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Research Paper

Autophagy induction by trehalose counteracts cellular prion infection

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Prion diseases are fatal neurodegenerative and infectious disorders for which no therapeutic or prophylactic regimens exist. In search of cellular mechanisms that play a role in prion diseases and have the potential to interfere with accumulation of intracellular pathological prion protein (PrP^{Sc}), we investigated the autophagic pathway and one of its recently published inducers, trehalose. Trehalose, an alpha-linked disaccharide, has been shown to accelerate clearance of mutant huntingtin and α-synuclein by activating autophagy, mainly in an mTOR-independent manner. Here, we demonstrate that trehalose can significantly reduce PrP^{Sc} in a dose- and time-dependent manner while at the same time it induces autophagy in persistently prion-infected neuronal cells. Inhibition of autophagy, either pharmacologically by known autophagy inhibitors like 3-methyladenine, or genetically by siRNA targeting *Atg5*, counteracted the anti-prion effect of trehalose. Hence, we provide direct experimental evidence that induction of autophagy mediates enhanced cellular degradation of prions. Similar results were obtained with rapamycin, a known inducer of autophagy, and imatinib, which has been shown to activate autophagosome formation. While induction of autophagy resulted in reduction of PrP^{Sc}, inhibition of autophagy increased the amounts of cellular PrP^{Sc}, suggesting that autophagy is involved in the physiological degradation process of cellular PrP^{Sc}. Preliminary in vivo studies with trehalose in intraperitoneally prion-infected mice did not result in prolongation of incubation times, but demonstrated delayed appearance of PrP^{Sc} in the spleen. Overall, our study provides the first experimental evidence for the impact of autophagy in yet another type of neurodegenerative disease, namely prion disease.

Introduction

Prion diseases comprise a group of fatal neurodegenerative and infectious disorders, including Creutzfeldt-Jacob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), kuru, fatal familial insomnia (FFI) and variant CJD (vCJD) in humans, scrapie in sheep and goats, chronic wasting disease (CWD) of mule deer and elk, and bovine spongiform encephalopathy (BSE) in cattle. The central feature of these disorders is the conformational change of the host-encoded, cellular prion protein (PrP^{c}) to an abnormal, partially proteinase K resistant and infectious isoform (PrP^{Sc}), which finally accumulates in brains of diseased individuals.[1-5] Normally folded and mature PrP^{c} is attached to the outer leaflet of the plasma membrane via a glycosyl-phosphatidyl-inositol (GPI) anchor and conversion of PrP^{c} into PrP^{Sc} seems to take place near the cell surface or along the early endocytic pathway, probably in caveolae-like domains (CLDs) or in rafts (reviewed in ref. 6). Preceding PrP^{c} localization at the cell surface seems to be essential for conversion of PrP^{c} into PrP^{Sc},[7-9] and studies in mice suggest that the conversion process requires direct interaction of the two PrP isoforms, possibly in a complex with as yet unidentified auxiliary factors.[1]

The neuropathological triad in prion diseases consists of spongiform changes in the brain, glia activation, and neuronal loss.[10,11] Besides apoptosis, the occurrence of multivesicular bodies and autophagic vacuoles in the cytoplasm of prion-infected cultured cells[12] and in neurons derived from brain biopsy samples of TSE affected humans[13] or animals[14-17] was reported. Degradation of organelles or cytoplasmic proteins can be mediated by macroautophagy (referred to hereafter as autophagy), in which double membrane structures called autophagosomes/autophagic vacuoles are formed. Autophagosomes then fuse with lysosomes to generate autophago-lysosomes in which contents are degraded by acidic lysosomal hydrolases.[18] This mechanism is conserved among eukaryotes and has been characterized from yeast to man.[19]

Several studies suggest a crucial role of autophagy in neurodegenerative diseases, including Alzheimer disease, Parkinson disease, tauopathies and polyglutamine expansion diseases like Huntington disease.[20-30] Trehalose is an alpha-linked disaccharide synthesized 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.[31] Many of its protective effects are apparently caused by its ability to act as a chemical chaperone.[32] It was shown that trehalose accelerates the clearance of aggregate-prone proteins, such as mutant huntingtin and α-synuclein, by activating autophagy mainly in an mTOR-independent manner.[33]

In this work we show that autophagy not only plays a role in neurodegenerative diseases involving cytosolic aggregate-prone or

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aggregated protein, but also in disease types in which protein aggregates accumulate primarily within vesicular cellular compartments, as is the case in prion diseases.[34] We provide here 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 trehalose or other inducers of autophagy and autophagosome formation like rapamycin and imatinib. The anti-prion effect was prevented by simultaneous application of potent autophagy inhibitors like 3-MA and by silencing the *Atg5* gene with siRNA. Therefore, our data show that the observed increased cellular degradation of prions is a result of induced autophagy. Furthermore, autophagy may play a role in the physiological degradation process of cellular PrP^{Sc}, as we detected increased amounts of PrP^{Sc} in cells treated with autophagy inhibitors alone. Proof of evidence was also obtained in in vivo studies. 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.

Results

Trehalose induces autophagy and reduces PrP^{Sc}. Intrigued by the finding that clearance of mutant huntingtin and α-synuclein is accelerated by activating autophagy in an mTOR-independent manner,[33] we 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 (Fig. 1A).

We observed a reduction of PrP^{Sc} when cells were treated with 100 mM trehalose (Fig. 1B, upper, lane 11). 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 autophagosome membranes and the amount of LC3-II correlates with the extent of autophagosome formation.[35] 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 (Fig. 1B, lower, lanes 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, we analyzed 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 analyzed by immunoblotting (Fig. 1C). Quantification revealed a time-dependent reduction of PrP^{Sc} upon trehalose treatment (Fig. 1D). Long-term treatment of cells with 100 mM trehalose (14 days) showed that reduction of PrP^{Sc} is not a transient phenomenon (Fig. 1C, right-hand). To analyze 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 (Fig. 1E).

PrP^{c} levels in uninfected N2a cells were not influenced by treatment with 100 mM trehalose (Suppl. Fig. S1A). Moreover, in ScN2a cells treated with 100 mM trehalose followed by a solubility assay via ultracentrifugation, levels of soluble PrP^{c} in the supernatant fraction of trehalose-treated cells were comparable to that of untreated cells, while clearly reduced amounts of insoluble PrP^{Sc} were detected in the pellet fraction (Suppl. Fig. S1B), confirming the fact that trehalose-induced autophagy leads to reduction of insoluble PK-resistant PrP^{Sc} aggregates.

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.

Trehalose activates autophagosome formation. So far we have shown 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 Fig. 1A). To characterize the observed induction of autophagy in more detail, cells were treated with 100 mM trehalose for 48 h and subsequently analyzed by immunoblotting (Fig. 2A, lanes 1 and 2). Of note, 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,[36] in the same experimental set. The used dose of bafilomycin A1 is saturating for LC3-II levels in this assay (data not shown). ScN2a cells were either mock-treated or treated with 100 mM trehalose for 48 h with simultaneous bafilomycin A1 treatment and analyzed by immunoblotting (Fig. 2A, lanes 3 and 4). Treatment of ScN2a cells with 100 mM trehalose increased LC3-II levels about 7-fold (Fig. 2B), which is typically achieved upon induction of autophagy.[35] Cotreated cells show a significantly increased amount of LC3-II compared to cells treated with bafilomycin A1 or trehalose alone (Fig. 2B), confirming that trehalose activates autophagy in our cell culture model beyond autophagosome-lysosome fusion, at the level of autophagosome formation.

To further investigate induced autophagosome formation by trehalose, confocal laser microscopy was performed with N2a and ScN2a cells stably overexpressing LC3 fused to green fluorescent protein (N2aGFP-LC3 and ScN2aGFP-LC3) (Suppl. Fig. S2). Cells treated with trehalose or imatinib, which served as positive control for autophagosome formation,[37] clearly showed punctate 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 colocalized 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.

Changes in endogenous autophagic activity affect cellular levels of PrP^{Sc}. In order to further examine the impact of trehalose-induced autophagy on degradation of PrP^{Sc}, we investigated the effect of 3-MA, an inhibitor of phosphatidylinositol-3-kinase[38] known to inhibit cellular autophagy, on the anti-prion effect of trehalose.

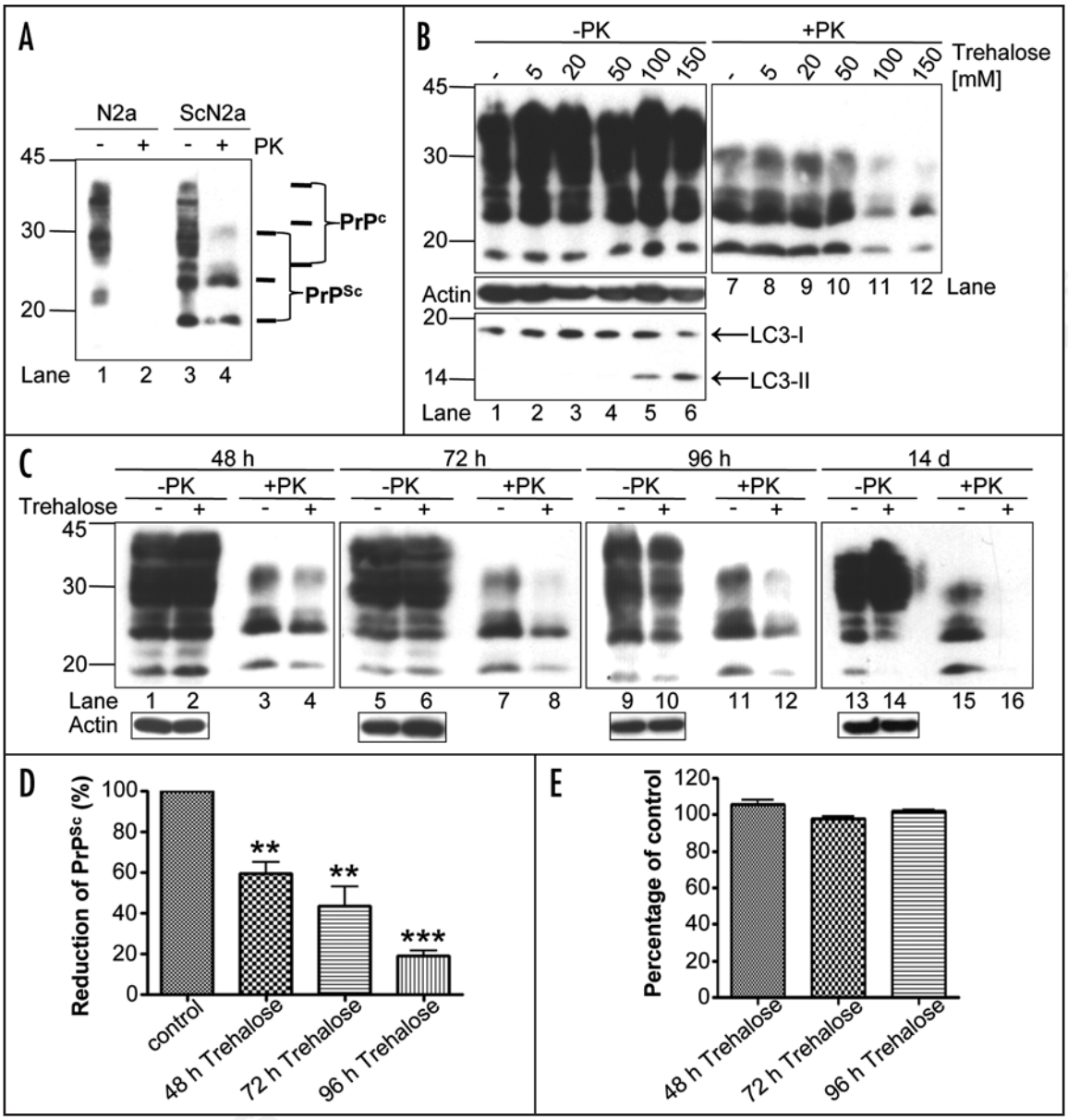

Figure 1. Dose- and time-dependent reduction of PrP^{Sc} and induction of autophagy by trehalose. (A) N2a and ScN2a cells were proteinase K (PK) digested and analyzed by immunoblotting using anti-PrP monoclonal antibody (mAb) 4H11. In contrast to uninfected N2a cells, PrP^{Sc} (19, 24 and 27–30 kDa) is detected in prion-infected ScN2a cells (compare lanes 2 and 4). (B) 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 visualized by immunoblotting using anti-PrP mAb 4H11 (upper). Lysates of ScN2a cells treated for 48 h with trehalose were probed with anti-LC3 mAb in immunoblot to analyze induction of autophagy (lower). Trehalose induces cellular PrP^{Sc} reduction and induces autophagy in a dose-dependent manner. (C) 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 visualized by immunoblotting using anti-PrP mAb 4H11. (D) 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}. (E) Trypan blue assay to analyze 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.

ScN2a cells were either mock-treated, treated with 100 mM trehalose, or cotreated with 100 mM trehalose and 10 mM 3-MA for 48 h and analyzed by immunoblotting (Fig. 3A and B). Compared to cells treated with trehalose alone (lane 2), induction of autophagy by trehalose was strongly impaired by cotreatment with 3-MA (lane 3). The same approach was used to analyze the effect of 3-MA on the anti-prion effect of trehalose. In fact, ScN2a cells cotreated with 100 mM trehalose and 10 mM 3-MA showed no reduction of PrP^{Sc} (Fig. 3C, compare lanes 7 and 8). As pharmacological inhibition of autophagy might be affected by nonwarranted side effects of the drug used, we additionally used siRNA to knock down the *Atg5* gene, an essential gene implicated in the autophagic pathway. The siRNA designed to downregulate the expression of *Atg5* (Fig. 3D and E) was reproducibly able to impair the PrP^{Sc} reducing effect of trehalose. This provides evidence that PrP^{Sc} reduction upon trehalose treatment depends on the autophagic activity in the cell.

Taken together, our data show the potential of trehalose to enhance cellular autophagy, which then likely mediates lysosomal degradation of PrP^{Sc} in prion-infected cells.

Interestingly, we observed a moderate though significant increase in the amount of PrP^{Sc} when cells were treated with 3-MA alone (Fig. 3C, compare lanes 4 and 8; F). This phenomenon was confirmed by immunoprecipitation assays of radioactively labelled PrP^{Sc} (Fig. 3G). ScN2a cells treated with 10 mM 3-MA for 24 h showed a clear increase in the amount of PrP^{Sc} when compared to mock-treated cells (Fig. 3G, compare lanes 2 and 3) indicating an impaired degradation of PrP^{Sc}.

To analyze whether the effect of activated autophagy on PrP^{Sc} degradation is restricted to the mTOR-independent pathway induced by trehalose, we tested the effect of rapamycin, a known inducer of the mTOR-dependent autophagic pathway. In fact, slightly lower levels of PrP^{Sc} (~50% reduction) were observed in ScN2a cells treated with 0.2 μM rapamycin for 48 h (Fig. 4A, compare lanes 5 and 7). PrP^{Sc} was not detected in cells treated with the autophagosome formation inducer imatinib,[37] which served as a positive control for reduction of PrP^{Sc} (Fig. 4A, lane 8). In addition, an increase in LC3-II levels (~3 fold) was detected in rapamycin-treated cells (Fig. 4C, compare lanes 1 and 3, D).

Taken together, we provide experimental evidence that various inducers of autophagy have the potential to reduce cellular PrP^{Sc}. In contrast, inhibition of autophagy seems to result in increased levels of PrP^{Sc}, indicating that autophagy may be involved in the physiological degradation process of cellular PrP^{Sc}.

Trehalose treatment of intraperitoneally prion-infected mice delays appearance of PrP^{Sc} in the spleen. To study whether trehalose has the potential to interfere with prion infection in in vivo models, we continuously applied trehalose, and sucrose as a control, to the drinking water of mice which were intraperitoneally infected with a high dose of prions. First, individual mice were sacrificed and tested for the amount of PrP^{Sc} in the spleen (Fig. 5). Immunoblot analysis of spleens obtained from trehalose- or sucrose-treated mice revealed reduced PrP^{Sc} deposition in the trehalose-treated group at time points 30 and 60 days post infection (Fig. 5A, B and D). However, at day 90 post infection PrP^{Sc} levels were equal between sucrose- and trehalose-treated animals (Fig. 5C 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.

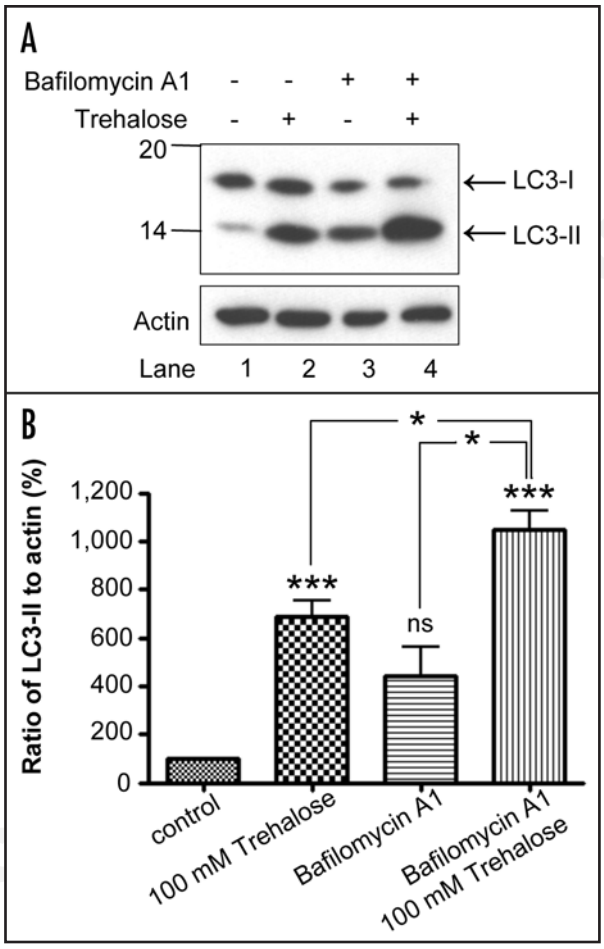

Figure 2. Characterization of trehalose-activated autophagy. (A) ScN2a cells were either mock-treated (control, lane 1), treated with 100 mM trehalose for 48 h (lane 2), with bafilomycin A1 (200 nM) for 4 h prior to lysis of cells (lane 3), or treated with 100 mM trehalose for 48 h with simultaneous bafilomycin A1 (200 nM) treatment for 4 h prior to lysis of cells (lane 4). Cells were analyzed by immunoblotting using anti-LC3 mAb. (B) 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.

Discussion

Impairment of autophagy in the CNS is associated with neuronal death and neurological abnormalities. Mice with defects in autophagy developed neurodegenerative symptoms in the CNS without the expression of disease-causing, aggregate prone proteins.[39,40] Previous studies also implicated macroautophagy as a key pathway for removal of aggregated intracellular proteins, including mutant huntingtin, α-synuclein and ataxin-3.[41] However, data on autophagy in prion diseases are still very limited.[13-17,42-44] Electron microscopy studies pointed towards a possible contribution of autophagy in prion disease-associated neurodegeneration, whereas others observed in prion-infected brain tissue a slight reduction in expression of genes mediating autophagy.[44]

In recent studies, we have shown that treatment of prion-infected cells with imatinib activates lysosomal degradation of PrP^{Sc}.[45] In another study, we demonstrated that imatinib can induce the formation of autophagosomes.[37] These two studies, however, did not determine whether the activation of the autophagic pathway per se has an anti-prion effect as a consequence. In the present study we utilized 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.[33] We used here well-established prion infection cell culture models to investigate the impact of autophagy on cellular PrP^{Sc}.

Our data demonstrate that the degradation of PrP^{Sc} which is induced by trehalose is mediated by activation of autophagy. As soon as trehalose-induced autophagy was constrained by 3-MA or

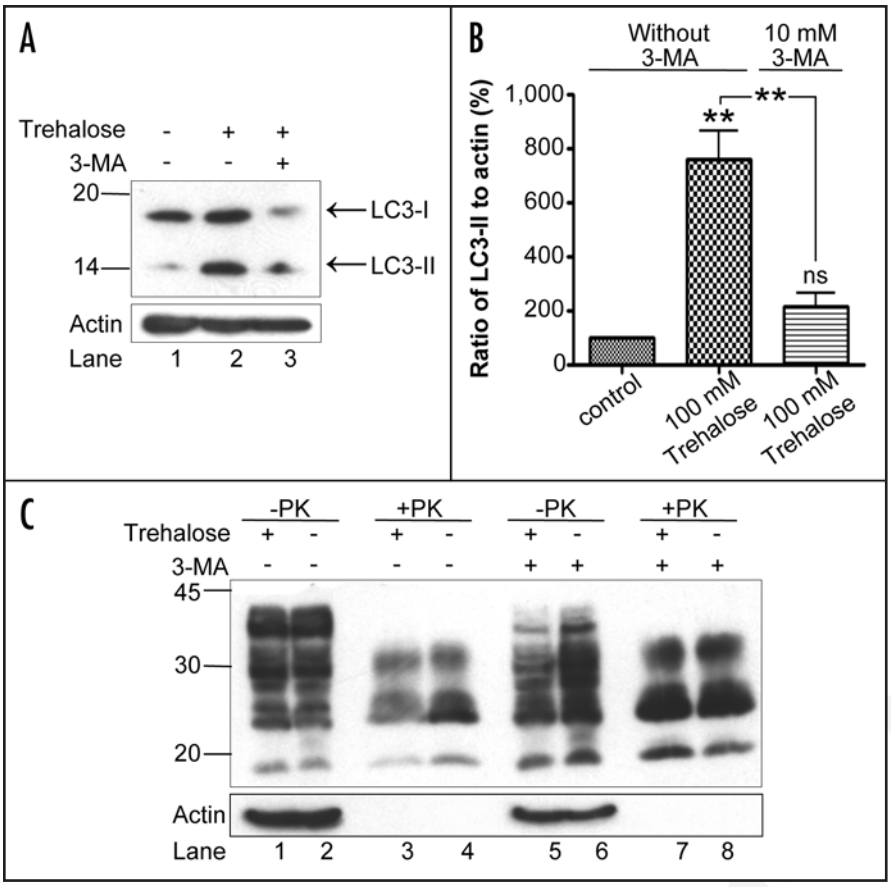

Figure 3A–C. Altered cellular PrP^{Sc} levels caused by changes in autophagy activity. (A) ScN2a cells were either mock-treated, treated with 100 mM trehalose or cotreated with 100 mM trehalose and 10 mM 3-MA for 48 h. Cells were analyzed by immunoblotting using anti-LC3 mAb. (B) The level of endogenous LC3-II in trehalose-treated cells, with or without cotreatment 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 cotreated with 100 mM trehalose and 10 mM 3-MA for 48 h. Upon PK digestion, PrP was visualized by immunoblotting using anti-PrP mAb 4H11. Reduction of PrP^{Sc} is not observed when cells are cotreated with trehalose and 3-MA.

wortmannin (data not shown), the effect on PrP^{Sc} was abolished. Similar results were obtained when the *Atg5* gene, an essential member of the autophagic machinery, was genetically silenced prior to trehalose treatment. These observations provide experimental evidence that the effect of trehalose on PrP^{Sc} depends on the autophagy machinery. The fact that the anti-prion effect is not restricted to mTOR-independent activation of autophagy, as rapamycin treatment also led to a detectable, although weak PrP^{Sc} reduction, strongly confirms the relationship between activated autophagy and anti-prion effects. Our results also suggest that autophagy is involved in the physiological degradation process of PrP^{Sc} during cellular prion infection. 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, indicating that repression of endogenous autophagy can compromise the physiological degradation of cellular PrP^{Sc}. As a consequence, more PrP^{Sc} may be available for the conversion process, leading subsequently to an increase in cellular PrP^{Sc}.

Even though trehalose treatment 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. Previously, similar results were obtained in mice treated with imatinib.[46] 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.[47] 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 prion disease scenarios. An obvious goal of our future studies is to reveal whether drug-induced autophagy can be used as a novel avenue for therapy against prion diseases.

Of note, our work expands 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. We show here that autophagy, as a cytosolic, nonspecific 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}.[1,34]

The beneficial or, eventually, also deleterious effects of autophagy in prion disease remain elusive. To account for the neuro-protective effects of autophagy[39,40] and the ability of autophagy to reduce PrP^{Sc} in prion-infected cells and to some extent also in vivo, it is quite possible that cells impaired in autophagy might be more susceptible to persistent prion infection. On the other hand, it remains to be determined whether a moderate basal level of cellular autophagy, that is likely to be present even after 3-MA treatment, is 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.[48] Hence, although strongly induced autophagy has the potential to reduce or clear prions and PrP^{Sc} seeds, the possibility that a moderate autophagy supports PrP^{Sc} seed production at certain stages of prion infection, and thereby is promoting prion disease, has to be considered and remains the subject of further investigations. Thus, a combination of anti-prion and prion-promoting effects of

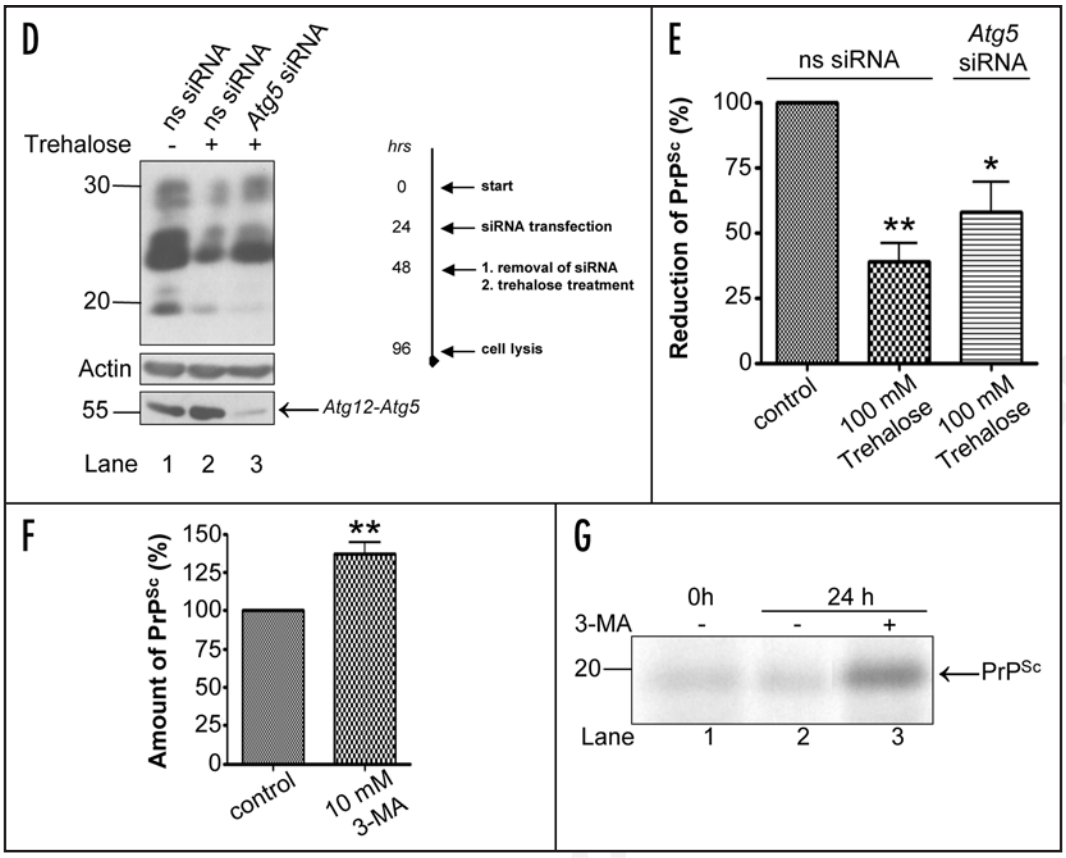

Figure 3D–G. Altered cellular PrP^{Sc} levels caused by changes in autophagy activity. (D) ScN2a cells were either transfected with nonsilencing (ns) siRNA (lane 2) or siRNA targeting *Atg5* (lane 3) and then treated with 100 mM trehalose. As a control, cells were transfected with ns siRNA and left untreated (lane 1). Chronological course of the experiment is schematically shown on the right. To confirm successful knockdown of *Atg5*, lysates were probed with anti-ATG5 mAb by immunoblotting (lower). PrP^{Sc} was visualized by immunoblotting using anti-PrP mAb 4H11. (E) 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. (F) 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. (G) ScN2a cells were metabolically labelled and chased in medium with or without 10 mM 3-MA. After 0 or 24 h of chase, cells were PK digested and prior to immunoprecipitation using anti-PrP pAb A7 a solubility assay was performed. Samples were deglycosylated and analyzed on SDS-PAGE followed by autoradiography. Arrow on the right indicates deglycosylated PrP^{Sc}. 3-MA treatment increases cellular PrP^{Sc} in ScN2a cells.

autophagy cannot be excluded, a scenario which reminds us of the seeming paradox about the role of autophagy in cell death or cancer biology.[49]

Materials and Methods

Imatinib (Novartis Pharmaceuticals Corporation; Basel, Switzerland) was dissolved in DMSO at a stock solution of 10 mM and stored at -20°C. Pefabloc proteinase inhibitor and FuGene transfection reagent were obtained from Roche Diagnostics GmbH (Mannheim, Germany), proteinase K was from Roth (Karlsruhe, Germany). Trehalose, 3-methyladenine (3-MA), rapamycin and all other chemicals were from Sigma (Munich, Germany). Immunoblotting was done using the enhanced chemiluminescence blotting technique (ECL plus) from Amersham Corporation (Buckinghamshire, UK). (^{35}S)-Met/Cys (Promix; 1,000 Ci/mmol) and Protein A-Sepharose were obtained from Amersham. Anti-PrP mAb 4H11 and anti-PrP pAb A7 have also been described previously.[45] Mouse monoclonal anti-β-actin antibody was from Sigma, anti-LC3 mAb and mouse anti-ATG5 mAb were obtained from nanoTools (nanoTools Antikörpertechnik GmbH & Co., KG, Teningen, Germany). Cell culture media and solutions were obtained from Invitrogen (Karlsruhe, Germany).

Cell culture. The mouse neuroblastoma cell line N2a (American Type Culture Collection CCL-131) and ScN2a cells (persistently infected with prion strain RML) were maintained in OptiMEM medium containing 10% fetal calf serum (FCS), penicillin/streptomycin and glutamine in a 5% CO_2 atmosphere. Specific prion infectivity in vivo of these cultured cells infected with RML prions has been described.[50] Drug treatment of cells was performed by adding 100 mM trehalose, 10 mM 3-MA, 10 μM imatinib, 200 nM rapamycin or 200 nM bafilomycin A1 to the culture media.

Immunoblot analysis. Immunoblot analyses were performed as previously described.[50] Confluent cells were lysed in cold lysis buffer (10 mM Tris-HCl, pH 7.5; 100 mM NaCl; 10 mM EDTA; 0.5% Triton X-100; 0.5% sodium deoxycholate (DOC)) for 10 min. For proteinase K (PK) treatment, post-nuclear lysates were divided into two halves. One half was incubated with PK (20 μg/ml) for 30 min at 37°C and digestion was stopped by addition of proteinase

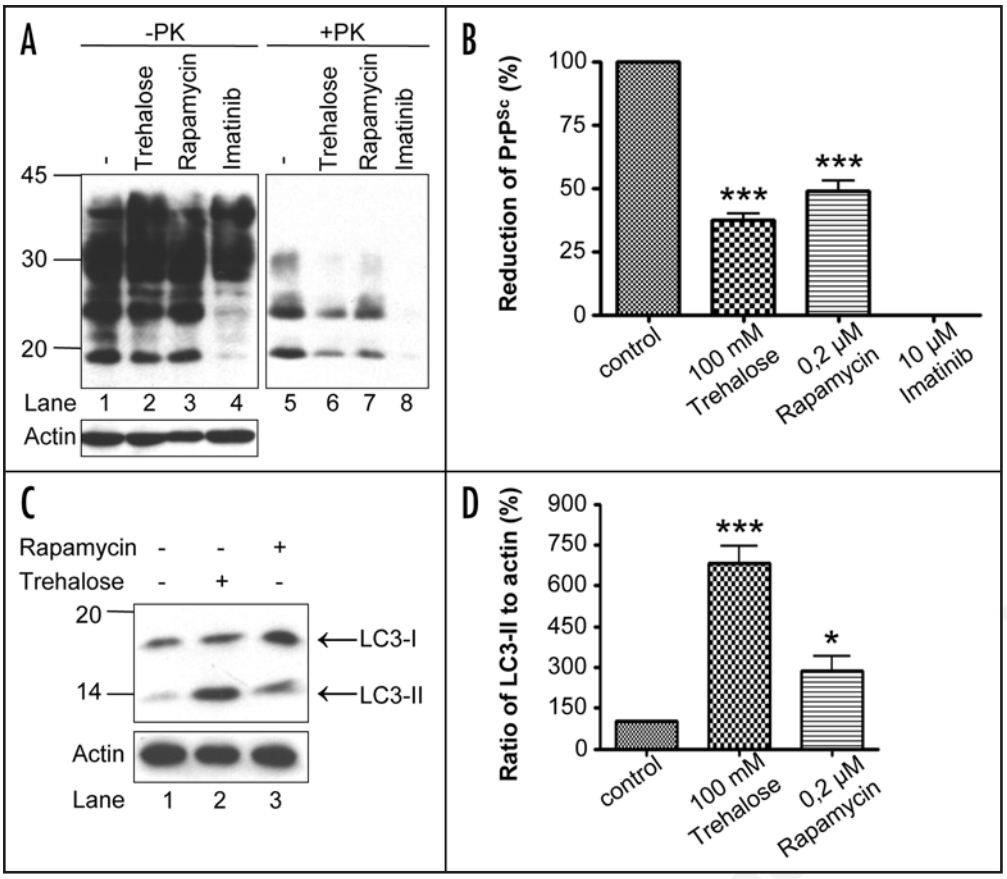

Figure 4. Additional autophagy inducers which can reduce PrP^{Sc}. (A) ScN2a cells were treated for 48 h with either 100 mM trehalose, 0.2 µM rapamycin, 10 µM imatinib, or no treatment was accomplished. PrP was visualized by immunoblotting using anti-PrP mAb 4H11. Less PrP^{Sc} is detected in cells treated with the different autophagy-inducers when compared to mock-treated cells. (B) PrP^{Sc} signals in compound-treated cells are expressed as percentage of control and represent the mean ± S.E. of three independent experiments (***$p < 0.001$). Compared to mock-treated cells, less PrP^{Sc} is detected in cells treated with trehalose or rapamycin, respectively. Total clearance of PrP^{Sc} was observed in imatinib treated cells. (C) ScN2a cells were either mock-treated (control, lane 1), treated with 100 mM trehalose (lane 2), or treated with 0.2 µM rapamycin (lane 3) for 48 h. Cells were analyzed by immunoblotting using anti-LC3 mAb. (D) Levels of endogenous LC3-II in trehalose- and rapamycin-treated cells are expressed as percentage of control and represent the mean ± S.E. of three independent experiments. Both trehalose (~7-fold) and rapamycin (~3-fold) increase the level of endogenous LC3-II.

inhibitors (0.5 mM Pefabloc) and directly precipitated with methanol. The sample without PK treatment was directly supplemented with proteinase inhibitors and precipitated with methanol. After centrifugation for 30 min at 2,000 xg (4°C), the pellets were re-dissolved in TNE buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA) and gel loading buffer (7% SDS, 30% glycerine, 20% Et-SH, 0.01% Bromphenol blue in 90 mM Tris-HCl pH 6.8) was added. After boiling for 5 min an aliquot was analyzed on 12.5% SDS-PAGE. Proteins were electrotransferred to polyvinylidene difluoride (PVDF) membrane (Amersham). Membranes were blocked with nonfat dry milk (5%) in Tris-buffered saline (TBST) (0.05% Tween 20, 100 mM NaCl, 10 mM Tris-HCl; pH 7.8), incubated overnight with the appropriate antibody at 4°C and stained using enhanced chemiluminiscence blotting (ECL plus) kit from Amersham.

Transfection of siRNA. siRNA oligonucleotides were from Qiagen (Hilden, Germany). Transfection of siRNA was performed with Lipofectamine 2000 according to the protocol provided by the manufacturer (Invitrogen). Antibiotic-free medium was used for experiments with transfected cells.

Metabolic radiolabeling and immunoprecipitation assay. Metabolic radiolabeling and immunoprecipitation assays were done as previously described.[45] Confluent cells were washed twice with phosphate-buffered saline (PBS) and incubated for 1 h in RPMI medium without methionine/cysteine containing 1% FCS. The medium was then supplemented with 800 mCi/ml of (^{35}S) L-Met/Cys (Amersham) for 16 h. Due to the long pulse-chase period addition of 1% FCS is needed and has no negative influence on labeling efficiency in our hands. ScN2a cells were either mock-treated or treated with 10 mM 3-MA. After 0 and 24 h cells were washed twice in cold PBS and lysed in cold lysis buffer (100 mM NaCl; 10 mM Tris-HCl, pH 7.8; 10 mM EDTA; 0.5% Triton X-100; 0.5% DOC). Insoluble material was removed by centrifugation at 10,000 xg for 1 min. Then lysates with and without PK treatment were supplemented with proteinase inhibitors and N-lauryl sarcosine to 1%, and ultracentrifuged in a Beckman TL-100 table ultracentrifuge for 1 h at 100,000 xg (TLA-45 rotor; 4°C). Pellet fractions were resuspended in 100 µl RIPA buffer (0.5% Triton X-100; 0.5% DOC; in PBS) with 1% sodium dodecyl sulfate (SDS) and boiled for 10 min. The pellet fraction was diluted with 900 µl RIPA buffer (supplemented with 1% sarcosyl) and the primary antibody A7 (1:300) was incubated overnight at 4°C. Protein A-Sepharose beads were then added for 60 min at 4°C. The immuno-adsorbed proteins were washed in cold RIPA buffer supplemented with 1% SDS, deglycosylated with PNGaseF at 37°C, and analyzed on 12.5% SDS-PAGE followed by autoradiography.

Animal studies. Intraperitoneal infections of mice were performed as previously described.[44] Trehalose or the control disaccharide sucrose was thereafter continuously administered as 4% solutions via the drinking water (*ad libitum*). Spleens of individual mice were analyzed in immunoblot at different time points for PrP^{Sc} and incubation times to clinical prion infection monitored as described.[44]

Trypan blue assay. To assess viability of cells upon 100 mM trehalose treatment, trypan blue assay was performed. Thereby, treatment of cells with trypan blue allows detection of the number of viable cells by staining unviable cells. Cells were cultured for 48, 72 or 96 h in medium containing 100 mM trehalose or no trehalose. Then, cells were mixed in an 1:2 ratio with trypan blue and subsequently stained and unstained cells were counted. Percentage of viable cells

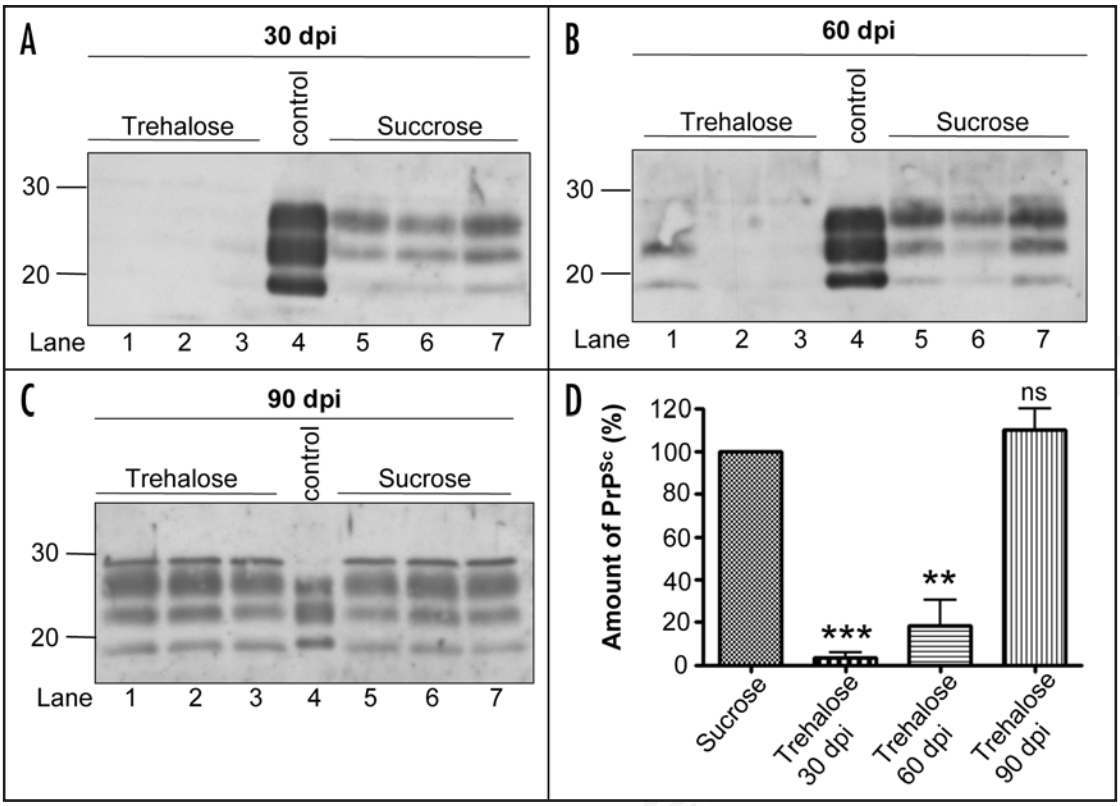

Figure 5. PrP^{Sc} in spleens of trehalose- and sucrose-treated mice. Immunoblot detection of PrP^{Sc} in spleens of trehalose- and sucrose-treated mice 30 (A), 60 (B) and 90 (C) days post infection (dpi), respectively. Lanes 1–3 denote trehalose-treated mice, lane 4 is a positive control for PrP^{Sc}, 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.

was calculated by determining the ratio of unstained (viable) cells to stained (not viable) and unstained cells. In each experiment at least 600 cells were counted.

Statistical analysis. 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, California) using the unpaired two-tailed t test for pairwise comparisons. Statistical significance was expressed as follows: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (ns: not significant). LC3-II was measured relative to actin signals to determine endogenous LC3-II levels.

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/AguibAUTO5-3-Sup.pdf

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