Autophagy, Prion Infection and their Mutual Interactions

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Abstract

Prion diseases are infectious and fatal neurodegenerative disorders of man and animals which are characterized by spongiform degeneration in the central nervous system. Prion propagation involves the endocytic pathway and endosomal and lysosomal compartments are implicated in trafficking and re-cycling as well as final degradation of prions. Shifting the equilibrium between propagation and lysosomal clearance to the latter impairs cellular prion load. This and earlier findings of autophagic vacuoles in correlation to prion infections both in *in vitro* and *in vivo* studies prompted us and others to analyze the role of autophagy in prion infection. Autophagy is a fundamental cellular bulk degradation process for e.g. organelles or cytoplasmic proteins which has many implications for physiology and patho-physiology of cells and whole organisms. In various neurodegenerative disease models mainly protective functions of autophagy were recently described. In this review, we focus on recent findings which correlate autophagy and its manipulations with prion infection scenarios, and discuss perspectives and future directions. The findings summarized here add to the knowledge of the role of autophagy in neurodegeneration and provide interesting new insight into how non-cytosolic aggregated proteins might be subjected to autophagic clearance.

Introduction

Prion diseases are infectious neurodegenerative disorders that can affect humans and animals. Examples for animal prion diseases are scrapie in sheep and goat, bovine spongiform encephalopathy (BSE) in cattle, transmissible mink encephalopathy (TME) in mink, and chronic wasting disease (CWD) in elk and deer. Prion diseases in humans are Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker (GSS) syndrome, fatal familiar insomnia (FFI), kuru, and the new variants of CJD (vCJD and secondary vCJD). The disease is characterized by a rapidly progressing course that leads inevitably to death, usually within a few months. Typically, this is preceded by a long incubation time entirely free of symptoms, lasting for years to many decades in humans. Severe loss of neurons is a key characteristic for all prion diseases, accompanied by strong astrogliosis and mild microglia activation. This results in a progressive spongiform degeneration of the central nervous system (CNS) which manifests itself in ataxia, behavioral changes and, in humans, a highly progressive loss of intellectual abilities (Aguzzi and Polymenidou, 2004; Collinge, 2005; Prusiner, 1998; Weissmann, 2004).

According to the protein-only hypothesis these diseases are caused by prions, *proteinaceous infectious* particles devoid of encoding nucleic acid (Prusiner, 1982). They consist mainly, if not solely, of an abnormally folded isoform (PrP^{Sc}) of the normal, host-encoded prion protein PrP^{c} (Aguzzi and Polymenidou, 2004; Cohen et al., 1994; Prusiner, 1998). Prions have self-propagating capacities in that they are able to catalyze a profound conformational switch from PrP^{c} into an aggregated structure resulting finally in the accumulation of misfolded and aggregated PrP^{Sc} in the brain. Hence, prion diseases share profound similarities with other protein misfolding and neurodegenerative diseases like Alzheimer's, Huntington's and Parkinson's disease (Aguzzi and Haass, 2003). Yet, prions are unique as they are not only able to replicate their conformation but are also naturally and experimentally transmissible within and to some extent between species.

In prion-infected cultured neuronal cells (Schatzl et al., 1997) and also in brain biopsy materials of prion-infected patients (Liberski et al., 2008; Sikorska et al., 2004) the appearance of multi-vesicular bodies and autophagic vacuoles has been reported. This prompted us and others to investigate in detail whether there is a link between autophagy and prion infection, in particular as the impact of autophagy in various other neurodegenerative diseases has been recently described (Bursch and Ellinger, 2005; Rubinsztein, 2006).

In this review we focus on the cellular mechanism of autophagy, its regulation, some well-established methods to monitor autophagy, its implications in disease with emphasis on neurodegenerative disorders, and finally, its putative role in prion infection scenarios.

Introducing 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 deliver cytoplasmic cargo to lysosomes (Figure 1). 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) (Figure 1). 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).

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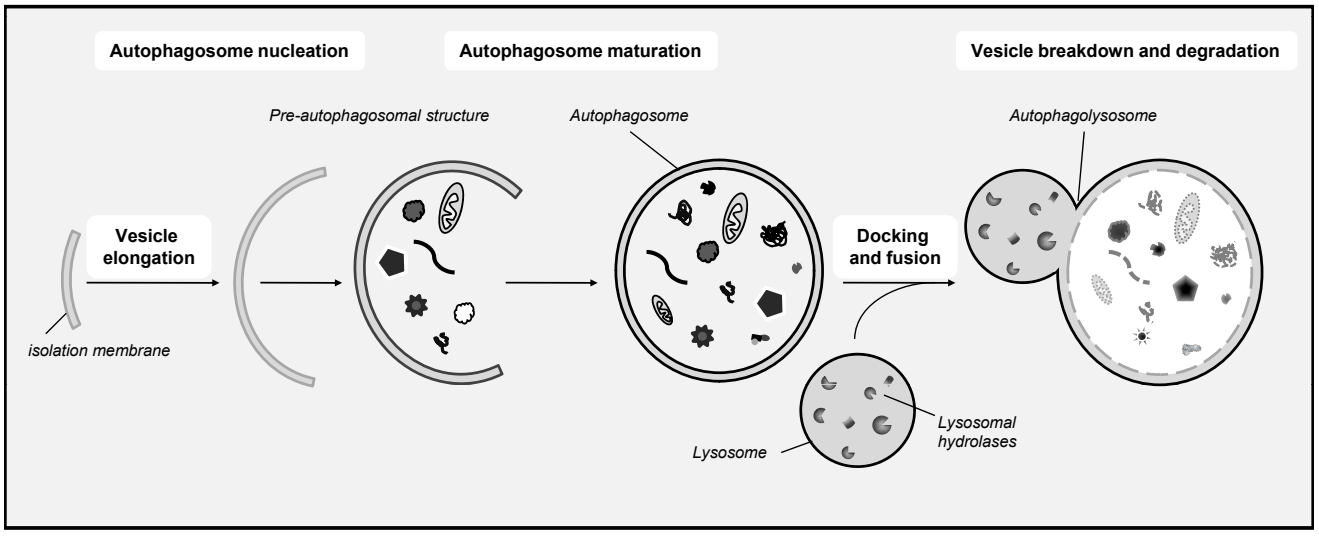

Figure 1. Schematic representation of main cellular steps in macroautophagy.
Autophagosome nucleation starts with an isolation membrane in the cytosol which matures to an autophagosome vesicle. By docking and fusion to lysosomes the content of this autophagolysosome gets access to the lysosomal degradation machinery.

Regulation and manipulation of 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. 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 (IP_3R), 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).

Monitoring autophagy

Basal and induced levels of autophagy are important with regard to its role in human health and disease. With the rapidly advancing research in the autophagy field a range of biochemical and morphological methods has been developed to monitor autophagy [e.g. reviewed in (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).

Autophagy in physiology and patho-physiology

Among recent advances, an exciting finding is the striking pleiotropy of autophagy. 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 us 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).

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).

Autophagy and neurodegenerative diseases

As mentioned above, autophagy occurs at basal, constitutive levels in the cell. Recent studies have highlighted the importance of basal autophagy in intracellular quality control. The demand for basal autophagy is tissue-specific, though. 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., 2007b; Nakai et al., 2007). Several studies suggest 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., 2005; 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). A number of *in vivo* studies during the last years 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 ubiquitin-positive 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).

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 or degenerating neurons (Petersen et al., 2001). Available data state, beyond any doubt, that autophagy has a beneficial effect of protecting against neurodegeneration. There are several hypotheses about how autophagy can prevent neurodegeneration. However, this is not yet fully understood. One idea is that autophagy eliminates aggregated and aggregate-prone proteins (Bjorkoy et al., 2005; Iwata et al., 2005; Komatsu et al., 2007a; Pankiv et al., 2007; Ravikumar et al., 2002). Concerning Alzheimer's disease and the involvement of autophagy, another hypothesis is that impaired autophagic flux provides a novel site for Aβ peptide production (Yu et al., 2005). Thus, it is reasonable to assume that autophagy could be a therapeutic target for treatment of these neurodegenerative diseases because of its protective role (Rubinsztein et al., 2007).

Recent detailed studies underlined that degradation of disease-related mutant proteins is highly dependent on autophagy, in addition to the ubiquitin–proteasome system. Examples include studies performed with extended polyglutamine-containing proteins that cause various neurodegenerative diseases (Martinez-Vicente and Cuervo, 2007; Rubinsztein, 2006; Rubinsztein et al., 2005). It was shown that the clearance of aggregate-prone proteins, such as mutant huntingtin fragments or mutant forms of α-synuclein causing Huntington´s and Parkinson´s disease, respectively, can be mediated by autophagy (Ravikumar et al., 2002; Webb et al., 2003). Animal models of Huntington´s disease and of other proteinopathies revealed that treatment with rapamycin, a known inducer of autophagy, accelerates the clearance of toxic proteins (Berger et al., 2006; Iwata et al., 2005; Qin et al., 2003; Ravikumar et al., 2004; Shibata et al., 2006). Induction of autophagy, mediated by lithium and trehalose, has been seen to accelerate the clearance of mutant huntingtin and α-synucleins (Sarkar et al., 2007a; Sarkar et al., 2005). The beneficial effect of up-regulated autophagy has also been described for other diseases associated with aggregate-prone proteins, such as Alzheimer´s disease, forms of motor neuron disease caused by mutations in superoxide dismutase 1 (SOD1), and forms of peripheral neuropathy caused by mutations in peripheral myelin protein 22 (PMP22) (Berger et al., 2006; Fortun et al., 2003; Kabuta et al., 2006).

Moreover, up-regulation of autophagy by regulatory protein kinase complex Target of Rapamycin (TOR) inhibitors such as rapamycin and its analogue CCI-779 protects against neurodegeneration seen in polyglutamine disease models in Drosophila and mice (Ravikumar et al., 2004). Recently, small-molecule enhancers of rapamycin were identified (Sarkar et al., 2007b), which improve the clearance of mutant huntingtin and α-synuclein and protect against neurodegeneration in a Drosophila Huntington's disease model. Of note, the effects of small molecule enhancers of rapamycin are independent of TOR, which adds to the possibility of using them in combination with rapamycin for therapeutic purposes. However, it is important to put into consideration that the potential lying in manipulating autophagy as a therapeutic approach for neurodegenerative diseases strongly depends on complex disease-specific factors. On the cellular level, a profound understanding of how the autophagic machinery is involved

during the pathogenic course of the disease will be relevant. On the whole organism level, the impact of autophagy manipulation will depend on the dynamics of the disease in terms of dissemination and the changes that occur in the organism.

Autophagy and 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) 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., 2000; Dandoy-Dron et al., 1998; 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*; $NP^{0/0}$) 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 recently described. 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., 2008; Liberski et al., 2002; Liberski et al., 2004). 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

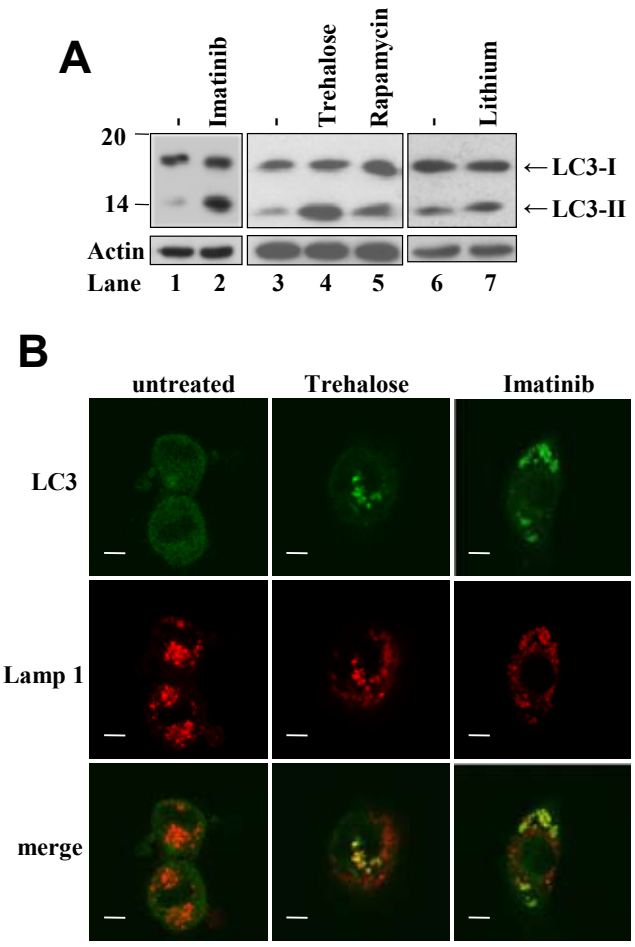

Figure 2. Monitoring autophagosome formation.
(A) ScN2a cells were treated with 10 µM imatinib, 100 mM trehalose, or 200 nM rapamycin for 48 h, or 10 mM lithium for 24 h. Mock-treated cells served as controls. Subsequently, cell lysates were probed in SDS-PAGE using anti-LC3 antibody. Higher amounts of LC3-II are detected in compound-treated cells compared to mock-treated controls, indicating induction of autophagy and autophagosome formation.
(B) ScN2a cells were transfected with GFP-LC3 and either left untreated, treated with 100 mM trehalose or 10µM imatinib for 48 h. Cells were stained with anti-lamp 1 and cy2-conjugated secondary antibody and subsequently analyzed by confocal microscopy. Both autophagosome formation (upper panels) and co-localization of autophagosomes and lysosomes (lower panels) is observed in compound-treated cells.

mechanism, leading even to degradation of prions. 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.

Previously, our group showed that imatinib, a drug used to treat chronic myelogenous leukemia, is activating lysosomal degradation of PrP^{Sc} (Ertmer et al., 2004) and is at the same time a potent inducer of autophagy and/or autophagosome formation (Ertmer et al., 2007). In prion-infected mice, imatinib treatment 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. 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). In line with studies on mutant forms of huntingtin or α-synuclein (Sarkar et al., 2007a; Sarkar et al., 2005) we could recently show that both lithium and trehalose enhance the clearance of PrP^{Sc} in prion-infected cells by induction of autophagy (Aguib et al., 2009; Heiseke et al., 2009). As mentioned, a common method to monitor autophagosome formation is to analyse the level of LC3-II which is associated with autophagosome membranes (Kabeya et al., 2000). Chemical compounds used in our studies all increased the amount of LC3-II in prion-infected cells in immunoblot analysis (Figure 2A). LC3 fused to green fluorescent protein (GFP) is another tool to measure induction of autophagosome formation. Prion-infected neuroblastoma (ScN2a) cells treated with autophagy inducing drugs exhibited punctuate GFP staining (exemplarily shown in Figure 2B for trehalose and imatinib), indicating the association of GFP-LC3 with autophagosomal membranes as a result of induction of autophagy.

In our studies we provided the first direct evidence that induction of autophagy results in degradation of cellular PrP^{Sc}. 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}, we show that autophagy is not only an important clearance route for cytosolic aggregate-prone proteins. Besides compounds inducing autophagy in an mTOR-independent manner (e.g. lithium, trehalose), we studied rapamycin, a drug widely used to activate autophagy by inhibiting mTOR. Rapamycin also reduced the level of cellular PrP^{Sc}, showing that both autophagy inducing pathways, mTOR-dependent and -independent, may be involved in the degradation of PrP^{Sc}. Reduction of PrP^{Sc} in prion-infected ScN2a cells upon treatment with several autophagy inducing drugs is shown in Figure 3.

PrP^{c} localizes via a glycosylphosphatidylinositol (GPI)-anchor at the outer leaflet of the plasma membrane in cholesterol- and sphingolipid-rich microdomains (Taraboulos et al., 1995) and can move laterally to detergent-soluble domains within the plasma-membrane for subsequent internalization (Sunyach et al., 2003). Therefore, it is possible that upon internalization PrP^{c} is in reach of the autophagic

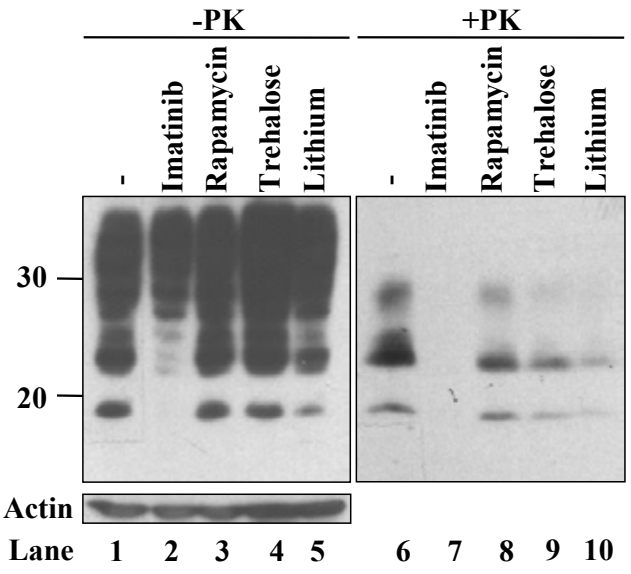

Figure 3. Reduction of PrP^{Sc} upon treatment of cells with different autophagy inducing compounds.
ScN2a cells were either left untreated or treated with 10 μM imatinib, 200 nM rapamycin, 100 mM trehalose, or 10 mM lithium for 48 h and cell lysates subsequently analyzed by SDS-PAGE using anti-PrP monoclonal antibody 4H11. Upon PK digestion (lanes 6-10) less PrP^{Sc} is observed in compound-treated cells compared to untreated control cells.

degradation machinery. We showed that lithium is not only reducing PrP^{Sc} but also levels of PrP^{c} in an autophagy-dependent manner. We observed no reduction of PrP^{c} in lithium-treated, autophagy-deficient fibroblasts whereas the level of PrP^{c} was slightly but significantly reduced in wild-type fibroblasts upon lithium treatment. In the recent past it has been shown that reduction of PrP^{c} by shedding of the protein from the membrane or by down-regulation of PrP^{c} reduces conversion of PrP^{c} into its pathogenic isoform PrP^{Sc} by limiting the amount of PrP^{c} substrate available for conversion (Aguib et al., 2008; Heiseke et al., 2008; Marella et al., 2002; Parkin et al., 2004). Therefore, reduction of PrP^{c} by lithium-induced autophagy may indirectly contribute to reduction of PrP^{Sc} by autophagy. As we did not observe reduced levels of PrP^{c} upon treatment of cells with other autophagy inducing compounds this phenomenon seems to be compound-specific and the exact molecular mechanism remains to be deciphered.

To test whether autophagy-inducing compounds are candidates for therapeutic approaches against prion infection we treated intraperitoneally prion-infected mice (Aguib et al., 2009; Heiseke et al., 2009). Rapamycin treatment of prion-infected mice initiated in the last third of incubation time (i.e. day 100 p.i.), mimicking a pre-clinical therapeutic situation, showed a significant prolongation of prion incubation times as compared to mock-treated control mice. Similar findings were obtained with lithium, although less pronounced (Heiseke et al., 2009). Trehalose treatment did not prolong incubation times, but clearly showed effects on the appearance of PrP^{Sc} in spleens (Aguib et al., 2009). Depending on when trehalose treatment was started,

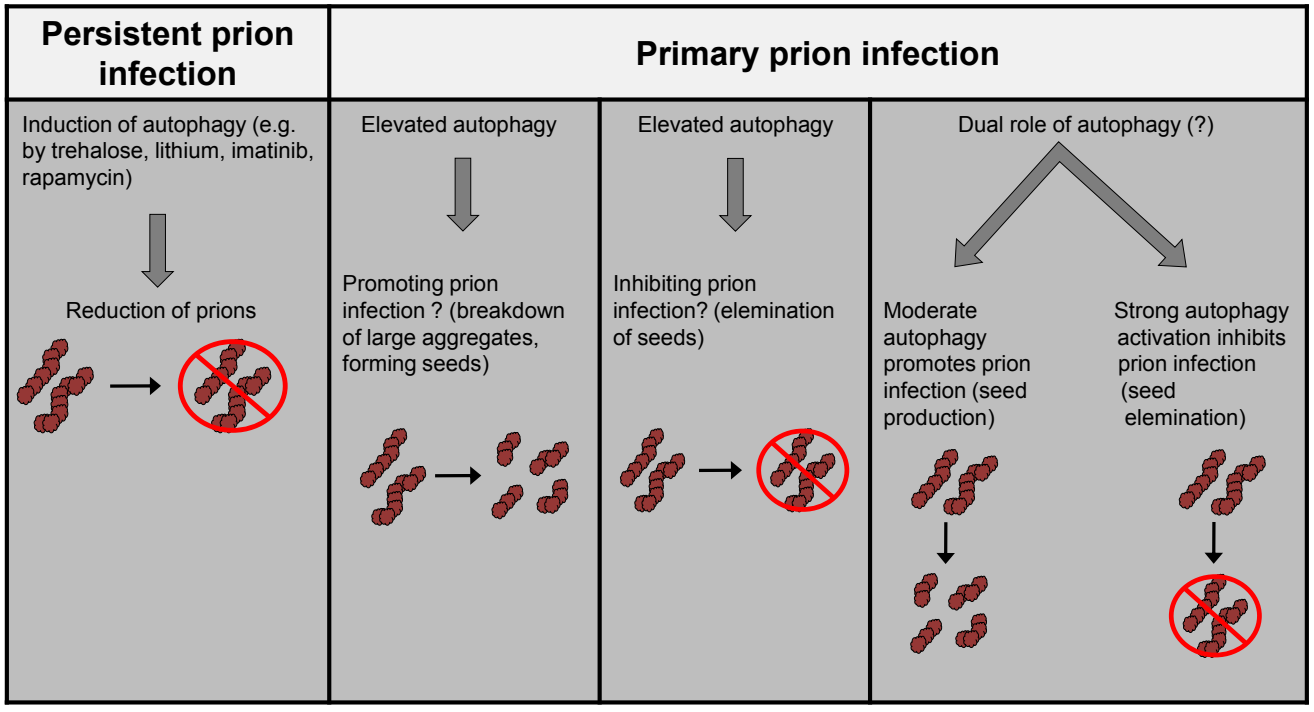

Figure 4. Model of established and putative actions of autophagy in prion infection scenarios.

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. Overall, although still preliminary, these *in vivo* studies strongly indicate that autophagy inducing compounds are beneficial in prion disease scenarios and ask for further and more complex studies, including also combination of drugs.

More than one role of autophagy in prion infection?

It is conceivable that the basic process of autophagy has a physiological role in prion infection and might be used by cells for controlling or counter-acting cellular prion infection. In line with this assumption, we observed an increased amount of PrP^{Sc} in cells in which autophagy was reduced by treatment with autophagy inhibitors (Aguib et al., 2009). The increased PrP^{Sc} level may result in an increased conversion of PrP^{c} into PrP^{Sc}, subsequently leading to more cellular PrP^{Sc}. In addition, when we assayed for altered regulation of autophagy in primary infection models, a correlation of prion infection and increase in autophagy was obtained (Heiseke, Aguib and Schatzl, personal communication). Manipulation of autophagy in primary prion infection, either increasing or decreasing autophagy, also had reproducible effects on primary prion infection.

Besides more detailed *in vitro* studies appropriate *in vivo* experiments are required to elucidate the impact of autophagy on prion infection and to validate whether autophagy plays a general role in prion disease scenarios. 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.

Although there is now good evidence from our work 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 we started to test whether increase or decrease of basal autophagy is a modifier of prion infection and susceptibility to prion infection. 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. Experimental evidence for this was obtained when we tried to infect fibroblasts from $Atg5^{-/-}$ mice with murine prions (Heiseke, Aguib and Schatzl, personal communication). Whereas wild-type fibroblasts could readily be infected with prions, knockout fibroblasts were significantly less infectable. Re-introduction of *Atg5* via lentiviral transduction clearly improved this deficiency

in primary prion infection (Heiseke, Aguib and Schatzl, personal communication). This provides reliable evidence that a certain level of autophagy can be both a positive modifier of prion infection and a supporting factor, which in certain scenarios is even essential for prion propagation. As outlined in Figure 4, a combination of anti-prion and prion-promoting effects of autophagy cannot be excluded as well. This remains us with a scenario reminding of the seeming paradox of the role of autophagy in cell death and cancer biology (Mizushima et al., 2008).

Conclusions and future directions

The systematic analysis of autophagy in prion infection scenarios is still rather incomplete. From what is found so far there is good experimental evidence from *in vitro* and partly also *in vivo* studies that induction of autophagy, e.g. by chemical compounds, 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. The exact molecular mechanisms are still incompletely understood, in particular as the very vast majority of PrP^{Sc}/prions reside within endosomal and lysosomal vesicles. On the other hand this clearly shows that not only cytosolic materials are prone to autophagic degradation. In future work it has to be studied how prion propagation, prion trafficking and re-cycling, and finally prion clearance are interconnected with the autophagic pathway. In addition, more work is needed to elucidate whether induction of autophagy in this context has to be mTOR-dependent, -independent, or both. Finally, bringing this knowledge to translational research and bridging therapeutic anti-prion concepts will be extremely difficult, as it is with basically all other anti-prion strategies so far. Besides suboptimal pharmacokinetics and possible side effects the ultralarge obstacle rests with ineffective passing of the blood-brain-barrier. This does not exclude effects in post-exposure situations in peripheral prion infection scenarios and a sophisticated combination of compounds which target divers 'anti-prion' pathways still is a reasonable goal.

The biological function of autophagy *per se* in prion infection and disease is still searching answers. Preliminary data indicate that the cellular level of autophagy can be a modifier of susceptibility to prion infection, although at present it is still difficult to dissect whether changes in autophagy are a pre-requisite or consequence of cellular prion infection. Questions like this are readily accessible in *in vitro* studies. As mentioned, autophagy might also 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., 2009) 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. Faced with early lethality in *Atg* knockout mice, one way to go might be crossing the available conditional knockout mice, which are neuron-specifically floxed, with alternative Cre deleter mice to gain both postnatal knockout and prolonged life time which then allows performing standard prion incubation time assays.

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 a molecular level.

Acknowledgements

This work was supported by SFB-596 (project A8), APRI Canada, and was performed within the framework of European Union FP6 Network of Excellence 'Neuroprion'. Y.A. was supported by grants from Technische Universität München (TUM) and the German Academic Exchange Service (DAAD).

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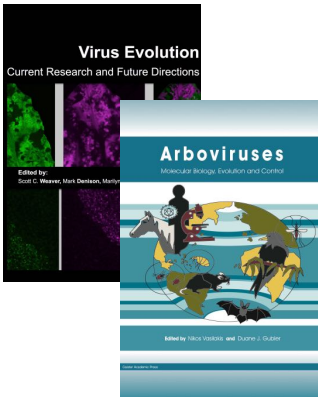

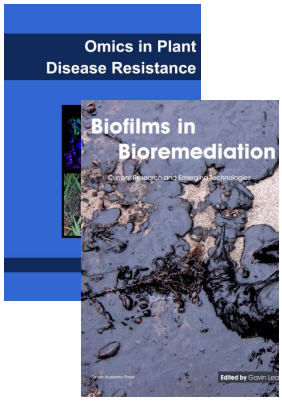

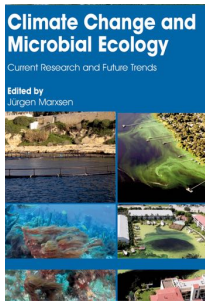

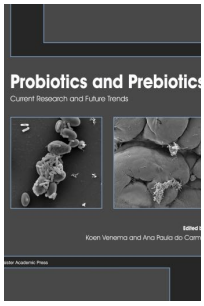

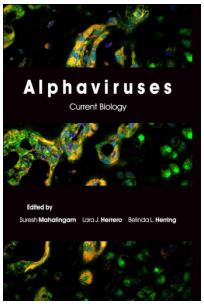

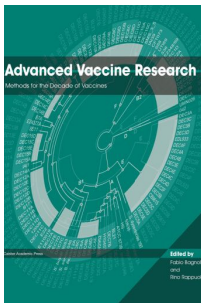

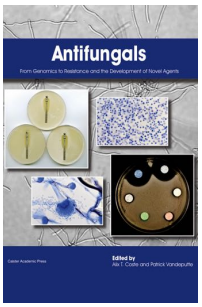

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