The molecular basis for neuronal death in prion disease is not established but putative pathogenic roles for both disease-related prion protein (PrPSc) and accumulated cytosolic PrP have been proposed. Here we report that only prion-infected neuronal cells become apoptotic after mild inhibition of the proteasome, and this is strictly dependent upon sustained propagation of PrPSc. While cells overexpressing PrPC developed cytosolic PrP aggregates, this did not cause cell death. In contrast, only in prion-infected cells, mild proteasome impairment resulted in the formation of large cytosolic perinuclear aggresomes that contained PrPSc, heat shock chaperone 70 (Hsc70), ubiquitin, proteasome subunits and vimentin. Similar structures were found in the brains of prion-infected mice. PrPSc aggresome formation was directly associated with activation of caspase 3 and 8 resulting in apoptosis. These data suggest that neuronal propagation of prions invokes a neurotoxic mechanism involving intracellular formation of PrPSc aggresomes. This in turn triggers caspase-dependent apoptosis, and further implicates proteasome dysfunction in the pathogenesis of prion diseases.

Prion diseases are rare fatal neurodegenerative disorders which include Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle and scrapie in sheep. The molecular hallmark of these disorders is the accumulation of abnormal prion protein conformers (PrPSc) derived from normal cellular host prion protein (PrPC)(1). The cause of neurodegeneration in these disorders is not well understood, and a major gap exists in the understanding of how the conversion of PrPC to PrPSc ultimately kills neurons. While PrPC is absolutely required for prion conversion and neurotoxicity (2), knockout of PrPC in adult brain (3) and in embryonic models (4;5) has no overt phenotypic effect effectively excluding loss of PrPC function in neurons as a significant mechanism in prion neurodegeneration. However, there is much evidence that also argues against the direct neurotoxicity of PrPSc or prions (whether or not they are identical). PrPC-null tissue remains healthy and free of pathology when exposed to PrPSc (6;7) and there is no direct correlation between neuronal loss and PrPSc plaques in CJD brains (8). Similarly, prion diseases in which PrPSc is barely detectable have been described (9-11), and sub-clinical infection where high levels of PrPSc accumulate in the absence of clinical symptoms are also recognised (12). In such ‘sub-clinical disease states’ the majority of the accumulated PrPSc may be inert; alternatively, PrPSc may not be the toxic entity, but instead a toxic oligomeric PrP intermediate species (PrPL for lethal) may be produced during prion conversion (12). Either this intermediate species or PrPSc itself may then only elicit neurotoxic effects when present at sufficient concentrations in particular sub-cellular compartments.

Various mechanisms have been proposed to explain neuronal death in prion disease [reviewed in (13)], which is thought to occur via an apoptotic mechanism (14-16). In vitro studies have suggested that both full-length PrPSc (16;17) and shorter peptide fragments (18) are toxic when applied to primary cultured neurons. Other mechanisms suggested relate to altered PrPC trafficking. It has been described that PrPC may assume a transmembrane topology (CtermPrP) the
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concentration of which has been suggested to correlate with neurotoxicity (19). More recently, it was proposed that prion-associated toxicity involves altered trafficking of PrPC, where inhibition of the ubiquitin-proteasome system (UPS) results in extensive PrPC accumulation in the cytoplasm and associated neuronal cell death (20). However, the data is conflicting, with evidence both for (21;22), and against (23-25) this cytoplasmic accumulation of PrPC having neurotoxic sequelae. One of the major drawbacks of many of these studies on cytosolic PrPC is the high levels of proteasome inhibition used which may limit any physiological relevance to the situation in vivo (26).

The concept that UPS inhibition may contribute to neurodegeneration is not new. Degradation of intracellular proteins via the UPS is a highly complex and tightly regulated process that plays a major role in a variety of cellular processes (27). Aberrations in this system have been implicated, either as a primary or secondary event, in the pathogenesis of a range of neurodegenerative diseases including Parkinson’s disease, Alzheimer’s disease, and Huntington’s disease (28). The degradative capacity of the UPS in the nervous system is known to become impaired in these neurodegenerative diseases as well as during the ageing process itself [(29); and reviewed in (48)].

We therefore set out to examine further the nature of the PrP species responsible for neurotoxicity and to what extent low level or ‘physiological’ UPS inhibition may be involved in prion disease pathogenesis. We chose to study these effects both in cell models of prion infection as well as examining overexpression of native PrPC. We found that while neuronal cells overexpressing PrPC developed cytosolic PrPC aggregates under conditions of mild proteasome inhibition, this did not cause cell death. However under similar conditions we found that neuronal propagation of prions invokes a neurotoxic mechanism involving intracellular formation of compartmentalised cytosolic PrPSc aggresomes that triggers caspase-dependent apoptosis and implicates proteasome dysfunction in the pathogenesis of prion diseases. The aggresome has emerged as a key organelle in the clearance of toxic cytoplasmic misfolded protein aggregates (30). Interestingly, we found evidence for similar structures in vivo in brains of prion-infected mice.

Materials and Methods

Cell culture and scrapie infection
GT-1 and N2aPD88 mouse neuronal cells were infected with mouse-adapted RML scrapie prions or wild-type CD-1 mouse brain homogenate as described (31;32) and passaged to remove the initial brain inoculum. Cells were cultured in Opti-MEM (Invitrogen) with 10% FCS supplemented with 1% penicillin-streptomycin and maintained at 37°C in 5% CO2. Cultures were tested for the presence of newly generated PrPSc by the scrapie cell assay (SCA)(32). The SCA was used to determine the percentage of infected cells (32). Cells were diluted in duplicate so that no more than 1000 cells were seeded into one well of a scrapie cell assay plate. To determine the number of infected cells, the assay was developed using the standard colour reaction as described (32). To determine the total number of cells, the assay was developed using a 1 in 10 dilution of Trypan Blue as an indicator of cell viability. The percentage of infected cells was calculated as the number of infected cells expressed as a percentage of the total number of cells. Both ScN2aPD88 and ScGT-1 cells were cured of PrPSc with 0.5 µg/ml anti-PrP antibody ICSM18 (D-Gen Ltd, London) for 14 days. Confirmation of clearance of PrPSc was determined using the scrapie cell assay as described (32).

Generation of N2amoPrPC cells
Exon 3 (bp7-1316) encoding the full ORF of mouse PRNP was cloned into the Not I - Cla I sites of the retroviral vector LNCX2 (Clontech, CA, USA). This vector was then packaged into the GP-E86 line using Fugene 6 (Roche), and selected using G418. Viral supernatant was used to infect N2aPD88 and GT-1 cells with 4 µg/ml polybrene (Sigma). At 24 hours post-retroviral transduction, stable exon 3 moPRNP- expressing clones were selected using G418. Expression levels were quantified by immunoblotting as described below.

Cell death and apoptosis assays
For cell death studies, dose-response curves were established using proteasome inhibitors and concentrations causing ~20% cell death in wild-type cells were chosen. Analysis of cell death
using the LDH kit was as recommended by the manufacturer (Alexis, Nottingham, UK). Quantification of apoptosis using annexin-V and PI staining, and caspase 3 and 8 assays were performed according to manufacturer’s instructions (Oncogene, MA, USA). For caspase inhibition experiments, cells were pre-incubated for 2 hours with z-DEVD-fmk or z-IETD-fmk before addition of proteasome inhibitors. Annexin V-FITC binding was quantified using flow cytometry (FACS). A total of 10,000 cells per sample were analysed for cell death by FACS Calibur (Becton Dickinson, CA) with the Cell Quest software.

**SDS-PAGE and immuno-blot analysis**

Cells were harvested and brain tissue homogenized on ice in PBS, freeze-thawed three times and treated with benzonase (50U/ml) to digest DNA. Protein concentration was determined by BCA assay (Pierce, Northumberland, UK). The equivalent of 25µg of total protein was loaded onto 16% SDS-PAGE mini-gels (Novex, Paisley, UK) and analysed by electrophoresis and immuno-blotting as described (33). For proteinase K (PK)(Roche, UK) digestion, lysates were incubated with PK at 1µg/mg protein (cells) or 5µg/mg protein (brain) at 37°C for 90 min. Equivalent protein loading was confirmed in non-PK treated lanes by stripping membranes and re-probing with anti-β-actin antibody (Sigma).

**Immunofluorescence and confocal analysis**

Cells were fixed onto poly-L-lysine coated glass coverslips using 4% Paraformaldehyde for 20 min at room temperature (RT), washed three times with PBS, and then permeabilised in methanol at –20°C for 15 min. Cells were then incubated in 10% normal goat serum for 30 min. Incubation with primary antibody was at 37°C for 1 hour. After washing, cells were incubated for 45 min with the appropriate secondary antibody at 37°C, washed several times in PBS and mounted in Antifade (Sigma) containing 1µg/ml 4',6-diamidino-2-phenylindole (DAPI; Sigma). To remove PrP<sup>C</sup> and reveal PrP<sup>Sc</sup>, cells were exposed to 98% formic acid for 5 min after fixing and before permeabilisation. For confocal analysis, images were obtained using an LSM510 confocal microscopy system (Zeiss). A 63x oil immersion objective was used for all imaging. For some experiments, ICSM18 was conjugated to a fluorescent Alexa-488 FITC secondary antibody (Molecular Probes). Details of all antibodies are available in on-line supplementary data Table S1.

**Subcellular fractionation and PrP analysis**

Analysis of PrP solubility and aggregation was performed with modifications to published procedures (20;34). Briefly, after 24 hours lactacystin treatment, cells were harvested, washed in ice-cold PBS, freeze-thawed three times and then centrifuged at 100,000 x g for 10 min at 4°C to remove cellular debris. The supernatant was collected, adjusted with an equal volume of 2 x lysis buffer (100mM Tris, pH 7.4, 300mM NaCl, 4mM EDTA, 1% Triton-X-100, 1% deoxycholate) and incubated with benzonase (50U/ml) for 20 min at 4°C before centrifugation at 100,000 x g for 45 min. Proteins in the supernatant were precipitated at –20°C with methanol, air dried and then boiled in 2 x SDS-sample buffer, whilst proteins in the pellet fraction were boiled in 2 x SDS-sample buffer. Each fraction was analysed by immunoblotting as described above.

**Affinity purification of PrP<sup>Sc</sup> aggregates**

Magnetic tosyl-activated beads were coated with either mouse monoclonal antibody to vimentin or Brc126, an IgG isotype control antibody, according to manufacturer’s instructions (Dynal, Bromborough, UK). ScGT-1 cells (with or without proteasome inhibition) were harvested in ice-cold PBS, freeze-thawed three times before treatment with benzonase (50U/ml) on ice for 15 min. Equivalent aliquots of sample (10µl containing 25µg total cell protein) were incubated with vimentin antibody-coated beads, Brc126-coated beads, or magnetic beads alone (25µl bead bed volume) for 2 hours at 37°C on an orbital shaker. Beads were concentrated in a magnetic particle concentrator (Dynal) and washed three times with PBS (3 x 5 min 500µl PBS). Washed beads were resuspended in 20µl PBS and analysed after PK digestion (Final protease concentration of 1µg/mg protein, 90 min, 37°C) or in the absence of protease digestion. Beads were treated with 2 x sample buffer at 100°C for 10 min and the supernatant analysed by immunoblotting with biotinylated anti-PrP monoclonal antibody ICSM35 (D-Gen Ltd, London). For analysis of brain, 10% brain homogenate was prepared in PBS.
and freeze-thawed three times before digestion with benzonase (50U/ml on ice, 15 min), and centrifugation at 1000 x g for 10 min to remove cellular debris. Aliquots of supernatant (25µl) were adjusted with an equal volume of 2 x lysis buffer (100mM Tris, pH 7.4, 300mM NaCl, 4mM EDTA, 1% Triton-X-100, 1% deoxycholate) and incubated with either vimentin antibody-coated beads, Brc126 antibody-coated beads or magnetic beads alone (25µl bead bed volume) for 2 hours at 37°C on an orbital shaker. Beads were concentrated in a magnetic particle concentrator (Dynal) and washed three times with PBS (3 x 5 min 500µl PBS). Washed beads were resuspended in 20µl PBS and analysed after PK digestion (Final protease concentration 5µg/mg protein, 90 min, 37°C) or in the absence of protease digestion. Beads were treated with 2 x sample buffer at 100°C for 10 min and the supernatant analysed by immunoblotting with biotinylated anti-PrP monoclonal antibody ICSM35.

Statistical analysis
Data were analysed by two tailed non-parametric Mann Whitney U-Test and significance was expressed as follows: *P<0.01; **P<0.001; ***P<0.0001 unless otherwise specified. For all graphs bars represent means ± SD; and * P < 0.01, ** P < 0.001; *** P < 0.0001 (two-tailed Mann Whitney U-test).

Image acquisition
Fluorescence images were obtained using a confocal microscope (Zeiss microscope LSM510 META) equipped with “plan-Apochromat” 63x/1.40 Oil DIC objective at room temperature and is controlled by Zeiss LSM software. Fluorescence was recorded at 488nm using 30mW Ar-laser for excitation or at 543nm using 1mW HeNE-laser for excitation. Zeiss Immersol™ 518 F was used as imaging medium. Images not requiring confocal analysis were obtained using an Axioplan 2 MOT microscope (Zeiss) with filters for FITC, Rhodamine and DAPI and Plan Neofluar 10x/0.30 Ph1 objective at room temperature. An AxioCam MRm (Zeiss) camera was used and was controlled using the Axiovision Control software (Zeiss).

RESULTS

PrPSc infection sensitises both GT-1 and N2aPD88 cells to mild proteasome inhibition
Mouse hypothalamic neuronal GT-1 (31) and highly prion-susceptible N2aPD88 cells (32) were infected with mouse-adapted RML scrapie prions or wild-type CD-1 brain homogenate, and passaged to remove the brain inoculum (31;32) (Fig 1A). Cells mock-infected with wild-type CD-1 brain homogenate were negative on the SCA indicating that they were not prion-infected (unpublished data). Cells were then treated with a range of doses of the irreversible proteasome inhibitor lactacystin (Fig 1B – graph i). Significant differences in cell death was observed. At 1µM lactacystin a highly significant difference (p<0.0001) was observed in cell death in the ScGT-1 cells (52%) compared to uninfected GT-1 cells (17%) (Fig 1B – graph ii). Mock-infected GT-1 cells resulted in similar levels of cell death to wild-type GT-1 cells confirming the specificity of prion infection in sensitising cells to mild proteasome inhibition (Fig S1 supplementary data). This concentration of lactacystin was selected to mimic the degree of proteasome impairment that may occur in vivo (26;35). These results were reproduced with another specific proteasome inhibitor, epoxomicin (Fig S2 supplementary data). To ensure the effects we observed were not confined to a sub-group of ScGT-1 cells known to have an apoptotic phenotype after scrapie infection (31), experiments were repeated using scrapie-infected clonal N2aPD88 cells (32). Again, RML-infected N2aPD88 cells were significantly more susceptible to induction of apoptosis after mild proteasome inhibition compared to uninfected N2aPD88 cells (p<0.0001) (Fig 1C - graphs i and ii). Analysis by the SCA (32) showed that ~52% and ~42% of the ScGT-1 and ScN2aPD88 cell populations were scrapie-infected (unpublished data) which is consistent with the percentages of cell death seen in Fig 1B - graph ii and Fig 1C – graph ii.

Curing prion-infected cells with anti-PrP monoclonal antibodies abrogates the neurotoxic effect of proteasome inhibition
To investigate whether the sensitivity of scrapie-infected cells was due to PrPSc, cells were treated for 14 days with 0.5µg/ml of anti-PrP monoclonal
antibody ICSM18 (36), and confirmed to have undetectable levels of infectivity using the SCA (unpublished data). Curing cells of prion infection abrogated the sensitivity to proteasome inhibition and resulted in the same degree of cell death as uninfected GT-1, mock-infected GT-1 and N2aPD88 cells (Figs 1B, 1C, Fig S1 supplementary data). Thus prion propagation appears to sensitise these neuronal cells to mild proteasome inhibition.

**Cytosolic accumulation of detergent-insoluble PrP^C aggregates is not neurotoxic after mild proteasome inhibition**

To further investigate whether our findings were due to PrP^Sc, we generated N2aPD88 cells overexpressing ~3-fold full-length wild-type mouse PrP (N2a^moPrPC)(Fig 2A). In non-lactacystin treated N2a^moPrPC cells, the PrP^C was localised on the cell surface, in the lysosomal system and in an ER-Golgi pattern with partial ER co-localisation (63) (Fig 2B). After low dose lactacystin treatment, the pattern of PrP^C immunostaining changed in the N2a^moPrPC cells, with the majority of PrP^C deposition now in the cytoplasm co-localising with the cytosolic protein Hsc70 (Fig 2C).

However, despite the presence of cytosolic PrP^C, there was no significant difference in cell death when compared to wild-type N2aPD88 cells (Fig 2D – graph i), suggesting that at low level proteasome inhibition, cytoplasmic accumulation of PrP^C is not neurotoxic. Notably, in our N2a^moPrPC cells there appeared to be a neuroprotective effect of PrP^C overexpression (p<0.001) after treatment with 10µM lactacystin compared to wild-type N2aPD88 cells (Fig 2D – graph ii) as previously reported (24;25). At high doses of lactacystin (10µM) there were high levels of cell death in both the scrapie-infected and uninfected N2aPD88 (~90% in ScN2aPD88 and ~75% in N2aPD88)(Fig 1C – graph i) in agreement with previous studies (20). To assess whether the cytosolic PrP^C observed in our lactacystin-treated cells had formed detergent-insoluble aggregates, we performed subcellular fractionation with analysis of detergent solubility and aggregation status of PrP^C in cytosolic and membrane fractions (Fig 2E). With no proteasome treatment, all the PrP^C expressed in our N2a^moPrPC was fully detergent-soluble (Fig 2E lane 1); after low dose lactacystin treatment (1µM) there was an increase in detergent-insoluble aggregated PrP^C isolated in the pellet fraction (Fig 2E lane 4). After high dose lactacystin treatment (10µM) all the PrP^C was aggregated and detergent-resistant (Fig 2E lane 6).

We then infected our N2a^moPrPC cells with RML scrapie prions, and observed correlation between the presence of PrP^Sc and neurotoxic effect after 1µM lactacystin treatment (Fig 2F). Thus the presence of PrP^Sc, rather than cytoplasmic aggregates of wild-type PrP^C, appears to be associated with apoptosis after mild proteasome inhibition.

**Prion infection induces caspase 3 and 8 dependent apoptosis which is abrogated by specific caspase inhibitors**

To evaluate cell death after mild proteasome inhibition, nuclear DNA fragmentation analysis, annexin-V and PI staining was undertaken in lactacystin-treated ScGT-1 cells, and apoptosis quantified using FACS analysis. These results demonstrated that the ScGT-1 cells were dying by apoptosis (Figs 3A and 3B). Apoptosis may be initiated through a number of different pathways, and in vivo studies in prion disease have suggested that caspase 3 and 8 dependent pathways are activated (37;38). To study this process further a time-course study of caspase 3 and 8 activation was undertaken. From 1 hour there was a highly significant rise in caspase 3 and 8 activities in the ScGT-1 cells versus uninfected cells in a time-dependent manner (Figs 3C and 3D). At 24 hours there was a 120% increase in caspase-8 activation compared to uninfected GT-1 cells (Fig 3D); this finding supports in vivo data suggesting that caspase 8 mediated apoptotic cell death plays a significant role in prion-mediated neuronal cell death (39). Apoptotic cell death was completely abrogated in the scrapie-infected cells using cell-permeable specific caspase 3 and 8 inhibitors (DEVD-fmk and IETD-fmk respectively) supporting their pivotal role in scrapie-mediated neuronal cell death (Fig 3E).

PrP^Sc, but not aggregated PrP^C, forms large cytosolic perinuclear aggresomes which appear directly neurotoxic.
Aggresomes are located near the microtubule-organising centre at the centrosome (40) reflecting the fact that aggresome formation needs an intact microtubule network (40;41). They are also distinguished by a cage of the intermediate filament protein vimentin which is an invariant feature of these structures (40;42). We used stringent formic acid pre-treatment of cells to remove PrP<sup>C</sup> immunoreactivity, and to reveal PrP<sup>Sc</sup> deposits, in our scrapie-infected cells (Fig 4A). Double-labeling immunostaining demonstrated that after mild proteasome inhibition, PrP<sup>Sc</sup> accumulates in ScGT-1 cells as large cytoplasmic perinuclear aggresomes and co-localises with vimentin, Hsc70, 20S proteasome and ubiquitin (Figs 4B-4E). Similar structures were found in lactacystin-treated ScN2aPD88 cells (Fig S3 supplementary data). Using confocal microscopy, cytoplasmic localisation was confirmed by colocalisation with the cytosolic chaperone Hsc70 (Fig 4D) and the absence of immunostaining with markers for the ER and nucleus (unpublished data).

To confirm that PrP<sup>Sc</sup> itself was a major constituent of these aggresomes we performed an affinity purification of the ScGT-1 aggresomes using vimentin antibody-coated magnetic beads. Vimentin is a type-III intermediate filament that normally displays an extended cytoplasmic distribution. In aggresome-containing cells, vimentin is redistributed to form a cage-like structure wrapped around the exterior of the inclusion (41); it has been suggested that this contributes to the stability of the aggresome (42). Vimentin antibody-coated beads purified PrP<sup>Sc</sup> from lactacystin-treated ScGT-1 cells (Fig 5A lane 2), the specificity of this interaction was confirmed using isotype control antibody-coated beads which did not purify PrP<sup>Sc</sup> (Fig 5A lanes 3-4) and beads alone (Fig 5A lanes 5-6). Previous reports have suggested that cytosolic PrP<sup>C</sup> forms aggresomes after cyclosporin A treatment (43), we therefore performed immunostaining for aggresomes in our N2a<sub>amo</sub>/Pr<sup>PC</sup> cells. Importantly, we found that cytoplasmic PrP<sup>C</sup> aggregates did not form aggresomes (unpublished data). Mock-infected cells also did not form aggresomes and were indistinguishable from wild-type uninfected cells (unpublished data).

The role of aggresomes in cellular neurotoxicity is controversial; their formation in cells has been reported to be a neuroprotective mechanism to sequester toxic misfolded proteins (44), whereas others suggest that they are a toxic species (45;46). To investigate this further, we used agents which inhibit the formation of aggresomes by disrupting retrograde microtubule-mediated transport (47). Prior to treatment with colchicine, nocodazole and cytochalasin D we undertook dose-response curves in N2aPD88 and GT-1 cells to optimise treatment of cells with these agents (Fig S4 Supplementary data). Colchicine is an anti-mitotic agent that disrupts microtubule function. Treatment with colchicine (5 µg/ml) prevented cell death in prion-infected cells after low proteasome inhibition (p<0.0001)(Fig 5B - graph i). To ensure that colchicine treatment had also prevented aggresome formation we performed immunofluorescence which confirmed that the prevention of PrP<sup>Sc</sup> aggresome formation had abrogated cell death (Fig 5C – panels 1 and 2). We also examined the effect of nocodazole treatment on the formation of PrP<sup>Sc</sup> aggresomes; nocodazole is an agent that also disrupts microtubule dynamics (47). This supported the argument that the effect of colchicine treatment was via this mechanism, as cell death was also abrogated by 0.5 µM nocodazole treatment (p<0.0001)(Fig 5B - graph ii) with prevention of aggresome formation (Fig 5C – panel 3). To ensure that the abrogation of cell death induced by PrP<sup>Sc</sup> aggresomes by the microtubule-disrupting agents was specific, we treated our cells with 50ng/ml cytochalasin D which disrupts actin microfilaments which are not involved in aggresome formation (47). Treatment with cytochalasin D did not affect cell death (Fig 5B – graph iii) nor cleared aggresomes (Fig 5C – panel 4) confirming our data that clearance of PrP<sup>Sc</sup> aggresomes selectively abrogates cell death. To ensure that colchicine and nocodazole were not exerting their anti-apoptotic effect through clearance of PrP<sup>Sc</sup>, we treated ScGT-1 cells for 5 days with these drugs which had no effect of PrP<sup>Sc</sup> levels in these cells assessed using the SCA (unpublished data).

Formation of PrP<sup>Sc</sup> aggresomes is temporally associated with caspase 3 and 8 activation
To examine whether aggresome formation is directly related to caspase activation we measured
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caspase 3 and 8 activities after treatment with colchicine and nocodazole to prevent aggresome formation. This treatment also abrogated caspase activation in prion-infected cells (Fig 6A) suggesting a direct relationship between the formation of PrP^Sc aggresomes and caspase activation leading to apoptosis. We then performed a time-course analysis of PrP^Sc aggresome formation and caspase 3 and 8 activation in ScGT-1 cells after mild proteasome inhibition (Figs 6B-6F – panels 1-3). At time zero, there are no PrP^Sc aggresomes (Fig 6B - panel 3) and no evidence of caspase 8 (Fig 6B - panel 1) or caspase 3 activation (Fig 6B - panel 2). PrP^Sc aggresome formation initiates as small perinuclear structures at 6 hours (Fig 6C - panel 3) which directly correlates with the presence of de novo caspase 8 and 3 immunostaining in the cells at the same time-point (Fig 6C – panels 1 and 2). After 12 hours the size of the PrP^Sc aggresome increases (Fig 6D - panel 3) with marked caspase 8 and 3 immunostaining in the cells containing PrP^Sc aggresomes (Fig 6D - panels 1 and 2). By 18 and 24 hours there is widespread intra-cellular caspase 8 and 3 immunostaining and large perinuclear PrP^Sc aggresomes seen (Fig 6E and F - panels 1-3). These data are supported by the time-course study of caspase 3 and 8 activation in ScGT-1 cells (Figs 3C and D) where from 6 hours onwards there was a significant increase in caspase 3 and 8 activity levels (p<0.001; Figs 3C and D). These data support a direct relationship between the formation of PrP^Sc aggresomes and caspase 8- and 3-dependent apoptosis in neuronal cells after mild proteasome inhibition.

PrP^Sc is also associated with aggresome-like structures in vivo

To assess whether PrP^Sc aggresome structures occurred in vivo we attempted to affinity purify PrP^Sc from terminal scapie-infected CD-1 mouse brains using vimentin antibody-coated magnetic beads (Figs 7A and 7B). We demonstrate that there is a specific association between PrP^Sc and the intracellular protein vimentin in vivo as we were able to affinity-purify PrP^Sc from scrapie-infected CD-1 mouse brain using vimentin antibody-coated beads (Fig 7A lane 3). Using densitometry and quantitative immunoblotting the estimated proportion of total brain PrP^Sc recovered by the vimentin beads is ~4% which represents the intracellular PrP^Sc associated with aggresomes in these terminal scrapie-infected mouse brains (Fig 7B lane 7). Isotype-control antibody or beads alone did not isolate PrP^Sc (Fig 7A lanes 1 and 4). There was no association between PrP^C and vimentin in uninfected CD-1 brain again confirming the specificity of the interaction with PrP^Sc and vimentin (Fig 7A lanes 5 and 6).

DISCUSSION

This study aimed to define further the cellular basis of neurotoxicity in prion-mediated neuronal death and the subcellular compartments in which toxicity may be generated. To investigate the role of the UPS in prion-mediated toxicity, we studied much milder levels of proteasome inhibition than reported in previous studies (20-22;34). Levels of proteasome impairment that we investigated are more compatible with the loss of proteasome activity associated with either senescence (45;48) or that may be seen in prion-infected brain (26;35). We chose to study two separate mouse prion-propagating neuronal cell lines (N2aPD88 and GT-1) (31;32) to allow validation of the data in different prion-infected cell systems. There are very few cell lines able to stably propagate prions in vitro, and these two neuronal cell systems are well-characterised and represent a valuable tool for analysis (49). We confirmed that both the N2aPD88 and GT-1 cells are able to propagate low levels of scrapie infectivity and suffer no obvious cytotoxic effects (31;32;50;51). This may be due to possible cell-specific properties of enhanced degradative capacity where accumulation of PrP^Sc does not occur to a level where it may become neurotoxic (52), which may account for the reason these neuronal cells are uniquely able to stably propagate low levels of mouse prions.

Here we show that prion-infected N2aPD88 and GT-1 neuronal cells were significantly more susceptible to cell death when treated with low dose proteasome inhibitors than uninfected or mock-infected cells. These cells underwent caspase 3 and 8 dependent apoptotic cell death that was abrogated by specific caspase 3 and 8 inhibitors. Curing these cells of prion infection with an anti-PrP antibody abrogated neurotoxicity; however when the same cells were re-infected
with prions, apoptosis occurred under conditions of mild proteasome inhibition. Therefore neurotoxicity was dependent on continued PrPSc propagation. To investigate whether apoptosis was due to a non-specific cellular proteinopathy we studied N2aPD88 cells overexpressing PrPc. Under the same low level of proteasome inhibition, these cells developed large cytoplasmic PrPc aggregates, but did not undergo apoptosis. Neurotoxicity occurred only when these PrPc-overexpressing cells were infected with prions, arguing that prion infection was a pre-requisite for apoptosis under these conditions.

Under conditions of mild proteasome impairment, both prion-infected cell lines accumulated large cytoplasmic perinuclear aggresomes containing PrPSc, heat shock protein 70, ubiquitin, proteasome subunits and vimentin, characteristic of these structures. PrPSc aggresome formation was temporally associated with caspase 3 and 8 activation and subsequent apoptosis. Inhibition of aggresome formation with different microtubule-inhibitors abrogated both caspase activation and cell death, indicating that aggresome formation triggers apoptosis. Importantly PrPSc was associated with vimentin in RML prion-infected mouse brain suggesting similar PrPSc aggresome structures may have relevance in vivo. Recently granular deposits of disease-related prion protein have also been reported in the cell body of neurons suggesting intraneuronal prion aggregates may play a role in CJD pathogenesis (53).

Our data suggest a neurotoxic mechanism in prion disease where formation of intra-neuronal cytosolic PrPSc-containing aggresomes is associated with caspase 3 and 8 dependent apoptosis. They support a role for UPS dysfunction in the neuropathogenesis of prion disease, but not a role for cytosolic aggregation of wild-type PrPc. While we confirm in this study that high levels of proteasome inhibition (20) can result in accumulation of misfolded cytosolic PrPc and resultant neurotoxicity, such a degree of proteasome inhibition is unlikely to occur in vivo during prion pathogenesis (26;45;48).

It has been proposed that aggresome formation is a specific and active cellular response to cope with excessive levels of misfolded and aggregated proteins (40-42). In support of the role for aggresomes in processing intracellular misfolded protein aggregates, proteasome components and molecular chaperones are actively recruited to aggresomes. Here we have identified for the first time the formation of cytosolic PrPSc aggresomes, and shown their presence is deleterious to neuronal cells. Aggresomes contain ubiquitin, chaperones and proteasome components thereby lowering the degradative ubiquitin and proteasome-dependent proteolysis in the cell in a negative feedback loop resulting in an auto-catalytic chain leading to the induction of apoptosis (41;46) Accumulation of misfolded proteins at the centrosomes may also severely impair their function and therefore interfere with cell division (54). Recently it has also been shown that UPS impairment by protein aggregates is global, and that the capacity of the entire cellular UPS is compromised by the presence of aggregates that are restricted to either the cytoplasmic or nuclear compartments (55). In our cell system prion aggresome formation appears directly related to caspase 8 activation, which then proteolytically activates downstream caspase 3 and induces neuronal apoptotic cell death. Similar intra-neuronal caspase 8 mediated apoptosis in response to aggregated proteins has been described in Huntington’s disease (56) and Alzheimer’s disease (57). Whether PrPSc aggresome structures occur in vivo is not known, but our data showing a specific association between PrPSc and the aggresome-associated intracellular protein vimentin in RML-infected CD-1 mouse brain, suggests this may be the case.

We also propose that it may specifically be the cytosolic accumulation of PrPSc aggresomes that is particularly pro-apoptotic. In support of this, inhibition of specific lysosomal cysteine proteases in GT-1 cells inhibited the degradation of PrPSc and resulted in an accumulation of compartmentalised lysosomal-associated PrPSc (58;59), however this was not cytotoxic (58). While pathogenic prion protein mutants have been reported to form intracellular aggresomes in response to proteasome inhibition (43;60) and misfolded cytosolic PrP has been reported to form aggresome-like structures after cyclosporin A treatment (43), there have been no reports to date of PrPSc aggresome formation; nor the effects of aggresomes on cell viability in prion disease. How
PrP\textsuperscript{Sc} may enter the cytoplasm to form aggresomes has not been established, but little is known about the exact details of cellular PrP\textsuperscript{Sc} trafficking [recently reviewed in (61)]. Possible sites of entry include retrotranslocation from the ER (62), as has been described for PrP\textsuperscript{Sc} and some pathogenic prion mutants (34;63-65), during its intracellular trafficking pathway, or by intracellular trafficking from outside the cell. PrP\textsuperscript{Sc} may then accumulate in the cytoplasm when the proteasome is inhibited as may occur in ageing or during prion infection \textit{in vivo} (35;48), and generate toxic aggresome structures as demonstrated in this study. A fundamental question raised by the present study is whether PrP\textsuperscript{Sc} accumulation in aggresomes is accompanied by concomitant accumulation of a distinct neurotoxic PrP\textsuperscript{L} species. Detailed physico-chemical characterisation of PrP\textsuperscript{Sc} aggresome formation will be required to pinpoint the neurotoxic PrP entity. The neurotoxic intracellular mechanism suggested by the present study is likely to be part of a multi-factorial prion disease pathogenesis which may also involve synaptic dysfunction, and alterations to cellular membrane permeability (66). Given the critical role that has emerged for the UPS in protein misfolding disorders (28), combined with the age-dependent decrease in UPS activity, the design of drugs that improve UPS function in neurons may help provide effective intervention to slow or prevent these diseases in which toxic proteins are misfolded.
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The abbreviations used are: BSE, bovine spongiform encephalopathy; CJD, Creutzfeld-Jakob disease; CNS, central nervous system; COL, colchicine; DAPI, 4',6-Diamidino-2-phenylindole; EPOX, epoxomicin; ERAD, endoplasmic reticulum associated degradation; Hsc70, heat shock chaperone 70; LAC, lactacystin; LAMP-1, lysosome associated membrane protein-1; LDH, lactate dehydrogenase; NOC, nocodazole; NS, not significant; PI, propidium iodide; PK, proteinase K; PRNP, prion protein gene; PrP, prion protein; PrP L, PrP Lethal; SCA, scrapie cell assay; UPS, ubiquitin-proteasome system; z-DEVD-fmk, Z-Asp-Glu-Val-Asp-fluoromethylketone; z-IETD-fmk, Z-Ile-Glu-Thr-Asp-fluoromethylketone

**FIGURE LEGENDS**

**Fig 1.** Mild proteasome inhibition sensitises prion-infected GT-1 and N2aPD88 cells to apoptosis and curing the cells of prions abrogates this effect.  
*A) Cell lysates from wild type mouse hypothalamic neuronal GT-1 cells (lanes 1 and 2), RML scrapie-infected GT-1 cells (lanes 3 and 4), wild type mouse neuroblastoma N2aPD88 cells (lanes 5 and 6) and RML scrapie-infected N2aPD88 cells (lanes 7 and 8) were incubated in the absence (-) or presence (+) of proteinase K; and immunoblotted using anti-PrP antibody ICSM35 to demonstrate PrPSc infection in these cells.  
B,i) Dose-response curves for proteasome inhibition by lactacystin (LAC) in GT-1 cells, ScGT-1 cells or ScGT-1-18 cells. Cell death was determined after 24h by measuring LDH release (n=12).  
B,ii) The percentage of cell death after mild LAC treatment (1µM) is significantly different (p<0.0001; n=12) in ScGT-1 cells compared to uninfected GT-1 cells or antibody-cured ScGT-1-18 cells.  
C,i) Dose-response curves for proteasome inhibition by lactacystin (LAC) in N2aPD88 cells, ScN2aPD88 cells or antibody-cured ScN2aPD88-18 cells. Cell death was determined after 24h by measuring LDH release (n=12).  
C,ii) The percentage of cell death after mild LAC treatment (1µM) is significantly different (p<0.0001; n=12) in ScN2aPD88 cells compared to uninfected N2aPD88 cells or antibody-cured ScN2aPD88-18 cells. Analysis by the SCA showed that ~52% and ~42% of the ScGT-1 and ScN2aPD88 cell populations were scrapie-infected which is consistent with the percentages of cell death seen in Fig 1B) ii and 1C) ii. Prefix Sc denotes scrapie infection; suffix -18 indicates the clonal line that has been cured of prion infection with anti-PrP antibody ICSM18.

**Fig 2.** Cytosolic accumulation of detergent-insoluble PrP<sup>C</sup> aggregates after mild proteasome inhibition is not neurotoxic.  
*A) PrP expression in N2aPD88 cells before (lane 1) or after transfection with wild-type mouse PrP<sup>C</sup> to produce N2a<sup>moPrPC</sup> cells (lane 2).  
B) Intracellular localisation of PrP<sup>C</sup> in untreated N2a<sup>moPrPC</sup> cells. Anti-PrP monoclonal antibody ICSM18 (red) and markers for the lysosome (LAMP-1, green), ER (anti-α-PDI, green) and nucleus (DAPI, blue). PrP is located in lysosomes (top panel), in the ER and Golgi (middle panel) and on the cell surface (bottom panel).  
C) PrP<sup>C</sup> aggregates in N2a<sup>moPrPC</sup> cells co-localise with cytosolic Hsc70 and are predominantly located in the cytoplasm after lactacystin (LAC) treatment (1µM; 24h). Anti-PrP monoclonal antibody ICSM18 (red), DAPI nuclear staining (blue), cytosolic chaperone Hsc70 (green).  
*D, i) Cell death in both N2aPD88 and N2a<sup>moPrPC</sup> cells after LAC treatment (1µM;
24h) is not significantly different, but levels of death in both cell types are significantly different from LAC treated ScN2aPD88 cells (p<0.0001). D, ii) After treatment with 10µM LAC for 24 hours, N2aamoPrPC cells showed a significantly lower percentage of cell death compared to wild type N2aPD88 cells (p<0.001; n=12) suggesting that overexpressed cytosolic PrP C may have a neuroprotective effect.

E) Subcellular fractionation of PrP (S, soluble supernatant; P, insoluble pellet). In the absence of LAC treatment, PrP in N2aamoPrPC cells is detergent-soluble (lane 1); after low dose (1µM) LAC treatment a proportion of PrP becomes insoluble and is isolated in the pellet fraction (lane 4); after high dose (10µM) LAC treatment all PrP becomes detergent-insoluble and aggregated and is found only in the pellet fraction (lane 6). F) Inflicting these N2aamoPrPC cells with RML scrapie prions produces a significant (p<0.0001; n=6) increase in cell death after 1µM LAC treatment indicating that the presence of PrPSc sensitises these cells to apoptosis. Scale bars = 20µm.

Fig 3. Prion infection induces caspase 3 and 8 dependent apoptosis which is abrogated by specific caspase inhibitors. A) ScGT-1 cells undergo apoptosis after 1µM LAC treatment. Nuclear fragments (DAPI, blue) are shown by arrows. The percentage of apoptotic cells (annexin V-positive, green) and necrotic cells (propidium iodide-positive, red; annexin V-positive, green) were determined at 0, 12 and 24 hours (scale bar top panel = 20µm ; scale bar bottom panel = 126µm). B) The percentage of apoptotic cells increases by ~30% after LAC treatment as quantified by FACS analysis (p<0.03; n=4). C) During the course of apoptosis the activity of caspase 3 and 8 activation increased in a time-dependent manner. From 1 hour there was a highly significant increase in caspase 3 activity (p<0.001) in the ScGT-1 cells as compared to uninfected GT-1 cells. D) There was also a highly significant increase in caspase 8 activation over time (p<0.001). E) Cell death was completely abrogated in ScGT-1 cells (p<0.0001; n=12) after treatment with highly specific caspase 3 or 8 inhibitors.

Fig 4. PrPSc forms large cytoplasmic perinuclear aggresomes. A) To analyse the subcellular localisation of PrPSc, cells were treated with 98% formic acid for 2 and 5 minutes and then immunostained with anti-PrP antibody (ICSM18) to reveal PrPSc. In GT-1 cells, formic acid treatment removed all detectable PrP C after 2 minutes (top panel); in ScGT-1 cells, PrPSc is present on the cell surface and intracellularly (bottom panel). Nuclear staining with DAPI (blue). B-E) After treatment with 1µM LAC, PrPSc (green) accumulates in ScGT-1 cells in large perinuclear aggresomes and co-localises with ubiquitin (C), Hsc70 (D), 20S proteasome (E) and surrounded by a vimentin (B) cage. Scale bars = 20µm.

Fig 5. Aggresomes are composed of PrPSc and are neurotoxic to the cell. A) For co-immunoprecipitation experiments ScGT-1 cells were harvested 24 hours after treatment with 1µM lactacystin. PrPSc was co-precipitated from lactacystin-treated ScGT-1 cells by anti-vimentin antibody-coated beads (lanes 1 and 2) but not by Brc126 isotype control antibody-coated beads (lanes 3 and 4) or beads alone (lanes 5 and 6) demonstrating that aggresomes are composed of PrPSc. B) Cell death in lactacystin treated (1µM; 24h) ScGT-1 cells was abrogated in the presence of 5µg/ml colchicine (B,i) (p<0.0001; n=12), and reduced by ~20 % in the presence of 0.5µM nocodazole (B,ii) (p<0.0001; n=12), and was unaffected by 50ng/ml cytochalasin D (B,iii) (p=NS) indicating that clearance of aggresomes by colchicine and nocodazole ameliorates cell death. C) ScGT-1 cells were incubated for 24 hours in the presence of 1µM lactacystin (LAC) alone (panel 1) or in combination with colchicine (5µg/ml)(panel 2) or nocodazole (0.5µM)(panel 3). Treatment with colchicine or nocodazole prevented aggresome formation (panel 2 and 3). Treatment with cytochalasin D did not clear aggresomes (panel 4) confirming our data that clearance of PrPSc aggresomes selectively abrogates cell death. PrPSc-containing
aggresomes were detected using anti-PrP antibody ICSM18 (green) and anti-vimentin antibodies (red). Nuclear staining with DAPI (blue). Scale bars = 20µm

**Fig 6. PrP<sub>Sc</sub> aggresomes are temporally linked to caspase 3 and 8 activation.** *A*) Treatment with nocodazole (0.5µM) and colchicine (5µg/ml) which prevent aggresome formation abrogated caspase 3 and 8 activation in lactacystin (1µM) treated prion-infected cells. *B-F*) After formic acid treatment cells were immunostained with antibodies directed against PrP (ICSM18, red in panel 1 and 2; and green in panel 3), vimentin (red), activated caspase 8 (green) and activated caspase 3 (green) to demonstrate the time course relationship between aggresome formation and caspase 8 and 3 activation. Nuclear DAPI staining is blue. *B*) At time zero, there are no PrP<sub>Sc</sub> aggresomes (panel 3) and no evidence of caspase 8 (panel 1) or caspase 3 activation (panel 2); *C*) Early PrP<sub>Sc</sub> containing aggresomes are formed as small perinuclear structures after 6 hours (panel 3) with early caspase 8 and 3 activation (panels 1 and 2). *D*) After 12 hours the size of the PrP<sub>Sc</sub> aggresome increases (panel 3) with marked caspase 8 and 3 immunostaining in the cells containing PrP<sub>Sc</sub> aggresomes (panels 1 and 2). *E-F*) By 18 and 24 hours there is widespread intra-cellular caspase 8 and 3 immunostaining and large perinuclear PrP<sub>Sc</sub> aggresomes seen (panels 1-3). Scale bars = 40µm

**Fig 7. Association of PrP<sub>Sc</sub> and vimentin in scrapie-infected mouse brain.** *A,B*) Immunoblots developed with anti-PrP monoclonal antibody ICSM35. *A*) Co-immunoprecipitation experiments were performed using uninfected CD-1 mouse brain homogenate and RML-infected CD-1 mouse brain homogenate. Lanes 1-4 show PrP immunoreactivity from RML-infected CD-1 mouse brain homogenate after co-precipitation with vimentin antibody-coated magnetic beads (lanes 2 and 3 - show total PrP (lane 2) and PrP<sub>Sc</sub> (lane 3) affinity purified using vimentin coated beads); or isotype-control antibody-coated magnetic beads (lane 1 - negative) or magnetic beads alone (lane 4 - negative). Lanes 5 and 6 show that no PrP immunoreactivity is recovered from uninfected CD-1 mouse brain homogenate by co-precipitation with vimentin antibody-coated magnetic beads. *B*) Quantification of PrP<sub>Sc</sub> isolated from RML-infected CD-1 mouse brain. Lanes 1-5 show the amount of PrP<sub>Sc</sub> present in 12.5, 6.25, 3.13, 1.56, and 0.78µl of 10% RML-infected brain homogenate respectively. Lane 7 shows the amount of PrP<sub>Sc</sub> recovered by vimentin-antibody coated beads from 25µl of 10% RML-infected brain homogenate. Densitometry indicates recovery of ~4% of total PrP<sub>Sc</sub> representing intracellular PrP<sub>Sc</sub> bound to intracellular vimentin.
Figure 1

A

Cell death (%) vs. Lactacystin (µM)

B

Cell death (%) vs. Lactacystin (µM)

C

Cell death (%) vs. Lactacystin (µM)

(i) (ii) (i) (ii) (i) (ii)

GT-1 ScGT-1 ScGT-1-18 GT-1 ScGT-1 ScGT-1-18 GT-1 ScGT-1 ScGT-1-18

Vehicle only LAC treatment Vehicle only LAC treatment Vehicle only LAC treatment
Figure 2

(A) Western blot analysis showing the expression levels of β-actin and PrP in different lanes.

(B) Immunofluorescence images showing the localization of PrP, LAMP-1, α-PDI, and DAPI in N2aPD88 and N2a moPrPC cells with and without RML treatment.

(C) Immunofluorescence images showing the localization of PrP and Hsc70 in N2aPD88 and N2a moPrPC cells with and without RML treatment.

(D) Graphs showing the cell death percentages in different conditions: (i) Vehicle only vs. LAC treatment and (ii) N2aPD88 vs. N2a moPrPC with and without RML treatment.

(E) Western blot analysis showing the expression levels of PrP in different lanes treated with LAC at various concentrations.

(F) Graphs showing the cell death percentages in different conditions: N2aPD88, N2a moPrPC, and ScN2aPD88 with and without RML treatment.
Figure 3

A

Time (hours)
0 12 24

Vehicle only
1 µM LAC
1 µM LAC + caspase-3 inhibitor
1 µM LAC + caspase-8 inhibitor

B

% Apoptosis (FACS)

GT-1 ScGT-1

p<0.03

C

D

E

Caspase 3 activity (absorbance 415 nm)

GT-1 ScGT-1

Time (hours) 0 1 3 6 12 18 24

Caspase 8 activity (absorbance 415 nm)

GT-1 ScGT-1

Time (hours) 0 1 3 6 12 18 24

Cell death (%)

Vehicle only
1 µM LAC
1 µM LAC + caspase-3 inhibitor
1 µM LAC + caspase-8 inhibitor
Figure 4

A

GT-1

0

2

5

ScGT-1

Time (mins)

PrPSc Vimentin Merge

PrPSc Ubiquitin Merge

PrPSc HSc70 Merge

PrPSc 20s proteasome Merge

B

PrPSc

Vimentin

Merge

C

PrPSc

Ubiquitin

Merge

D

PrPSc

HSc70

Merge

E

PrPSc

20s proteasome

Merge
Figure 5

A

Panel 1

Panel 2

Panel 3

Panel 4

B

Cell death (%)

Vehicle only

1 µM LAC

1 µM LAC + 50 ng/ml Cytochalasin D

Vehicle only

1 µM LAC

1 µM LAC + 5 µg/ml Colchicine

Vehicle only

1 µM LAC

1 µM LAC + 0.5 µM Nocodazole

NS

Vehicle only

1 µM LAC

1 µM LAC + 50 ng/ml Cytochalasin D

B

(i)

(ii)

(iii)

C

PrPSc

Vimentin

Merge

Control

Colchicine

Nocodazole

Cytochalasin D

Panel 1

Panel 2

Panel 3

Panel 4
Figure 6A

Caspase activity (absorbance 415 nm)

|               | Vehicle only | LAC | LAC + COL | LAC + NOC |
|---------------|--------------|-----|-----------|-----------|
| **Caspase-3** |              |     |           |           |
| **Caspase-8** |              |     |           |           |

**Note:** Statistical significance indicated by **p < 0.01.
Figure 7

A

Lanes

1 2 3 4 5 6

kDa

36
30
16

PK

- - + - - +

B

kDa

36
30
16

PK

+ + + + + +

1 2 3 4 5 6 7
**Fig S1.** Proteasome inhibition with lactacystin sensitises prion-infected but not mock-infected GT-1 cells to apoptosis and curing the cells of prions abrogates this effect. Cell death was analysed by measuring LDH release in GT-1 cells (n=12) after 24 hours treatment with lactacystin. Mock infected GT-1 cells resulted in similar levels of cell death to wild-type GT-1 cells. There was a highly significant difference (p<0.0001) between the percentage of cell death in RML infected GT-1 cells (48%) when compared to wild-type GT-1 (17%) or mock infected GT-1 cells (16%). Curing the cells of prion infection with anti-PrP antibody abrogated the sensitivity to proteasome inhibition and resulted in the same degree of cell death as wild-type uninfected GT-1 cells.

**Fig S2.** Proteasome inhibition with epoxomicin sensitises prion-infected GT-1 cells to apoptosis and curing the cells of prions abrogates this effect. Dose-response curves for proteasome inhibition were established in GT-1 (-▲-) and ScGT-1 cells (-●-) using a range of doses of epoxomicin (0-10µM) and cell death was analysed after 24 hours treatment by measuring LDH release (Graph i; n=12). There was a highly significant difference (p<0.0001) between the percentage of cell death after epoxomicin treatment in the ScGT-1 cells (53%) as compared to uninfected GT-1 cells (31%)(Graph ii). Curing cells of prion infection with anti-PrP antibody abrogated the sensitivity to proteasome inhibition and resulted in the same degree of cell death as wild-type uninfected GT-1 (Graphs i and ii; *** p<0.0001)(antibody cured cells -□-).

**Fig S3.** PrPSc forms large cytoplasmic perinuclear aggresomes in ScN2aPD88 cells. ScN2aPD88 cells were treated with 1µM lactacystin for 24 hours and then treated with 98% formic acid for 5 minutes to remove PrP immunoreactivity. Cells were then immunostained with anti-PrP antibody (ICSM18) to reveal PrPSc. PrPSc (red) accumulates in ScN2aPD88 cells in large perinuclear aggresomes and is surrounded by a vimentin cage (green). Scale bars = 20µM.

**Fig S4.** Dose response curves for colchicine, nocodazole and cytochalasin D. Dose-response curves for the microtubule disrupting agents colchicine and nocodazole, and the actin microfilament-disrupting agent cytochalasin D were established in GT-1 (-▲-) and N2aPD88 cells (-■-) to optimise treatment of these cells with these agents. Cell death was determined by measuring LDH release after 24 hours (See Results, main text for further details) (n=12). Arrows demonstrate the dose chosen for all subsequent experiments.
Figure S1

Cell death (%) vs. Vehicle only and Lactacystin treatment for different conditions: wild-type GT-1, mock infected GT-1, RML infected GT-1, and ScGT-1-18.
Figure S2

(i) Cell death (%) vs. Epoxomicin (µM) for ScGT-1, ScGT-1-18, and GT-1.

(ii) Bar graph showing vehicle only and EPOX treatment for GT-1, ScGT-1, and ScGT-1-18.
Figure S3
Figure S4

(i) Cell death (%) vs. Colchicine (µg/ml)

(ii) Cell death (%) vs. Nocodazole (µM)

(iii) Cell death (%) vs. Cytochalasin D (µg/ml)
**Table S1  Antibodies used for immunofluorescence**

| Antibody                                      | Concentration |
|-----------------------------------------------|---------------|
| 20S proteasome, Affiniti Research, Exeter, UK | 1:100         |
| Ubiquitin (FK2), Affiniti Research, Exeter, UK| 1:100         |
| Activated caspase-8, Santa Cruz, Wiltshire, UK| 1:250         |
| Activated caspase-3, Affiniti Research, Exeter, UK| 1:250   |
| Hsc70, Santa Cruz, Wiltshire, UK              | 1:100         |
| LAMP-1, Santa Cruz, Wiltshire, UK             | 1:1000        |
| Anti-vimentin (Clone V9), Abcam, Cambridge, UK| 1:100         |
| Anti-PDI, Abcam, Cambridge, UK                | 1:100         |

**Table S1  Concentrations and source of antibodies used for immunofluorescence**
Disease-related prion protein forms aggresomes in neuronal cells leading to caspase-activation and apoptosis

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