci.* 13, 221-42. Review.

Poli G., Martino P.A., Villa S., Carcassola G., Giannino M.L., Dall'Ara P., Pollera C., Iussich S., Tranquillo V.M., Bareggi S., Mantegazza P. and Ponti W. (2004) Evaluation of anti-prion activity of Congo red and its derivatives in experimentally infected hamsters. *Arzneimittelforschung* 54, 406 -15

Pors H., von Eyben F.E., Sørensen O.S. and Larsen M. (1991) Longterm remission of multiple brain metastases with tamoxifen. *J. Neurooncol.* 10, 173-7.

Postina R. (2008) A closer look at alpha-secretase. *Curr. Alzheimer Res*. 5, 179-86. Review.

Prado M.A., Alves-Silva J., Magalhaes A.C., Prado V.F., Linden R., Martins V.R. and Brentani R.R. (2004) PrP^c on the road: trafficking of the cellular prion protein. *Journal of neurochemistry* **88,** 769-781.

Premzl M., Sangiorgio L., Strumbo B., Marshall Graves J.A., Simonic T. and Gready J.E. (2003) Shadoo, a new protein highly conserved from fish to mammals and with similarity to prion protein. *Gene* 314, 89-102.

Priola S.A. (1999) Prion protein and species barriers in the transmissible spongiform encephalopathies. *Biomed. Pharmacother*. 5, 27-33.

Priola S.A., Raines A. and Caughey W.S. (2000) Porphyrin and phthalocyanine antiscrapie compounds. *Science*. 287, 1503 -6.

Priola S.A., Chabry J. and Chan K. (2001) Efficient conversion of normal prion protein (PrP) by abnormal hamster PrP is determined by homology at amino acid residue 155. *J. Virol.* 75, 4673-80.

Priola S.A., Raines A. and Caughey W. (2003) Prophylactic and therapeutic effects of phthalocyanine tetrasulfonate in scrapie-infected mice. *J. Infect. Dis*. 188, 699 -705.

Priola S.A. & Caughey B. (1994) Inhibition of scrapie-associated PrP accumulation. Probing the role of glycosaminoglycans in amyloidogenesis. *Mol Neurobiol.* 8, 113-120.

Proske D., Gilch S., Wopfner F., Schätzl H.M., Winnacker E.L. and Famulok M. (2002) Prion-protein-specific aptamer reduces PrP^{Sc} formation. *Chembiochem.* 3, 717-25.

Prusiner S.B., McKinley M.P., Groth D.F., Bowman K.A., Mock N.I., Cochran S.P. and Masiarz F.R. (1981) Scrapie agent contains a hydrophobic protein. *Proc. Natl. Acad. Sci. USA*. 78, 6675-9.

Prusiner S.B. (1982) Novel proteinaceous infectious particles cause scrapie. *Science NY 216*, 136-144.

Prusiner S.B., Groth D.F., Bolton D.C., Kent S.B. and Hood L.E. (1984) Purification and structural studies of a major scrapie prion protein. *Cell* 38, 127-134.

Prusiner S.B., Gabizon R. and McKinley M.P. (1987) On the biology of prions. *Acta neuropathologica* 72, 299-314.

Prusiner S.B., Scott M., Foster D., Pan K.M., Groth D., Mirenda C., Torchia M., Yang S.L., Serban D., Carlson G.A.,, Hoppe‖ P.C., Westaway D., and DeArmond S.J. (1990) Transgenetic studies implicate interactions between homologous PrP isoforms in scrapie prion replication. *Cell* 63, 673-686.

Prusiner S.B. (1991) Novel properties and biology of scrapie prions. *Current topics in microbiology and immunology* 172, 233-257.

Prusiner S.B., Torchia M. and Westaway D. (1991) Molecular biology and genetics of prions--implications for sheep scrapie, "mad cows" and the BSE epidemic. Historical background. *The Cornell veterinarian* 81, 85-101.

Prusiner S.B., Groth D., Serban A., Koehler R., Foster D., Torchia M., Burton D., Yang S.L. and DeArmond S.J. (1993) Ablation of the prion protein (PrP) gene in mice prevents scrapie and facilitates production of anti-PrP antibodies. *Proceedings of the National Academy of Sciences of the United States of America* 90, 10608-10612.

Prusiner S.B. (1998) Prions. *Proceedings of the National Academy of Sciences of the United States of America 95*, 13363-13383.

Qin Z.H., Wang Y., Kegel K.B., Kazantsev A., Apostol B.L., Thompson L.M., Yoder J., Aronin N., and DiFiglia M. (2003) Autophagy regulates the processing of amino terminal huntingtin fragments. *Human molecular genetics 12*, 3231-3244.

Qu X., Yu J., Bhagat G., Furuya N., Hibshoosh H., Troxel A., Rosen J., Eskelinen E.L., Mizushima N., Ohsumi Y., Cattoretti G. and Levine B. (2003). Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *The Journal of clinical investigation 112*, 1809-1820.

Rambold A.S., Miesbauer M., Rapaport D., Bartke T., Baier M., Winklhofer K.F. and Tatzelt J. (2006) Association of Bcl-2 with misfolded prion protein is linked to the toxicpotential of cytosolic PrP. *Mol Biol Cell*. 17, 3356-68.

Rambold A.S., Müller V., Ron U., Ben-Tal N., Winklhofer K.F. and Tatzelt J. (2008) Stress-protective signalling of prion protein is corrupted by scrapie prions. *EMBO J*. 27, 1974-84

Rangel A., Burgaya F., Gavín R., Soriano E., Aguzzi A. and Del Río J.A. (2007) Enhanced susceptibility of Prnp-deficient mice to kainate-induced seizures, neuronal apoptosis, and death: Role of AMPA/kainate receptors. *J. Neurosci. Res*. 85, 2741-55.

Ravikumar B., Duden R. and Rubinsztein D.C. (2002) Aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy. *Hum. Mol. Genet*. 11,1107-17.

Ravikumar B., Vacher C., Berger Z., Davies J.E., Luo S., Oroz L.G., Scaravilli F., Easton D.F., Duden R., O'Kane C.J. and Rubinsztein D.C. (2004) Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. *Nat Genet.* 36, 585-95.

Reggiori F. and Klionsky D.J. (2002) Autophagy in the eukaryotic cell. *Eukaryotic cell 1*, 11-21.

Rideout H.J., Lang-Rollin I. and Stefanis L. (2004)Involvement of macroautophagy in the dissolution of neuronal inclusions. *Int. J. Biochem. Cell Biol.* 36, 2551-62.

Riek R., Hornemann S., Wider G., Billeter M., Glockshuber R. and Wüthrich K. (1996) NMR structure of the mouse prion protein domain PrP(121-321).*Nature* 382, 180-2.

Riek R., Hornemann S., Wider G., Glockshuber R. and Wüthrich K. (1997) NMR characterization of the full-length recombinant murine prion protein, mPrP(23-231). *FEBS Lett.* 413, 282-8.

Rieger R., Edenhofer F., Lasmézas C.I. and Weiss S. (1997) The human 37-kDa laminin receptor precursor interacts with the prion protein in eukaryotic cells. *Nat Med.* 3, 1383-8.

Riemer C., Burwinkel M., Schwarz A., Gültner S., Mok S. W., Heise I., Holtkamp N. and Baier M. (2008) Evaluation of drugs for treatment of prion infections of the central nervous system. *J. Gen. Virol*. 89, 594-7.

Riesner D. (2003) Biochemistry and structure of PrP(C) and PrP(Sc). *Br Med Bull.* 66, 21-33.

Rivera-Milla E., Stuermer C.A. and Málaga-Trillo E. (2003) An evolutionary basis for scrapie disease: identification of a fish prion mRNA. *Trends Genet*. 19, 72-5.

Robakis N.K., Sawh P.R., Wolfe G.C., Rubenstein R., Carp R.I. and Innis M.A. (1986a) Isolation of a cDNA clone encoding the leader peptide of prion protein and expression of the homologous gene in various tissues. *Proceedings of the National Academy of Sciences of the United States of America* 83, 6377-6381.

Robakis N.K., Devine-Gage E.A., Jenkins E.C., Kascsak R.J., Brown W.T., Krawczun M.S. and Silverman W.P. (1986b) Localization of a human gene homologous to the PrP gene on the p arm of chromosome 20 and detection of PrP-related antigens in normal human brain. *Biochemical and biophysical research communications* 140, 758-765.

Roberts B.T. and Wickner R.B. (2003) Heritable activity: a prion that propagates by covalent autoactivation. *Genes Dev.* 17, 2083-7.

Roberts P., Moshitch-Moshkovitz S., Kvam E., O'Toole E., Winey M. and Goldfarb D.S. (2003) Piecemeal microautophagy of nucleus in Saccharomyces cerevisiae. *Molecular biology of the cell* 14, 129-141.

Rockenstein E., Schwach G., Ingolic E., Adame A., Crews L., Mante M., Pfragner R., Schreiner E., Windisch M. and Masliah E. (2005) Lysosomal pathology associated with alpha-synuclein accumulation in transgenic models using an eGFP fusion protein. *J. Neurosci. Res.* 80, 247-59.

Rossi D., Cozzio A., Flechsig E., Klein M.A., Rulicke T., Aguzzi A. and Weissmann C. (2001) Onset of ataxia and Purkinje cell loss in PrP null mice inversely correlated with Dpl level in brain. *The EMBO journal 20*, 694-702.

Roucou X., Guo Q., Zhang Y., Goodyer C.G. and LeBlanc A.C. (2003) Cytosolic prion protein is not toxic and protects against Bax-mediated cell death in human primary neurons. *J. Biol. Chem.* 278, 40877-81.

Rubinsztein D.C., DiFiglia M., Heintz N., Nixon R.A., Qin Z.H., Ravikumar B., Stefanis L., and Tolkovsky A. (2005) Autophagy and its possible roles in nervous system diseases, damage and repair. *Autophagy 1*, 11-22.

Rubinsztein D.C. (2006) The roles of intracellular proteindegradation pathways in neurodegeneration. *Nature 443*, 780-786.

Rubinsztein D.C., Gestwicki J.E., Murphy L.O. and Klionsky D.J. (2007) Potential therapeutic applications of autophagy. *Nat. Rev. Drug Discov*. 6, 304-312.

Rubinsztein D.C., Cuervo A.M., Ravikumar B., Sarkar S., Korolchuk V., Kaushik S. and Klionsky D.J. (2009) In search of an "autophagomometer". *Autophagy*. 5, 585-9.

Safar J., Wille H., Itri V., Groth D., Serban H., Torchia M., Cohen F. E. and Prusiner S. B. (1998) Eight prion strains have PrP(Sc) molecules with different conformations. *Nat. Med.* 4, 125- 6.

Saito Y., Suzuki K., Hulette C.M. and Murayama S. (2004) Aberrant phosphorylation of alpha-synuclein in human Niemann-Pick type C1 disease. *J. Neuropathol Exp. Neurol.* 63, 323-8.

Saitoh T., Fujita N., Jang M.H., Uematsu S., Yang B.G., Satoh T., Omori H., Noda T., Yamamoto N., Komatsu M., Tanaka K., Kawai T., Tsujimura T., Takeuchi O., Yoshimori T. and Akira S. (2008) Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1β production. *Nature 456*, 264–268.

Saitoh T., Fujita N., Hayashi T., Takahara K., Satoh T., Lee H., Matsunaga K., Kageyama S., Omori H., Noda T., Yamamoto N., Kawai T., Ishii K., Takeuchi O., Yoshimori T. and Akira S. (2009). Atg9a controls dsDNAdriven dynamic translocation of STING and the innate immune response. *Proc. Natl. Acad. Sci. USA.*

Sakaguchi S., Katamine S., Nishida N., Moriuchi R., Shigematsu K., Sugimoto T., Nakatani A., Kataoka Y., Houtani T., Shirabe S., Okada H., Hasegawa S., Miyamoto T. and Noda T. (1996) Loss of cerebellar Purkinje cells in aged mice homozygous for a disrupted PrP gene. *Nature* 380, 528-531.

Sales N., Rodolfo K., Hassig R., Faucheux B., Di Giamberardino L. and Moya K. L. (1998) Cellular prion protein localization in rodent and primate brain. *Eur. J. Neurosci.* 10, 2464-2471

Salvati M., Cervoni L., Innocenzi G. and Bardella L. (1993) Tumori. Prolonged stabilization of multiple and single brain metastases from breast cancer with tamoxifen. *Report of three cases.* 79, 359-62.

Sarkar S., Floto R.A., Berger Z., Imarisio S., Cordenier A., Pasco M., Cook L.J. and Rubinsztein D.C. (2005) Lithium induces autophagy by inhibiting inositol monophosphatase. *J Cell Biol.* 26, 1101-11.

Sarkar S., Davies J.E., Huang Z., Tunnacliffe A., and Rubinsztein D.C. (2007a). Trehalose, a novel mTOR-independent autophagy enhancer, accelerates the clearance of mutant huntingtin and alpha-synuclein. *J. Biol. Chem. 282*, 5641–5652.

Sarkar S., Perlstein E.O., Imarisio S., Pineau S., Cordenier A., Maglathlin R.L., Webster J.A., Lewis T.A., O'Kane C.J., Schreiber S.L. and Rubinsztein D.C. (2007b). Small molecules enhance autophagy and reduce toxicity in Huntington's disease models. *Nat. Chem. Biol. 3*, 331–338.

Sarkar S., Krishna G., Imarisio S., Saiki S., O'Kane C.J. and Rubinsztein D.C. (2008). A rational mechanism for combination treatment of Huntington's disease using lithium and rapamycin. *Hum. Mol. Genet. 17*, 170–178.

Sawaya M.R., Sambashivan S., Nelson R., Ivanova M.I., Sievers S.A., Apostol M.I., Thompson M.J., Balbirnie M., Wiltzius J.J., McFarlane H.T., Madsen A.Ø., Riekel C. and Eisenberg D.(2007) Atomic structures of amyloid cross-beta spines reveal varied steric zippers. *Nature* 447, 453-7.

Scarlatti F., Bauvy C., Ventruti A., Sala G., Cluzeaud F., Vandewalle A., Ghidoni R. and Codogno P. (2004) Ceramide-mediated macroautophagy involves inhibition of protein kinase B and up-regulation of beclin 1. *J Biol. Chem.* 279, 18384-91

Scarlatti F., Granata R., Meijer A.J. and Codogno P. (2009) Does autophagy have a license to kill mammalian cells? *Cell Death Differ.* 16, 12-20.

Schätzl H.M., Laszlo L., Holtzman D.M., Tatzelt J., DeArmond S.J., Weiner R.I., Mobley W.C. and Prusiner S.B. (1997) A hypothalamic neuronal cell line persistently infected with scrapie prions exhibits apoptosis. *Journal of virology* 71, 8821-8831.

Schätzl H.M., Da Costa M., Taylor L., Cohen F.E. and Prusiner S.B. (1995) Prion protein gene variation among primates. *Journal of molecular biology* 245, 362-374.

Schätzl H.M. (2003) Der aktuelle Erkenntnisstand bei BSE und der Creutzfeldt-Jakob-Erkrankung. *Gesundheitswesen* 65 Sonderheft 1, 13-19.

Schäffler T. (2009) Analysis of different autophagy modulators and their impact on prion infection in cell culture models. *Master thesis. TU München.*

Schmitt-Ulms G., Legname G., Baldwin M.A., Ball H.L., Bradon N., Bosque P.J., Crossin K.L., Edelman G.M., DeArmond S.J., Cohen F.E. and Prusiner S.B. (2001) Binding of neural cell adhesion molecules (N-CAMs) to the cellular prion protein. *J. Mol. Biol*. 314, 1209-25.

Schenk D., Barbour R., Dunn W., Gordon G., Grajeda H., Guido T., Hu K., Huang J., Johnson-Wood K., Khan K., Kholodenko D., Lee M., Liao Z., Lieberburg I., Motter R., Mutter L., Soriano F., Shopp G., Vasquez N., Vandevert C., Walker S., Wogulis M., Yednock T., Games D. and Seubert P. (1999) Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. *Nature* 400, 173-7.

Schneider A. and Mandelkow E. (2008) Tau-based treatment strategies in neurodegenerative diseases. *Neurotherapeutics*. 5, 443-57. Review.

Scott M., Foster D., Mirenda C., Serban D., Coufal F., Wälchli M., Torchia M., Groth D., Carlson G., DeArmond S.J., Westaway D. and Prusiner S.B. (1989) Transgenic mice expressing hamster prion protein produce species-specific scrapie infectivity and amyloid plaques. *Cell.* 59, 847-57.

Scott M.R., Köhler R., Foster D. and Prusiner S.B. (1992) Chimeric prion protein expression in cultured cells and transgenic mice. *Protein Sci.* 1, 986-97.

Scott M., Groth D., Foster D., Torchia M., Yang S.L., DeArmond S.J. and Prusiner S.B. (1993) Propagation of prions with artificial properties in transgenic mice expressing chimeric PrP genes. *Cell.* 73, 979-88.

Scott M.R., Groth D., Tatzelt J., Torchia M., Tremblay P., DeArmond S.J. and Prusiner S.B. (1997) Propagation of prion strains through specific conformers of the prion protein. *J. Virol.* 71, 9032-44.

Seglen P.O., and Gordon P.B. (1982). 3-Methyladenine: specific inhibitor of autophagic/ lysosomal protein degradation in isolated rat hepatocytes. *Proceedings of the National Academy of Sciences of the United States of America* 79, 1889-1892.

Selenica M.L., Jensen H.S., Larsen A.K., Pedersen M.L., Helboe L., Leist M. and Lotharius J. (2007) Efficacy of small-molecule glycogen synthase kinase-3 inhibitors in the postnatal rat model of tau hyperphosphorylation. *Br. J. Pharmacol.* 152, 959-79.

Shmakov A.N. and Ghosh S. (2001). Prion proteins and the gut: une liaison dangereuse? *Gut* 48, 443- 447.

Shacka J.J., Klocke B.J., Young C., Shibata M., Olney J.W., Uchiyama Y., Saftig P. and Roth K. A. (2007) Cathepsin D deficiency induces persistent neurodegeneration in the absence of Bax-dependent apoptosis. *J. Neurosci.* 27, 2081-2090.

Shibata M., Lu T., Furuya T., Degterev A., Mizushima N., Yoshimori T., MacDonald M., Yankner B. and Yuan J. (2006). Regulation of intracellular accumulation of mutant Huntingtin by Beclin 1. *The Journal of biological chemistry 281*, 14474-14485.

Sikorska B., Liberski P.P., Giraud P., Kopp N. and Brown P. (2004) Autophagy is a part of ultrastructural synaptic pathology in Creutzfeldt-Jakob disease: a brain biopsy study. *The international journal of biochemistry & cell biology 36*, 2563-2573.

Silveira J.R., Raymond G.J., Hughson A.G., Race R.E., Sim V.L., Hayes S.F., and Caughey B. (2005) The most infectious prion protein particles. *Nature 437*, 257-261.

Singh R., Kaushik S., Wang Y., Xiang Y., Novak I., Komatsu M., Tanaka K., Cuervo A.M. and Czaja M.J. (2009) Autophagy regulates lipid metabolism. *Nature* 458, 1131-1135.

Skerra A. (2007) Alternative non-antibody scaffolds for molecular recognition. *Curr Opin Biotechnol.* 18:295-304. Review.

Sunyach C., Jen A., Deng J., Fitzgerald K.T., Frobert Y., Grassi J., McCaffrey M.W. and Morris R. (2003). The mechanism of internalization of glycosylphosphatidylinositol-anchored prion protein. *The EMBO journal 22*, 3591-3601.

Suzuki K. and Ohsumi Y. (2007). Molecular machinery of autophagosome formation in yeast, Saccharomyces cerevisiae. *FEBS letters 581*, 2156-2161.

Shmerling D., Hegyi I., Fischer M., Blattler T., Brandner S., Gotz J., Rulicke T., Flechsig E., Cozzio A., von Mering C., Hangartner C., Aguzzi A. and Weissmann C. (1998) Expression of amino-terminally truncated PrP in the mouse leading to ataxia and specific cerebellar lesions. *Cell* 93, 203-214.

Shyng S.L., Lehmann S., Moulder K.L. and Harris D.A. (1995) Sulfated glycans stimulate endocytosis of the cellular isoform of the prioni protein, PrP^c, in cultured cells. *J. Biol. Chem.* 270, 30221-29.

Silveira J.R., Raymond G.J., Hughson A.G., Race R.E., Sim V.L., Hayes S.F. and Caughey B. (2005) The most infectious prion protein particles. *Nature*. 437, 257-61.

Simoneau S., Rezaei H., Salès N., Kaiser-Schulz G., Lefebvre-Roque M., Vidal C., Fournier J.G., Comte J., Wopfner F., Grosclaude J., Schätzl H. and Lasmézas CI. (2007) In vitro and in vivo neurotoxicity of prion protein oligomers. *PLoS Pathog*. 3, e125.

Simons M., Schwärzler F., Lütjohann D., von Bergmann K., Beyreuther K., Dichgans J., Wormstall H., Hartmann T. and Schulz J.B. (2002) Treatment with simvastatin in normocholesterolemic patients with Alzheimer's disease: A 26-week randomized, placebo-controlled, double-blind trial. *Ann. Neurol.* 52, 346-50.

Sikorska B., Liberski P.P., Giraud P., Kopp N. and Brown P. (2004) Autophagy is a part of ultrastructural synaptic pathology in Creutzfeldt-Jakob disease: a brain biopsy study. Int. J. Biochem. Cell Biol. 36, 2563-73.

Soto C., Kascsak R.J., Saborío G.P., Aucouturier P., Wisniewski T., Prelli F., Kascsak R., Mendez E., Harris D.A., Ironside J., Tagliavini F., Carp R.I. and Frangione B. (2000) Reversion of prion protein conformational changes by synthetic beta-sheet breaker peptides. *Lancet. 355,* 192-197.

Sou Y.S., Waguri S., Iwata J., Ueno T., Fujimura T., Hara T., Sawada N., Yamada A., Mizushima N., Uchiyama Y., Kominami E., Tanaka K. and Komatsu M. (2008). The Atg8 conjugation system is indispensable for proper development of autophagic isolation membranes in mice. *Mol. Biol. Cell* 19, 4762–4775.

Sparkes R.S., Simon M., Cohn V.H., Fournier R.E., Lem J., Klisak I., Heinzmann C., Blatt C., Lucero M. and Mohandas T. (1986) Assignment of the human and mouse prion protein genes to homologous chromosomes. *Proceedings of the National Academy of Sciences of the United States of America* 83, 7358-7362.

Spencer B., Potkar R., Trejo M., Rockenstein E., Patrick C., Gindi R., Adame A., Wyss-Coray T. and Masliah E. (2009) Beclin 1 gene transfer activates autophagy and ameliorates the neurodegenerative pathology in alpha-synuclein models of Parkinson's and Lewy body diseases. *J. Neurosci.* 29, 13578–13588.

Spielhaupter C. and Schätzl H.M. (2001) PrP^c directly interacts with proteins involved in signaling pathways. *J Biol. Chem*. 276, 44604-12.

Stahl N., Borchelt D.R. and Prusiner S.B. (1990) Differential release of cellular and scrapie prion proteins from cellular membranes by phosphatidylinositol-specific phospholipase C. *Biochemistry* 29, 5405-12.

Stahl N., Baldwin M.A., Hecker R., Pan K.M., Burlingame A.L. and Prusiner S.B. (1992) Glycosylinositol phospholipid anchors of the scrapie and cellular prion proteins contain sialic acid. *Biochemistry.* 31, 5043-53.

Steele A.D., Emsley J.G., Ozdinler P.H., Lindquist S. and Macklis J.D. (2006) Prion protein (PrPc) positively regulates neural precursor proliferation during developmental and adult mammalian neurogenesis. *Proceedings of the National Academy of Sciences of the United States of America* 103, 3416-3421.

Stefanis L., Larsen K.E., Rideout H.J., Sulzer D. and Greene L.A. (2001) Expression of A53T mutant but not wild-type alpha-synuclein in PC12 cells induces alterations of the ubiquitin-dependent degradation system, loss of dopamine release, and autophagic cell death. *J. Neurosci.* 21:9549-60.

Stewart R.S. and Harris D.A. (2000) Most pathogenic mutations do not alter the membrane topology of the prion protein. *J. Biol. Chem.* 276, 2212-20.

Stewart L.A., Rydzewska L.H., Keogh G.F. and Knight R.S. (2008) Systematic review of therapeutic interventions in human prion disease. *Neurology* 70, 1272-81. Review.

Stoeckel J., Safar J., Wallace A.C., Cohen F.E. and Prusiner S.B. (1998) Prion protein selectively binds copper(II) ions. *Biochemistry*. 9, 7185-93.

Streit W.J., Walter S.A. and Pennell N.A. (1999) Reactive microgliosis. *Prog. Neurobiol.* 57, 563-81. Review.

Strumbo B., Ronchi S., Bolis L.C. and Simonic T. (2001) Molecular cloning of the cDNA coding for Xenopus laevis prion protein. FEBS Lett. 508, 170-4.

Sun L., Liu S.Y., Zhou X.W., Wang X.C., Liu R., Wang Q. and Wang J.Z. (2003) Inhibition of protein phosphatase 2A- and protein phosphatase 1-induced tau hyperphosphorylation and impairment of spatial memory retention in rats. *Neuroscience* 118, 1175-82.

Sunyach C., Jen A., Deng J., Fitzgerald K.T., Frobert Y., Grassi J., McCaffrey M.W. and Morris R. (2003) The mechanism of internalization of glycosylphosphatidylinositol-anchored prion protein. *EMBO* **15**, 3591-601.

Suzuki T., Kurokawa T., Hashimoto H. and Sugiyama M. (2002) cDNA sequence and tissue expression of Fugu rubripes prion protein-like: a candidate for the teleost orthologue of tetrapod PrPs. *Biochemical and biophysical research communications* 294, 912-917.

Suzumura A., Takeuchi H., Zhang G., Kuno R. and Mizuno T. (2006) Roles of glia-derived cytokines on neuronal degeneration and regeneration. *Ann. NY Acad. Sci.* 1088, 219-29. Review.

Takacs-Vellai K., Vellai T., Puoti A., Passannante M., Wicky C., Streit A., Kovacs A.L. and Müller F. (2005) Inactivation of the autophagy gene bec-1 triggers apoptotic cell death in C. elegans. *Curr. Biol.* 15, 1513-7.

Takahashi Y., Coppola D., Matsushita N., Cualing H.D., Sun M., Sato Y., Liang C., Jung J.U., Cheng J.Q., Mulé J.J., Pledger W.J. and Wang H.G. (2007). Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. *Nature cell biology 9*, 1142-1151.

Tamai K., Toyoshima M., Tanaka N., Yamamoto N., Owada Y., Kiyonari H., Murata K., Ueno Y., Ono M., Shimosegawa T., Yaegashi N., Watanabe M. and Sugamura K. (2008) Loss of hrs in the central nervous system causes accumulation of ubiquitinated proteins and neurodegeneration. *Am. J. Pathol.* **173**, 1806-1817.

Taraboulos A., Rogers M., Borchelt D.R., McKinley M.P., Scott M., Serban D. and Prusiner S.B. (1990a) Acquisition of protease resistance by prion proteins in scrapie-infected cells does not require asparagine-linked glycosylation. *Proc. Natl. Acad. Sci. USA* 87, 8262–8266.

Taraboulos A., Serban D. and Prusiner S.B. (1990b) Scrapie prion proteins accumulate in the cytoplasm of persistently infected cultured cells. *J. Cell Biol.* 110, 2117–2132.

Taraboulos A., Raeber A.J., Borchelt D.R., McKinley M.P. and Prusiner S. (1991) Brefeldin A inhibits protease resistant prion protein synthesis in scrapie-infected cultured cells. *FASEB J.* 5, A1177.

Taraboulos A., Raeber A.J., Borchelt D.R., Serban D. and Prusiner S. (1992) Synthesis and trafficking of prion proteins in cultured cells. *Mol. Biol. Cell.* 3, 851-863.

Taraboulos A., Scott M., Semenov A., Avrahami D. and Prusiner S.B. (1994) Biosynthesis of the prion proteins in scrapie-infected cells in culture. *Braz. J. Med. Biol. Res.* 27, 303-7.

Taraboulos A., Scott M., Semenov A., Avrahami D., Laszlo L. and Prusiner S.B. (1995) Cholesterol depletion and modification of COOH-terminal targeting sequence of the prion protein inhibit formation of the scrapie isoform. *The Journal of cell biology 129*, 121-132.

Tateishi J., Brown P., Kitamoto T., Hoque Z.M., Roos R., Wollman R., Cervenakova L. and Gajdusek D.C. (1995) First experimental transmission of fatal familial insomnia. *Nature* 376, 434-435.

Tayebi N., Callahan M., Madike V., Stubblefield B.K., Orvisky E., Krasnewich D., Fillano J.J. and Sidransky E. (2001) Gaucher disease and parkinsonism: a phenotypic and genotypic characterization. *Mol. Genet. Metab.* 73, 313-21.

Telling G.C., Scott M., Hsiao K.K., Foster D., Yang S.L., Torchia M., Sidle K.C., Collinge J., DeArmond S.J. and Prusiner S.B. (1994) Transmission of Creutzfeldt-Jakob disease from humans to transgenic mice expressing chimeric human-mouse prion protein. *Proc. Natl. Acad. Sci. U S A.* 91, 9936-40.

Telling G.C., Scott M., Mastrianni J., Gabizon R., Torchia M., Cohen F.E., DeArmond S.J. and Prusiner S.B. (1995) Prion propagation in mice expressing human and chimeric PrP transgenes implicates the interaction of cellular PrP with another protein. *Cell.* 83, 79-90.

Telling G.C., Parchi P., DeArmond S.J., Cortelli P., Montagna P., Gabizon R., Mastrianni J., Lugaresi E., Gambetti P. and Prusiner S.B. (1996) Evidence for the conformation of the pathologic 137 isoform of the prion protein enciphering and propagating prion diversity. *Science* 274, 2079- 82.

Thomas W.E. (1992) Brain macrophages: evaluation of microglia and their functions. *Brain Res. Rev.* 17, 61-74. Review.

Thompson A.J. and Barrow C.J. (2002) Protein conformational misfolding and amyloid formation: characteristics of a new class of disorders that include Alzheimer's and Prion diseases. *Curr. Med. Chem.* 9, 1751-62. Review.

Tian D.S., Liu J.L., Xie M.J., Zhan Y., Qu W.S., Yu Z.Y., Tang Z.P., Pan D.J. and Wang W. (2009) Tamoxifen attenuates inflammatory-mediated damage and improves functional outcome after spinal cord injury in rats. *J. Neurochem.* 109, 1658-67.

Tilly G., Chapuis J., Vilette D., Laude H. and Vilotte J.L. (2003) Efficient and specific down-regulation of prion protein expression by RNAi. *Biochem. Biophys. Res. Commun.* 305, 548 -51.

Tobler I., Gaus S.E., Deboer T., Achermann P., Fischer M., Rülicke T., Moser M., Oesch B., McBride P.A. and Manson J.C. (1996) Altered circadian activity rhythms and sleep in mice devoid of prion protein. *Nature* 380, 639-42.

Top S., Kaloun el B., Vessières A., Leclercq G., Laïos I., Ourevitch M., Deuschel C., McGlinchey M.J. and Jaouen G. (2003) Tamoxifen derivatives for delivery of the antitumoral (DACH)Pt group: selective synthesis by McMurry coupling, and biochemical behaviour. *Chembiochem.* 4, 754-61.

Trevitt C.R. and Collinge J. (2006) A systematic review of prion therapeutics in experimental models. *Brain.*129, 2241–2265.

Tribouillard D., Gug F., Galons H., Bach S., Saupe S.J. and Blondel M.(2007) Antiprion drugs as chemical tools to uncover mechanisms of prion propagation. *Prion* 1 :48-52.

Tsukada M., and Ohsumi Y. (1993) Isolation and characterization of autophagy-defective mutants of Saccharomyces cerevisiae. *FEBS letters 333*, 169-174.

Uversky V.N. and Eliezer D. (2009) Biophysics of Parkinson's disease: structure and aggregation of alpha-synuclein. Curr. Protein Pept. Sci.10, 483-99. Review.

Vana K. and Weiss S. (2006) A trans-dominant negative 37kDa/67kDa laminin receptor mutant impairs PrP(Sc) propagation in scrapie-infected neuronal cells. *Journal of molecular biology* 358, 57-66.

Várkonyi J., Rosenbaum H., Baumann N., MacKenzie J.J., Simon Z., Aharon-Peretz J., Walker J.M., Tayebi N. and Sidransky E. (2003) Gaucher disease associated with parkinsonism: four further case reports. *Am. J. Med. Genet. A.* 116A, 348-51.

Vella L.J., Sharples R.A., Lawson V.A., Masters C.L., Cappai R. and Hill A.F. (2007) Packaging of prions into exosomes is associated with a novel pathway of PrP processing. *J. Pathol.* 211, 582-90.

Ventruti A. and Cuervo A.M. (2007). Autophagy and neurodegeneration. *Current neurology and neuroscience reports 7*, 443-451.

Vey M., Pilkuhn S., Wille H., Nixon R., DeArmond S.J., Smart E.J., Anderson R.G., araboulos A. and Prusiner S.B. (1996) Subcellular colocalization of the cellular and scrapie prion proteins in aveolae-like membranous domains. *Proc. Natl. Acad. Sci. U S A.* 93, 14945-9.

Virchow R. Über eine im Gehirn und Rückenmark des Menschen aufgefundene Substanz mit der chemischen Reaktion der Cellulose. *Virchows Arch.* 6, 135–138 (1853) (in German).

Vorberg I., Raines A., Story B. and Priola S.A. (2004a) Susceptibility of common fibroblast cell lines to transmissible spongiform encephalopathy agents. *J. Infect. Dis.* 189, 431-9.

Vorberg I., Raines A. and Priola S.A. (2004b) Acute formation of protease-resistant prion protein does not always lead to persistent scrapie infection in vitro. *J. Biol. Chem.* 279, 29218-25.

Wadsworth J.D., Hill A.F., Joiner S., Jackson G.S., Clarke A.R. and Collinge J. (1999) Strainspecific prion-protein conformation determined by metal ions. *Nat.Cell Biol.* 1, 55-59.

Wadsworth J.D., Joiner S., Hill A.F., Campbell T.A., Desbruslais M., Luthert P.J. and Collinge J. (2001) Tissue distribution of protease resistant prion protein in variant Creutzfeldt- Jakob disease using a highly sensitive immunoblotting assay. *Lancet.* 358, 171-80.

Wadsworth J.D., Hill A.F., Beck J.A. and Collinge J. (2003) Molecular and clinical classification of human prion disease. *Br Med Bull*. 66, 241-54.

Wadsworth J.D. and Collinge J. (2007) Update on human prion disease. *Biochim. Biophys. Acta.* 1772, 598-609.

Wang C.W. and Klionsky D. J. (2003) The molecular mechanism of autophagy. *Mol. Med.* **9**, 65-76.

Wang Q.J., Ding Y., Kohtz D.S., Mizushima N., Cristea I.M., Rout M.P., Chait B.T., Zhong Y., Heintz N. and Yue Z. (2006) Induction of autophagy in axonal dystrophy and degeneration. *J. Neurosci.* 26, 8057–8068.

Ward H.W. (1973) Anti-oestrogen therapy for breast cancer: a trial of tamoxifen at two dose levels. Br. Med. J. 1, 13-4.

Watts J.C., Drisaldi B., Ng V., Yang J., Strome B., Horne P., Sy M.S., Yoong L., Young R.,Mastrangelo P., Bergeron C., Fraser P.E., Carlson G.A., Mount H.T., Schmitt-Ulms G. and Westaway D. (2007) The CNS glycoprotein Shadoo has PrP(C)-like protective properties and displays reduced levels in prion infections. *EMBO J.* 26, 4038-50.

Webb J.L., Ravikumar B., Atkins J., Skepper J.N. and Rubinsztein D.C. (2003) Alpha-Synuclein is degraded by both autophagy and the proteasome. *The Journal of biological chemistry 278*, 25009-25013.

Weissman C. (1996) Prionen, Neuartige Krankheitserreger. *Naturw. Rdsch.* 6, 211-214.

Weissmann C., Fischer M., Raeber A., Bueler H., Sailer A., Shmerling D., Rulicke T., Brandner S. and Aguzzi A. (1996) The use of transgenic mice in the investigation of transmissible spongiform encephalopathies. *International journal of experimental pathology* **77,** 283-293.

Weissmann C., Raeber A. J., Montrasio F., Hegyi I., Frigg R., Klein M. A. and Aguzzi A. (2001) Prions and the lymphoreticular system. *Philo.s Trans. R. Soc. Lond. B Biol. Sci.* 356, 177-84.

Weissmann C. (2004) The state of the prion. *Nature reviews* 2, 861-871.

Weissmann C. and Aguzzi A. (2005) Approaches to therapy of prion diseases. *Annual review of medicine* 56, 321-344.

Welch W.J. and Brown C.R. (1996) Influence of molecular and chemical chaperones on protein folding. *Cell Stress Chaperones* 1, 109-15.

Wells G.A., Scott A.C., Johnson C.T., Gunning R.F., Hancock R.D., Jeffrey M., Dawson M. and Bradley R. (1987) A novel progressive spongiform encephalopathy in cattle. *Vet Rec.* 121, 419-20.

Westaway D., Cooper C., Turner S., Da Costa M., Carlson G.A. and Prusiner S.B. (1994) Structure and polymorphism of the mouse prion protein gene. *Proceedings of the National Academy of Sciences of the United States of America* 91**,** 6418-6422.

White M.D., Farmer M., Mirabile I., Brandner S., Collinge J., Mallucci G.R. (2008) Single treatment with RNAi against prion protein rescues early neuronal dysfunction and prolongs survival in mice with prion disease. *Proc Natl Acad Sci U S A*. 105:10238-43.

Whittington M.A., Sidle K.C., Gowland I., Meads J., Hill A.F., Palmer M.S., Jefferys J.G. and Collinge J. (1995) Rescue of neurophysiological phenotype seen in PrP null mice by transgene encoding human prion protein. *Nat Genet.* 9, 197-201.

Wilesmith J.W. and Wells G. A. (1991) Bovine spongiform encephalopathy. *Curr. Top. Microbiol. Immunol.* 172, 21-38. Review.

Wilcock, G.K., Black S. E., Hendrix S.B., Zavitz K.H., Swabb E.A., Laughlin M.A.; Tarenflurbil Phase II Study investigators. (2008) Efficacy and safety of tarenflurbil in mild to moderate Alzheimer's disease: a randomised phase II trial. Lancet Neurol. 7, 483–493.

Will R.G., Ironside J.W., Zeidler M., Cousens S.N., Estibeiro K., Alperovitch A., Poser S., Pocchiari M., Hofman A. and Smith P.G. (1996) A new variant of Creutzfeldt-Jakob disease in the UK. *Lancet.* 347, 921-5.

Wille H., Michelitsch M.D., Guenebaut V., Supattapone S., Serban A., Cohen F. E., Agard D. A. and Prusiner S. B. (2002). Structural studies of the scrapie prion protein by electron crystallography. *Proc. Natl. Acad. Sci.* USA 99, 3563-3568.

Williams A., Jahreiss L., Sarkar S., Saiki S., Menzies F.M., Ravikumar B. and Rubinsztein D.C. (2006) Aggregate-prone proteins are cleared from the cytosol by autophagy: therapeutic implications. Curr. Top. Dev. Biol. 76:89-101.

Williams ES, Young S. (1980) Chronic wasting disease of captive mule deer: a spongiform encephalopathy. J Wildl Dis. 16:89-98.

Windl O., Dempster M., Estibeiro P. and Lathe R. (1995) A candidate marsupial PrP gene reveals two domains conserved in mammalian PrP proteins. *Gene* 159, 181-186.

Windl O., Dempster M., Estibeiro J.P., Lathe R., de Silva R., Esmonde T., Will R., Springbett A., Campbell T.A., Sidle K.C., Palmer M.S. and Collinge J. (1996) Genetic basis of Creutzfeldt-Jakob disease in the United Kingdom: a systematic analysis of predisposing mutations and allelic variation in the PRNP gene. *Human genetics* 98, 259-264.

Winslow A.R. and Rubinsztein D.C., (2008) Autophagy in neurodegeneration and development. *Biochim. Biophys. Acta* 1782, 723–729.

Wopfner F., Weidenhofer G., Schneider R., von Brunn A., Gilch S., Schwarz T.F., Werner T. and Schatzl H.M. (1999) Analysis of 27 mammalian and 9 avian PrPs reveals high conservation of flexible regions of the prion protein. *Journal of molecular biology* 289, 1163- 1178.

Xilouri M., Vogiatzi T., Vekrellis K., Park D. and Stefanis L. (2009) Abberant alpha-synuclein confers toxicity to neurons in part through inhibition of chaperone-mediated autophagy. *PLoS One* 4:e5515.

Yamamoto A., Tagawa Y., Yoshimori T., Moriyama Y., Masaki R. and Tashiro Y. (1998) Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells. *Cell Struct. Funct.* 23, 33-42.

Yorimitsu, T. and Klionsky, D.J. (2007). Eating the endoplasmic reticulum: quality control by autophagy. *Trends in cell biology 17*, 279-285.

Yoshimori, T. (2004). Autophagy: a regulated bulk degradation process inside cells. *Biochemical and biophysical research communications* 313, 453-458.

Yu W.H., Cuervo A.M., Kumar A., Peterhoff C.M., Schmidt S.D., Lee J.H., Mohan P.S., Mercken M., Farmery M.R., Tjernberg L.O., Jiang Y., Duff K., Uchiyama Y., Näslund J., Mathews P.M., Cataldo A.M. and Nixon R.A. (2005) Macroautophagy--a novel Beta-amyloid peptide-generating pathway activated in Alzheimer's disease. *The Journal of cell biology* 171, 87-98.

Yu W.H., Kumar A., Peterhoff C., Shapiro Kulnane L., Uchiyama Y., Lamb B.T., Cuervo A.M. and Nixon R.A.(2004) Autophagic vacuoles are enriched in amyloid precursor protein-secretase activities: implications for beta-amyloid peptide over-production and localization in Alzheimer's disease. *Int. J. Biochem. Cell Biol*. 36, 2531-40.

Yue Z., Jin S., Yang C., Levine A.J. and Heintz N. (2003). Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. Proceedings of the National Academy of Sciences of the United States of America 100, 15077-15082.

Yun S.W., Ertmer A., Flechsig E., Gilch S., Riederer P., Gerlach M., Schatzl H.M., and Klein M.A. (2007) The tyrosine kinase inhibitor imatinib mesylate delays prion neuroinvasion by inhibiting prion propagation in the periphery. J Neurovirol 13, 328-337.

Zeidler M., Johnstone E.C., Bamber R.W., Dickens C.M., Fisher C.J., Francis A.F., Goldbeck R., Higgo R., Johnson-Sabine E.C., Lodge G.J., McGarry P., Mitchell S., Tarlo L., Turner M., Ryley P. and Will R.G. (1997) New variant Creutzfeldt-Jakob disease: psychiatric features. *Lancet.* 350, 908-10.

Zerial M. and McBride H. (2001) Rab proteins as membrane organizers. *Nat Rev Mol Cell Biol*. 2, 107-17. Review.

Zhang X., Li L., Jung J., Xiang S., Hollmann C. and Choi Y.S. (2001) The distinct roles of T cellderived cytokines and a novel follicular dendritic cell-signaling molecule 8D6 in germinal center-B cell differentiation. *J. Immunol*. 167, 49-56.

Zhang J., Randall M.S., Loyd M.R., Dorsey F.C., Kundu M., Cleveland J.L. and Ney P.A. (2009) Mitochondrial clearance is regulated by Atg7-dependent and -independent mechanisms during reticulocyte maturation. *Blood* 114, 157–164.

Zhou, X., Babu, J. R., da Silva, S., Shu, Q., Graef, I. A., Oliver, T., Tomoda, T., Tani, T., Wooten, M. W. and Wang, F. (2007) Unc-51-like kinase 1/2-mediated endocytic processes regulate filopodia extension and branching of sensory axons. *Proc. Natl. Acad. Sci. U.S.A*. 104, 5842-5847.

7 Publications

▪ Peer-reviewed Publications

Heiseke A. *, **Aguib Y.***, and Hermann M. Schätzl. Autophagy, Prion Infection and their Mutual Interactions. (2010) *Curr Issues Mol Biol.* 12(2):87-98. **authors equally contributed*

Heiseke A *, **Aguib Y***, Riemer C., Baier M. and Hermann M. Schätzl. Lithium induces clearance of protease resistant prion protein in prion-infected cells by induction of autophagy. (2009) *J Neurochem.* 109(1):25-34. **authors equally contributed*

Aguib Y.*, Heiseke A.*, Gilch S., Riemer C., Baier M., Schätzl H.M., and Ertmer A. Autophagy induction by trehalose counteracts cellular prion-infection (2009) *Autophagy.* 5(3):361-9.

Schlapschy M., Fogarasi M., Gruber H., Gresch O., Schäfer C., **Aguib Y.**, Skerra A. Functional humanization of an anti-CD16 Fab fragment: obstacles of switching from murine {lambda} to human {lambda} or {kappa} light chains. (2008) *Protein Eng Des Sel.* 22(3):175-88.

Aguib Y., Gilch S., Krammer C, Ertmer A., Groschup M.H., Schätzl H.M. Neuroendocrine cultured cells counteract persistent prion infection by down-regulation of PrP^C. (2008) *Mol Cell Neurosci.* 38(1):98-109.

Gilch S., Schmitz F., **Aguib Y.**, Kehler C., Bülow S., Bauer S., Kremmer E., Schätzl H.M. (2007) CpG and LPS can interfere negatively with prion clearance in macrophage and microglial cells. *FEBS J.* **274**, 5834-44.

To be submitted

Aguib Y., Heiseke A., Schäffler T., Gilch S., Riemer C., Vorberg I., Baier M. & Schätzl H.M. The selective estrogen receptor modulator tamoxifen inhibits prion infection in vitro and in vivo by inducing autophagy.

Heiseke A., Aguib Y. , Gilch S., Ertmer A., Vorberg I. and Schätzl H.M. Basal Autophagy Enhances Primary Prion Infection.

▪ Further Publications

Schäffler T. Analysis of different autophagy modulators and their impact on prion infection in cell culture models' (2009). *Master's Thesis - Concept & supervision.*

Wauer T. Studying the regulation of PrP^C expression in neuroendocrine cells (2008). *Bachelor's Thesis - Concept & supervision.*

Aguib Y., Studies on the Susceptibilty of neuroendocrine cell lines to prion infection. (2006) *Master's Thesis.*

Aguib Y., Structure & function analysis on humanised recombinant variant of a anti-CD 16 Fab-fragment. (2004) *Bachelor's Thesis.*

▪ Conferences & Symposia

Poster presentation: **Y. Aguib***, A. Heiseke*, T. Schäffler*, T. Schwarzer, S. Gilch, I. Vorberg, M. Baier & H.M. Schätzl. Modulated autophagy and its impact on persistent and primary prion infection 'Between the involved and a paid helper'. *Autophagy in Stress, Development & Disease, Gordon Research Conference, Lucca (Barga), Italy.*

Oral presentation: **Aguib** Y.*, Heiseke A.* & Schätzl H.M. Autophagy & its modulations in prion infection. (2010) *Retreat Wilbad Kreuth, Germany.*

Poster presentation: **Y. Aguib***, A. Heiseke*, T. Schäffler*, T. Schwarzer, S. Gilch, I. Vorberg, M. Baier2 & H.M. Schätzl. Modulated autophagy and its impact on persistent and primary prion infections. (2009) *Prion Conference, Greece.*

Oral & poster presentation: Aguib Y.*, Heiseke A.* & Schätzl H.M. Autophagy induction by Trehalose counteracts cellular Prion Infection. (2009) *GfV- Virology meeting, Leipzig, Germany.*

Poster presentation: Aguib Y.*, Heiseke A.* & Schätzl H.M. Autophagy induction by Trehalose counteracts cellular Prion Infection. (2008) *Ringberg Symposium , Germany.*

Oral presentation: Aguib Y.*, Heiseke A.* & Schätzl H.M. Autophagy induction by Trehalose counteracts cellular Prion Infection. (2008) *SFB 596 -Meeting, Chiemsee, Germany.*

Poster presentation: **Aguib** Y.*, Heiseke A.*, Ertmer A., Elsässer H.P., Gilch S. & Schätzl H.M. Induction of cellular autophagy by imatinib reduces prions. Gordon Conference (2008): Autophagy in Stress, Development & Disease, *Ventura USA.*

Poster presentation: **Aguib** Y.*, Heiseke A.*, Ertmer A., Gilch S. & Schätzl H.M. Trehalose Reduces Prions by Induction of Cellular Autophagy, Gordon Conference (2008): Autophagy in Stress, Development & Disease, *Ventura USA.*

Poster presentation: **Aguib** Y., Gilch S., Ertmer A., Groschup M. and Schätzl H.M. Neuroendocrine cultured cells resolve transient prion infection by downregulation of PrP^c (2007) *Prion Conference, Edinburgh.*

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9 Curriculum Vitae

BIOGRAPHICAL

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EDUCATIONAL

1989 – 2001	Primary, secondary and high school at the DSB (Deutsche Schule der Borromäerinnen in Kairo)
05/2001	German final school examination – Abitur (First Class honors)
10/2001 – 07/2004	Study of Molecular Biotechnology, Bachelor of Science, TUM.
03 – 06 2004	Bachelor's Thesis, Department of biological chemistry, Professor Skerra, TUM.
10/2004 – 12/2006	Master's Studies, Molecular Biotechnology, TUM, Majors: Molecular Medicine and molecular biology of infectious diseases, Minor: MOT (Management, Organisation & Technologie) (First Class Honors).
03 – 12/2007	Master's Thesis, Institute of Virology, Professor Schätzl, TUM.
05/2007 – 03/2011	Doctoral Candidate at the Institute of Virology, Schätzl AG, TUM. (PI : Prof. Skerra, Department of biological chemistry, TUM.
Since 03/2011	Scientific Consultant and Project Manager to the TU München President.

HONORS & AWARDS

06/1999	PAD (Pädagogischer Austauschdienst, Kultusminiterium) Scholarship for Premium Scholars of german schools outside Germany.
10/2001 – 12/2006	DAAD Scholarship (Programm for Elite Graduates of german schools outside Germany)
04/2007 – 04/2008	Presidential Science & Engineering Research Fellowship, Karl Max v. Bauernfeind-Vereins e.V.
05/2008	Research Grant for doctoral candidates and young academics and scientists, DAAD.
11/2007 – 11/2008	Participation the Career building program 'Wissenschaftskarriere 2008' , TU München.
07/2008	Mayor's Award for distinguished Master's thesis at Center of Life and Food Sciences Weihenstephan, TUM.

FURTHER EXPERIENCES & APPOINTMENTS (Selection)

Since 02/2011	Member of the expert committee, consulting the Federal Foreign Office and the BMBF about supporting the democratic process in North Africa via Science and Education.
09/2011	Member of the Jury awarding the Theodor Berchem Preis for Outstanding Personalities in International Academic Cooperation.