

# Caspase-12 and endoplasmic reticulum stress mediate neurotoxicity of pathological prion protein

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**Prion diseases are characterized by accumulation of misfolded prion protein (PrP<sup>Sc</sup>), and neuronal death by apoptosis. Here we show that nanomolar concentrations of purified PrP<sup>Sc</sup> from mouse scrapie brain induce apoptosis of N2A neuroblastoma cells. PrP<sup>Sc</sup> toxicity was associated with an increase of intracellular calcium released from endoplasmic reticulum (ER) and up-regulation of several ER chaperones. Caspase-12 activation was detected in cells treated with PrP<sup>Sc</sup>, and cellular death was inhibited by overexpression of a catalytic mutant of caspase-12 or an ER-targeted Bcl-2 chimeric protein. Scrapie-infected N2A cells were more susceptible to ER-stress and to PrP<sup>Sc</sup> toxicity than non-infected cells. In scrapie-infected mice a correlation between caspase-12 activation and neuronal loss was observed in histological and biochemical analyses of different brain areas. The extent of prion replication was closely correlated with the up-regulation of ER-stress chaperone proteins. Similar results were observed in humans affected with sporadic and variant Creutzfeldt–Jakob disease, implicating for the first time the caspase-12 dependent pathway in a neurodegenerative disease *in vivo*, and thus offering novel potential targets for the treatment of prion disorders.**

**Keywords:** apoptosis/caspase-12/Creutzfeldt–Jakob disease/ER-stress/PrP<sup>Sc</sup>/scrapie

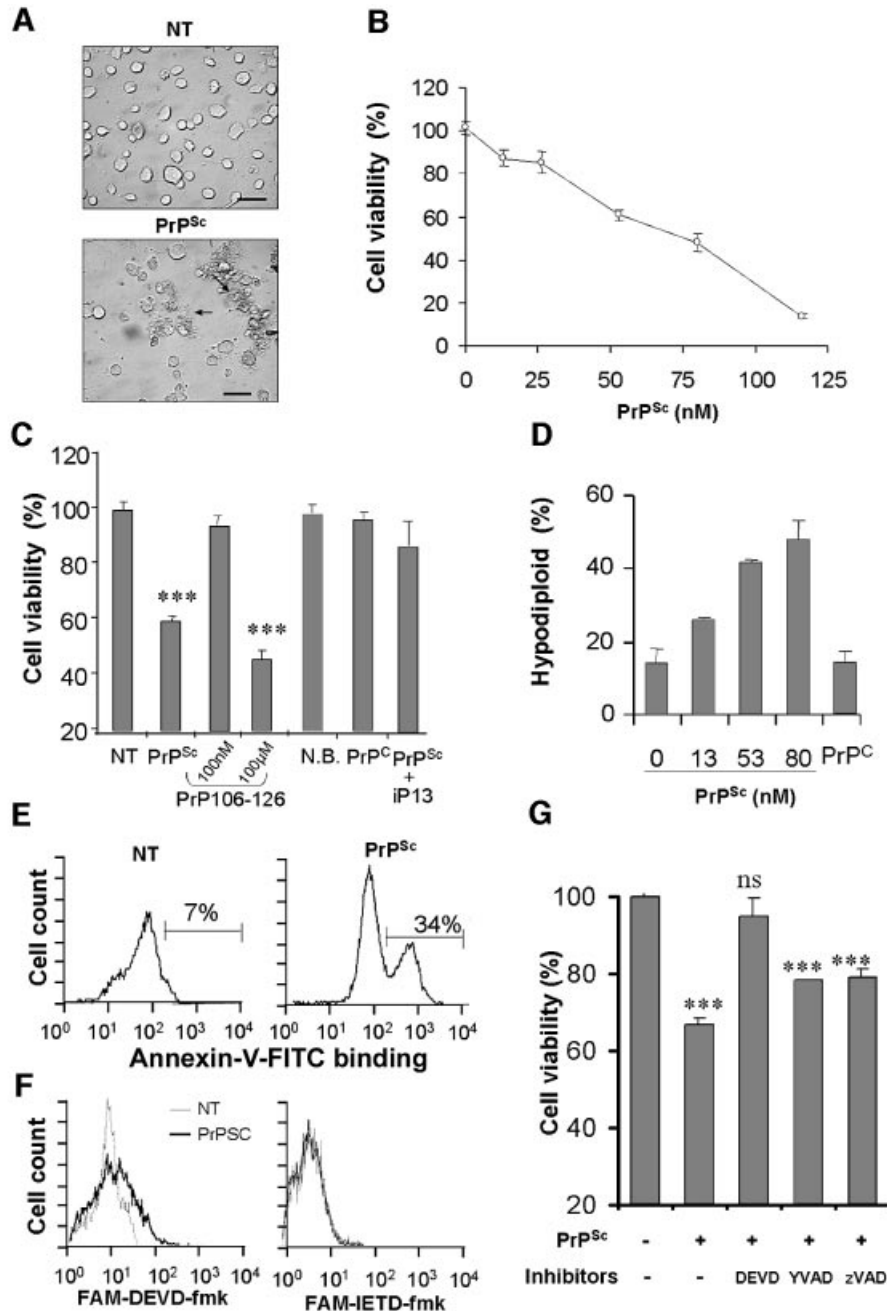
## Introduction

Transmissible spongiform encephalopathies (TSEs), also known as prion disorders, are a group of clinically diverse, but mechanistically similar neurological diseases, such as Creutzfeldt–Jakob disease (CJD) in humans and bovine spongiform encephalopathy (BSE), scrapie and chronic wasting disease in animals. The hallmark pathological features of TSEs are the spongiform degeneration of the brain, accompanied by extensive neuronal loss, astrogliosis, and cerebral accumulation of a misfolded and protease-resistant form of the prion protein (Prusiner, 1998). No amino acid sequence or

posttranslational differences have been detected between the normal host cell surface protein, PrP<sup>C</sup>, and its pathological form, PrP<sup>Sc</sup>. The conversion of PrP<sup>C</sup> into PrP<sup>Sc</sup> involves a conformational change whereby the  $\alpha$ -helical content diminishes and the amount of  $\beta$ -sheet increases (Prusiner, 1998).

Apoptosis is a programmed form of cell death, which plays a central role during development and homeostasis of multicellular organisms and has been implicated in a number of pathological conditions (Vaux and Korsmeyer, 1999; Reed, 2002). The central executioner molecules of apoptosis are a large family of cysteine proteases known as caspases (Budihardjo *et al.*, 1999; Takahashi, 1999). Activation of caspase-dependent apoptosis may be initiated by cell surface receptors or by mitochondrial stress (Budihardjo *et al.*, 1999). Recently, another apoptotic-regulatory pathway has been described, in which the induction of endoplasmic reticulum (ER) stress due to alteration of calcium homeostasis or accumulation of misfolded proteins, triggers the activation of an ER-resident caspase, termed caspase-12 (Nakagawa *et al.*, 2000).

Apoptosis has been described in the brains of patients affected by CJD, Fatal Familial Insomnia, scrapie-infected hamsters, mice and sheep (review in Hetz and Soto, 2003). Although a considerable effort has been made to understand the molecular basis of prion conversion, little is known about the mechanism by which this process is associated with the disease. Attempts to understand the molecular basis of neuronal dysfunction in prion diseases have led to the search for *in vitro* models to analyze the role of PrP<sup>Sc</sup> in cellular death. The prion protein fragment spanning the sequence 106–126 (PrP106–126), corresponding to a putative transmembrane region of PrP<sup>C</sup>, has been extensively used to induce cell death in neuronal cultures (Forloni *et al.*, 1993; O'Donovan *et al.*, 2001). The major drawback of using PrP106–126 as a model is that although this sequence is present in several longer peptides isolated from cerebral amyloid plaques of patients suffering from GSS (Ghetti *et al.*, 1996), the PrP106–126 peptide as such has never been found *in vivo*. Therefore, the relevance of the findings obtained with this peptide remains to be determined. The major aim of this study was to characterize the signaling pathways involved in neuronal death induced by highly purified PrP<sup>Sc</sup> from mouse brains infected with experimental scrapie. It has been reported earlier that purified PrP<sup>Sc</sup> leads to cell death (Post *et al.*, 2000). However, the mechanism of cell death was not studied. Our findings show that PrP<sup>Sc</sup>-induced apoptosis *in vitro* is associated with the induction of ER-stress and activation of caspase-12. Histological and biochemical analysis of brains from scrapie-sick mice and from humans affected by sporadic



**Fig. 1.** Purified PrP<sup>Sc</sup> from 139A-scrapie infected brains induces caspase-3 dependent apoptosis of N2A cells. (A) Morphological changes observed in N2A neuroblastoma cells treated with 50 nM PrP<sup>Sc</sup> for 48 h. (B) N2A cells were treated with different concentrations of PrP<sup>Sc</sup> and cell viability was analyzed after 48 h by MTS assay. (C) As controls, cells were incubated with: 165 nM of recombinant mouse PrP<sup>C</sup>; 50 nM PrP<sup>Sc</sup> pre-treated with 300 nM of  $\beta$ -sheet breaker peptide iPrP13 to decrease PrP<sup>Sc</sup>- $\beta$  sheet content; 100 nM or 100  $\mu$ M of aggregated PrP106-126 peptide for 7 days; or 0.5% (v/v) preparation of normal brain homogenate following the same procedure as to purify PrP<sup>Sc</sup> (N.B.). (D) Cells were incubated for 48 h with different concentrations of PrP<sup>Sc</sup>, 100 nM recombinant PrP<sup>C</sup> or left untreated. Subsequently, cells were stained with PI and hypodiploid cell population was quantified by FACS analysis. (E) Cells were treated with 66 nM PrP<sup>Sc</sup> for 6 h and then phosphatidylserine exposure on the cell surface was detected using annexin V-FITC. (F) Caspase-3 and caspase-8 activity *in situ* was determined by flow cytometry using the cell permeable substrates FAM-DEVD-fmk and FAM-IETD-fmk after treatment with PrP<sup>Sc</sup> for 20 h. Non-treated cells (gray line), or cells treated with 50 nM PrP<sup>Sc</sup> (black line). (G) Cells were incubated for 60 min with or without 100  $\mu$ M Ac-DEVD-fmk (DEVD), 100  $\mu$ M Ac-YVAD-fmk (YVAD) or 10  $\mu$ M zVAD-fmk (zVAD). Then, PrP<sup>Sc</sup> was added to a final concentration of 50 nM, and after 48 h cell survival was determined by the MTS assay. Data shown in (C, D and G) correspond to the means and standard deviations at two independent experiments performed in triplicate. Statistical analysis was performed by parametric *t*-test comparing each value with the untreated control.

CJD (sCJD) and variant CJD (vCJD) showed the presence of activated caspase-12 and ER-stress inducible chaperones in brain areas exhibiting extensive neuronal death.

These findings provide a novel target for TSE therapy and suggest a general mechanism of cell death in chronic neurodegenerative diseases.

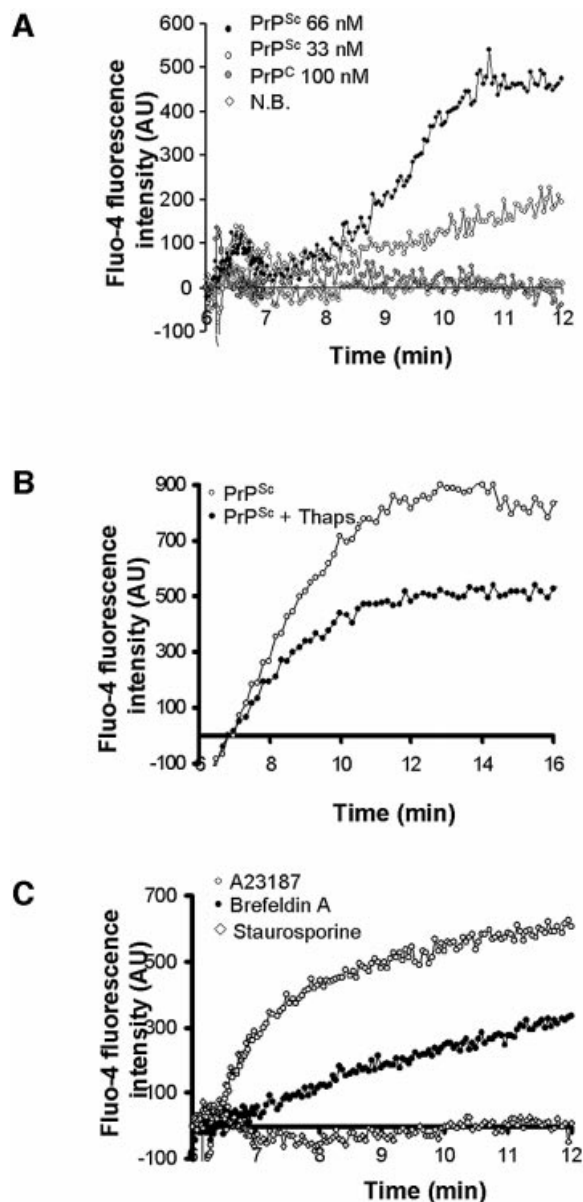
## Results

### Purified mouse PrP<sup>Sc</sup> induces caspase-3-dependent apoptosis in N2A cells

N2A cells were chosen to characterize neurotoxicity of PrP<sup>Sc</sup> purified from 139A scrapie-infected brains. N2A neuroblastoma cells were grown for 24 h in normal culture medium with low serum, and then different concentrations of PrP<sup>Sc</sup> were added to the medium. After 48 h of incubation, the cells treated with PrP<sup>Sc</sup> showed abnormal morphological changes, including cell shrinkage and aggregation of the cell bodies, followed by detachment of cells from the culture plate (Figure 1A). Quantification of cellular viability after PrP<sup>Sc</sup> treatment revealed that cellular death occurred in a concentration-dependent manner in the nanomolar range (Figure 1B). Several controls were performed. First, to rule out the possibility that toxicity might be caused by contaminants carried over from the purification procedure, cells were treated with equivalent volumes of a sample prepared under the same conditions, but starting with normal mouse brains. The results showed no effect of this preparation (Figure 1C). In addition, experiments in which cells were treated with recombinant mouse PrP<sup>C</sup> (165 nM) showed no effect on neuronal survival (Figure 1C), suggesting that PrP<sup>Sc</sup> toxicity is associated with its pathological conformation. In agreement with this hypothesis, pre-incubation of PrP<sup>Sc</sup> with a small peptide that disrupts the  $\beta$ -sheet structure (peptide iPrP13), leads to a significant decrease in the toxicity of the protein (Figure 1C). We have previously shown that iPrP13 decreases the  $\beta$ -sheet structure of PrP<sup>Sc</sup>, converting it into a protease-sensitive PrP<sup>C</sup>-like molecule (Soto *et al.*, 2000). Finally, treatment of the cells with 100 nM PrP106–126 did not produce any cell death (Figure 1C). Cytotoxicity with this peptide was observed only when cells were treated with 1000-fold higher concentration (100  $\mu$ M), compared with pure PrP<sup>Sc</sup>, and only after several days of incubation in culture (Figure 1C). These data suggest that PrP<sup>Sc</sup> neurotoxicity cannot simply be explained by a non-specific effect of an aggregated hydrophobic protein preparation.

Quantification of DNA content after PI-staining revealed that hypodiploid cells could be detected after PrP<sup>Sc</sup> treatment in a dose-dependent manner (Figure 1D). As controls, we used cells treated with recombinant PrP<sup>C</sup>, which showed no indication of apoptotic induction (Figure 1D). PrP<sup>Sc</sup>-induced apoptosis was additionally confirmed by phosphatidylserine externalization to the cell surface, which is commonly observed at early stages of apoptosis. Appearance of phosphatidylserine at the cell surface was observed in 34% of the cells 6 h after treatment with 66 nM PrP<sup>Sc</sup> (Figure 1E). Overall, these data indicate that nanomolar concentrations of purified PrP<sup>Sc</sup> induce cellular death by apoptosis.

To characterize the signalling pathways involved in PrP<sup>Sc</sup>-induced apoptosis, we studied the activation of different caspases using cell permeable fluorogenic substrates (FAM-YEVD-fmk, FAM-DEVD-fmk, and FAM-IETD-fmk) to detect caspase-1, caspase-3 and caspase-8 activity, respectively. After PrP<sup>Sc</sup> treatment, caspase-3 activation was observed progressively over time, reaching a maximum level at ~20 h (Figure 1F). However, no significant activation of caspase-8 or caspase-1 was



**Fig. 2.** PrP<sup>Sc</sup> induces a release of Ca<sup>2+</sup> from the endoplasmic reticulum. (A) N2A cells were loaded with the Ca<sup>2+</sup> dye Fluo-4 (10  $\mu$ g/ml final concentration). Changes in fluorescence intensity were measured by using the FLIPR1 setup after addition of 33 or 66 nM PrP<sup>Sc</sup>, 100 nM recombinant PrP<sup>C</sup>, or 0.5% (v/v) of a preparation from normal mouse brains (N.B.). Relative fluorescence intensity was plotted against time, and represents the average of three different experiments. (B) Cells were pre-treated with thapsigargin (5  $\mu$ M) for 15 min and calcium signals were analyzed over time after addition of 80 nM PrP<sup>Sc</sup>. (C) Control experiments showing that ER stress induced by brefeldin A (40  $\mu$ M) or A23187 (400 nM), but not by staurosporine (25 nM) promoted calcium release.

observed after 20 h of incubation with PrP<sup>Sc</sup> (Figure 1F, data not shown). Treatment of N2A cells with 100  $\mu$ M Ac-DEVD-fmk, a preferential caspase-3 inhibitor, completely abolished cellular death induced by 50 nM PrP<sup>Sc</sup> (Figure 1G). Treatment with 100  $\mu$ M Ac-YVAD-fmk, an inhibitor of interleukin-1 converting proteases, like caspase-1, or low doses of zVAD-fmk, a general caspase inhibitor that blocks mainly caspase-8 activation at this dose (Hetz *et al.*, 2002a), had no significant effect on PrP<sup>Sc</sup>-induced neuronal apoptosis.

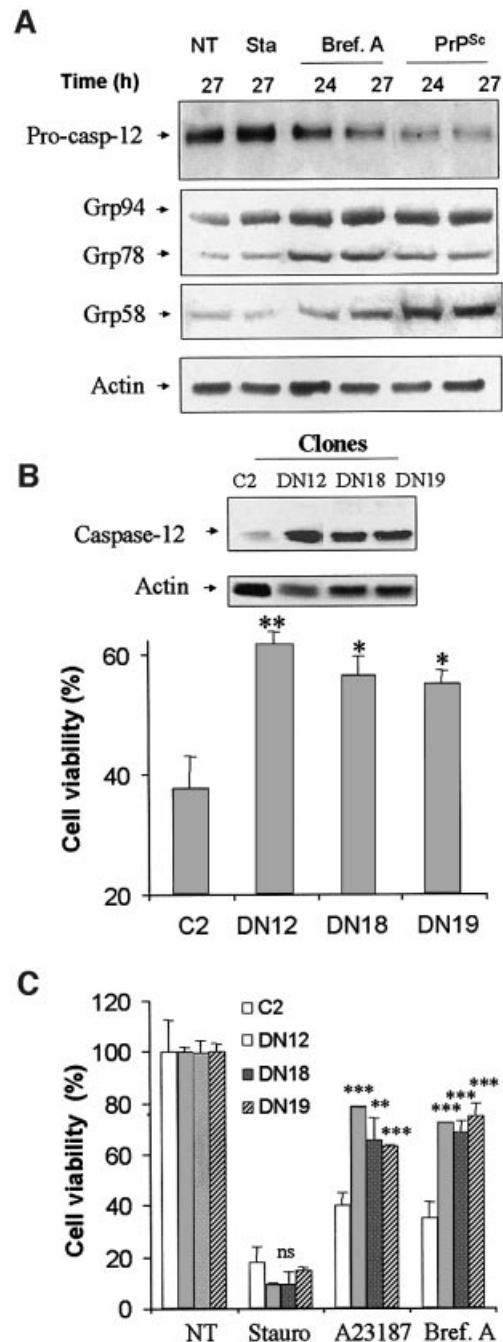
### PrP<sup>Sc</sup>-induced apoptosis is associated with calcium release from the ER

To investigate up-stream events involved in caspase-3 activation, we studied the effect of PrP<sup>Sc</sup> treatment on intracellular Ca<sup>2+</sup> levels. N2A cells were loaded with the Ca<sup>2+</sup>-responsive fluorophore Fluo-4, to monitor changes in intracellular calcium concentrations over time. After treatment with PrP<sup>Sc</sup>, a fast and sustained increase in Ca<sup>2+</sup> levels (within minutes) was observed in a dose-dependent manner (Figure 2A). As controls, cells were treated with 100 nM recombinant PrP<sup>C</sup> or equivalent volumes of a sample prepared from non-infected brains. No effects on intracellular calcium levels were observed under these conditions (Figure 2A). These experiments were performed in the absence of extracellular calcium, indicating that Ca<sup>2+</sup> increase stimulated by PrP<sup>Sc</sup> is released from intracellular stores, such as ER or mitochondria. In order to define the source of calcium, N2A cells were pre-treated with thapsigargin (5  $\mu$ M) to decrease the concentration of calcium in the ER before PrP<sup>Sc</sup> addition. Decreased calcium signals were observed in cells pre-treated with thapsigargin compared with the controls, indicating that PrP<sup>Sc</sup> induces an increase of intracellular calcium released mainly from the ER (Figure 2B). As positive controls, cells were treated with the ER-stress inducers brefeldin A (an inhibitor of ER-Golgi transport) and the ionophore A23187 (Figure 2C) at concentrations that induce apoptosis in N2A cells. No changes in intracellular Ca<sup>2+</sup> levels were observed after treatment of N2A cells with staurosporine (500 nM), which is known to induce apoptosis by the calcium-independent mitochondrial pathway (Figure 2C).

### PrP<sup>Sc</sup> induces ER-stress and activation of caspase-12

The main caspase associated with the ER apoptosis pathway is caspase-12, an ER-resident caspase, which upon activation by ER-stress and calcium release can

promote caspase-3 mediated apoptosis (Mehmet, 2000). Treatment of N2A cells with PrP<sup>Sc</sup> leads to activation of this protease, which is evidenced by the decrease of the pro-caspase-12 signal (Figure 3A, top panel). Similar results were observed when N2A cells were treated with lethal concentrations of brefeldin A, but not with staurosporine (Figure 3A, top panel). In addition, PrP<sup>Sc</sup> treatment was associated with a significant increase in the expression levels of the ER chaperone Grp58. A lower but still statistically significant induction of Grp94 and Grp78 was observed (Figure 3A, see Supplementary data for statistical analysis, available at *The EMBO Journal*



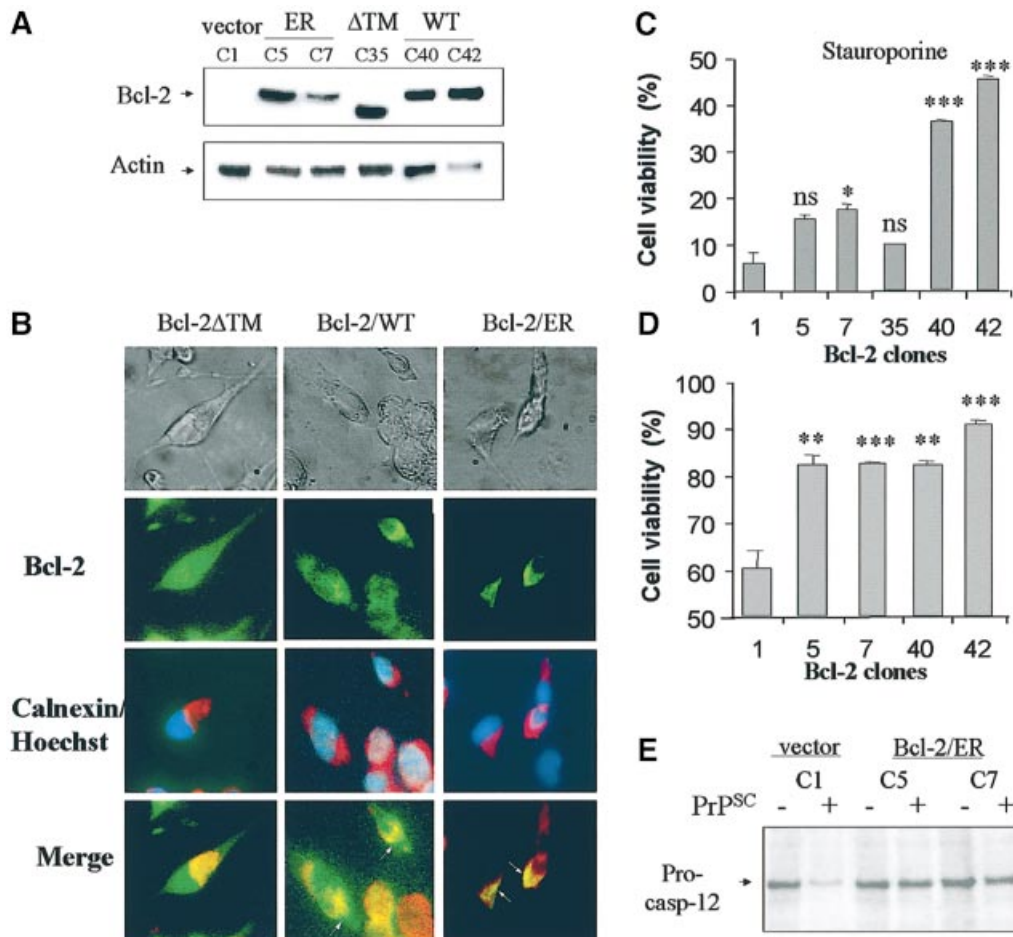
**Fig. 3.** Caspase-12 activation mediates PrP<sup>Sc</sup> neurotoxicity in N2A cells. (A) Cells were treated with PrP<sup>Sc</sup> (50 nM), staurosporine (25 nM) or brefeldin A (12  $\mu$ M), and the levels of pro-caspase-12, Grp58, Grp78, and Grp94 were determined by western blotting. As controls the levels of actin are shown. To determine the significance of the differences in expression level, data from at least three different experiments were analyzed by densitometry (see Supplementary data). Differences on the expression of pro-caspase-12 (at 24 and 27 h), Grp94 (at 24 and 27 h), Grp78 (at 27 h) and Grp58 (at 27 h) after treatment with PrP<sup>Sc</sup> were statistically significant ( $P < 0.005$ ). (B) Expression levels of caspase-12 dominant-negative in three different clones termed DN12, DN18 and DN19 were determined by western blotting. These clones as well as a clone transfected with vector alone (C2) were treated with 80 nM PrP<sup>Sc</sup> for 48 h. Results were analyzed statistically by non-parametric *t*-test comparing each result with values obtained with the control clone C2. (C) As controls, the same clones were treated with A23187 (200 nM), brefeldin A (12  $\mu$ M; Bref.A) or staurosporine (25 nM; Stauro), and cell viability was determined after 48 h by MTS analysis. In panels (B) and (C), values correspond to the average and standard deviation of three determinations. Results were statistically analyzed by *t*-test comparing the result of each treatment with the values obtained with the same treatment of clone C2 and results are shown in the figure, using the asterisk nomenclature as described in Materials and methods. Data were also analyzed by two-way ANOVA using clones and treatment as the variables. Results were significant with  $P < 0.0001$ .

Online). No induction of Grps was observed after treatment of cells with staurosporine (Figure 3A) or with equivalent concentrations of recombinant PrP<sup>Sc</sup> (data not shown).

To confirm the participation of caspase-12 in the PrP<sup>Sc</sup> apoptotic pathway we studied the effect of over-expression of a catalytic mutant of caspase-12 [caspase-12(C298A)], that has been shown to function as a dominant-negative (Rao *et al.*, 2002). N2A cells over-expressing the caspase-12 catalytic mutant were significantly more resistant to PrP<sup>Sc</sup> toxicity (Figure 3B). As controls, the same clones were treated with different concentrations of brefeldin A, A23187 or staurosporine. Cells over-expressing the dominant-negative caspase-12 were more resistant to insult induced by the ER-stress agents brefeldin A and A23187 (Figure 3C). However, no difference was observed when an agent inducing apoptosis by the mitochondrial pathway (staurosporine) was used (Figure 3C).

Recently, it has been described that under certain pathological conditions, Bcl-2 can regulate the induction

of apoptosis mediated not only by mitochondrial dysfunction, but also by ER-stress (Rudner *et al.*, 2001; Wang *et al.*, 2001). In order to characterize further the role of ER-mediated apoptosis in PrP<sup>Sc</sup>-induced cell death, N2A cells were stably transfected with a mutant form of Bcl-2 targeted to the ER (Bcl-2/ER) by replacing its transmembrane domain with the ER-insertional region of cytochrome b5 (Figure 4A). As controls we stably transfected cells with either a mutant form lacking the transmembrane domain (Bcl-2/ $\Delta$ TM), which is expressed in the cytoplasm and is inactive, or with wild-type Bcl-2 (Bcl-2/WT), which is expressed in both mitochondria and ER (Figure 4A). The specific distribution of each mutant was confirmed by co-staining the cells with antibodies against Bcl-2 and the ER marker calnexin (Figure 4B). Cells transfected with Bcl-2/ER completely overlapped in double stained preparations with a perinuclear distribution typical of the ER-Golgi network. As expected, Bcl-2/ $\Delta$ TM clones showed staining throughout the cell (including the nucleus), whereas Bcl-2/WT exhibited a



**Fig. 4.** Bcl-2 targeted to the ER decreases the sensitivity of N2A cells to PrP<sup>Sc</sup> toxicity and caspase-12 activation. (A) Cells were stably transfected with Bcl-2/ER, Bcl-2- $\Delta$ TM and Bcl-2-WT constructs or with empty vector, and Bcl-2 protein levels were analyzed by western blotting in different cellular clones. (B) In parallel, Bcl-2 distribution was detected by immunofluorescence in selected clones. Bcl-2 staining is shown in green; calnexin staining is shown in red and nuclear labeling with Hoechst33342 is shown in blue. (C) As controls, Bcl-2 transfected clones and control cells were treated with 150 nM staurosporine for 24 h. (D) The same N2A cell clones were treated with 50 nM PrP<sup>Sc</sup> for 48 h, and cell viability was assessed using MTS analysis. In the last two panels, statistical analysis was performed comparing the cell viability values for each transfected clone with the results obtained with the control clone C1. (E) Cells transfected with vector alone (C1 clone) or with Bcl-2/ER (clones 5 and 7) were treated with 50 nM PrP<sup>Sc</sup>. Caspase-12 activation was analyzed by western blot. The significance of the differences in pro-caspase-12 expression was estimated by densitometric analysis of the blots.

cytoplasmatic distribution, which partially colocalized with calnexin (Figure 4B).

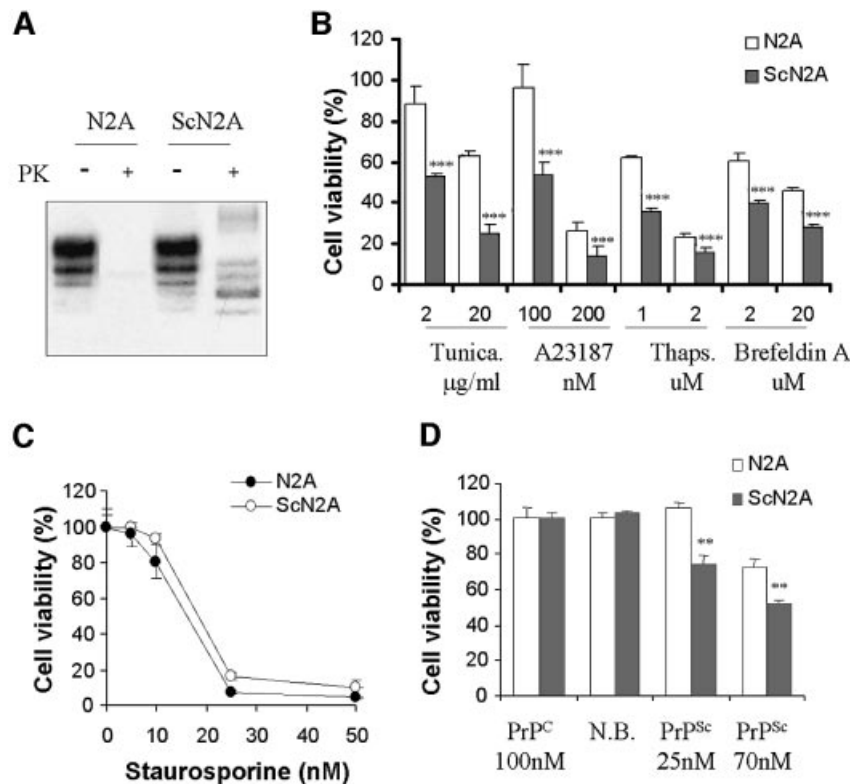
Treatment of the different cell clones with staurosporine showed that only the wild-type Bcl-2 expressing cells were significantly protected from the toxic effect of this agent (Figure 4C). The two clones expressing Bcl-2/ER (clones C5 and C7) showed significant protection against PrP<sup>Sc</sup> when compared with cells transfected with vector alone (Figure 4D). Moreover, cells expressing Bcl-2/WT, which is targeted to both mitochondria and ER, did not result in significantly further inhibition of neurotoxicity compared with Bcl-2/ER, suggesting that mitochondrial Bcl-2 does not contribute to inhibit apoptosis induced by PrP<sup>Sc</sup> (Figure 4D). Interestingly, the inhibition of cellular death in the clones expressing Bcl-2/ER was associated with a decrease in activation of caspase-12 (Figure 4E), further supporting the link between ER-stress and caspase-12 activation in the PrP<sup>Sc</sup> apoptotic pathway.

### PrP<sup>Sc</sup> replication sensitizes N2A cells to ER-stress-induced apoptosis

It has been described that a hypothalamic neuronal cell line persistently infected with scrapie prions has the ability to convert endogenous PrP<sup>C</sup> into PrP<sup>Sc</sup>, and exhibits typical morphological and biochemical features of apoptosis (Schatzl *et al.*, 1997). However, in most neuronal

lines chronically infected with prions, including N2A cells, there are no signs of cellular death (Rubenstein *et al.*, 1984; Butler *et al.*, 1988). In order to analyze the effect of PrP<sup>Sc</sup> replication on cellular dysfunction, we studied the vulnerability to toxic stress of N2A cells chronically infected with RML prions (ScN2A). These cells propagate protease-resistant prion protein (Figure 5A) and carry infectivity (Butler *et al.*, 1988). Even though there are no evident effects on cellular viability following prion infection, treatment with different ER-stress inducers, including thapsigargin, tunicamycin, brefeldin A and the ionophore A23187, revealed that ScN2A cells are more sensitive to ER-stress mediated cell death than non-infected N2A cells (Figure 5B). In control experiments, no differential effect was observed when ScN2A and N2A cells were treated with inducers of mitochondrial stress, such as staurosporine (Figure 5C) or serum deprivation (data not shown). These data suggest that PrP<sup>Sc</sup> replication induces ER-stress, and although this does not lead directly to cell death, it nevertheless sensitizes the cells to subsequent apoptotic stimuli, which target the ER-associated pathway.

A possible explanation for the lack of toxicity of endogenous PrP<sup>Sc</sup> produced in N2A cells is that since these cells divide rapidly, they do not accumulate enough PrP<sup>Sc</sup> to lead to cell death. To test this hypothesis, we analyzed



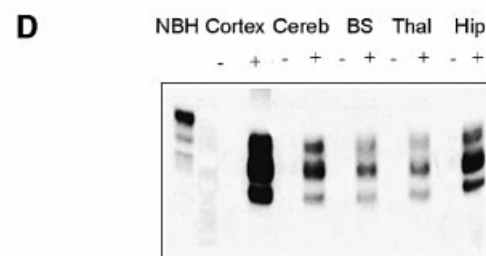
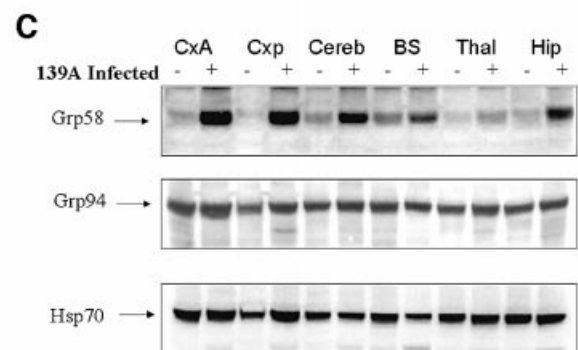
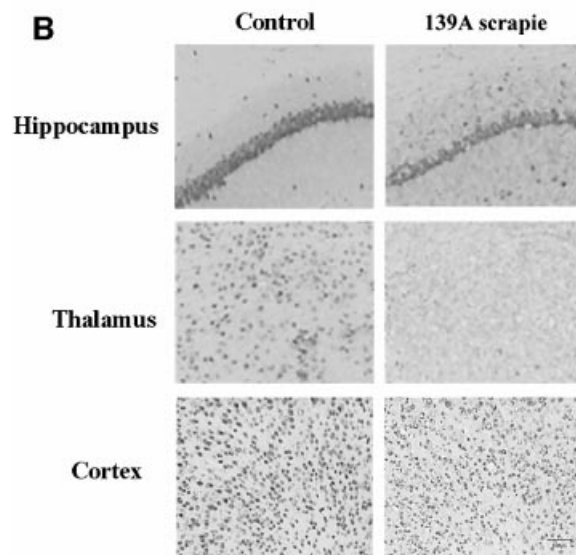
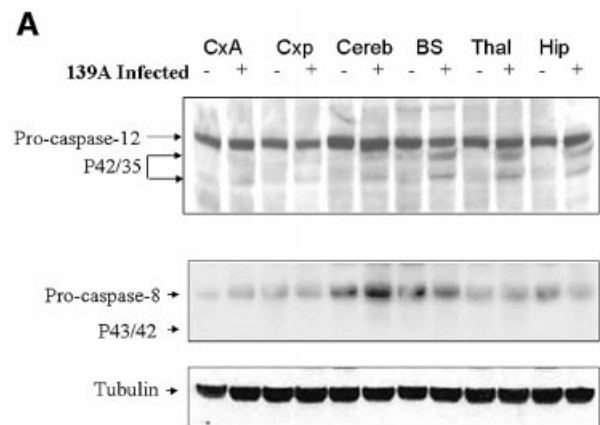
**Fig. 5.** PrP<sup>Sc</sup>-replication sensitizes N2A cells to ER-stress mediated apoptosis. (A) Scrapie-infected N2A cells replicate the protease-resistant PrP<sup>Sc</sup> form, whereas the non-infected cells contain only PK-sensitive PrP<sup>C</sup>, as analyzed by western blot. (B) ScN2A and N2A cells were treated with different concentrations of tunicamycin, A23187, thapsigargin (Thaps) or brefeldin A for 48 h, and cell viability was quantified using MTS. Filled bars represent infected cells and empty bars indicates non-infected cells. Data were analyzed by *t*-test comparing the values of infected and non-infected cells for each treatment ( $***P < 0.005$ ) and by two-way ANOVA using treatment and cells (infected and non-infected) as the variables ( $P < 0.0001$ ). (C) As controls, cells were treated with different concentrations of staurosporine for 48 h, and cellular viability was quantified by MTS analysis. (D) N2A and ScN2A cells were treated with PrP<sup>Sc</sup>, and cell viability was analyzed after 48 h using MTS. Data in panels (B, C and D) represent two independent experiments performed in triplicate. Parametric *t*-test was used to analyze statistical significance comparing for each condition the values of non-infected versus infected cells.

the extent of cell death induced by purified PrP<sup>Sc</sup> in infected and non-infected N2A. Addition of PrP<sup>Sc</sup> to the culture medium produced a higher toxicity in ScN2A cells than in non-infected cells (Figure 5D). Interestingly, low concentrations of PrP<sup>Sc</sup> (25 nM) that in non-infected cells do not produce toxicity, in ScN2A lead to significant cell death (Figure 5D). No effects on cellular viability were observed when infected and non-infected cells were treated with recombinant PrP<sup>C</sup> or an equivalent sample prepared from non-infected brains (Figure 5D). These data reinforce the hypothesis that PrP<sup>Sc</sup> damages the cells by the ER-stress pathway.

### Neuronal loss in murine scrapie is associated with the activation of caspase-12 and the up-regulation of Grp58

To validate the participation of caspase-12 during the development of prion disease *in vivo*, C57BL/6J mice were infected with 139A-scrapie and caspase-12 activation was analyzed by western blotting in different brain areas of symptomatic animals. Figure 6A shows that the active fragments of caspase-12 (p42/35) can be detected mainly in the hippocampus, thalamus and brain stem, whereas no clear signal was detected in cortex and cerebellum. This pattern was representative of the results obtained in several animals in late stages of the disease. No evidence of active caspase-12 fragments was ever detected in normal animals of the same age. The appearance of the active fragments was not paralleled by a noticeable decrease in pro-caspase-12 signal, most likely because of the high complexity of the brain extracts in terms of cellular type diversity and the selective neuronal cell death observed in scrapie. In control experiments, we analyzed the activation of caspase-8, observing no significant changes in the pro-caspase-8 form, and no increase in the levels of the active fragments (Figure 6A; p43/41). After histological analysis of the same brain areas it was possible to observe a correlation between the degree of caspase-12 activation and the extent of neuronal loss (Figure 6B). The highest levels of caspase-12 active fragments and neuronal death were found in the hippocampus, pyramidal cell layer and the thalamus, whereas in the cortex despite significant neuronal distortion little cell loss was observed, correlating with the absence of caspase-12 activation.

We then analyzed markers of ER-stress in the brain of these animals. Our data showed a dramatic up-regulation of the ER-stress inducible chaperon protein Grp58 in all brain areas. No significant changes were detected in the expression levels of the chaperones Grp94, calnexin,



**Fig. 6.** Neuronal loss in murine scrapie is associated with activation of caspase-12 and up-regulation of Grp58. (A) Activation of caspase-12 and caspase-8 was analyzed by western blotting in different brain areas, including anterior cortex (CxA), posterior cortex (Cxp), cerebellum (Cereb), brain stem (BS), thalamus (Thal) and hippocampus (Hip). (B) Neuronal loss in hippocampus, thalamus and cortex was measured by immunohistochemical staining with Neu-N, a neuronal-specific marker. (C) Expression levels of Grp58, Grp94 and Hsp70 were analyzed in the same brain areas described in (A). (D) PrP<sup>Sc</sup> levels were quantified after PK treatment of different brains areas from scrapie-infected and control animals. Results are representative of the data obtained with three different controls and scrapie-infected animals. The first lane shows a normal brain homogenate (NBH) before PK treatment.

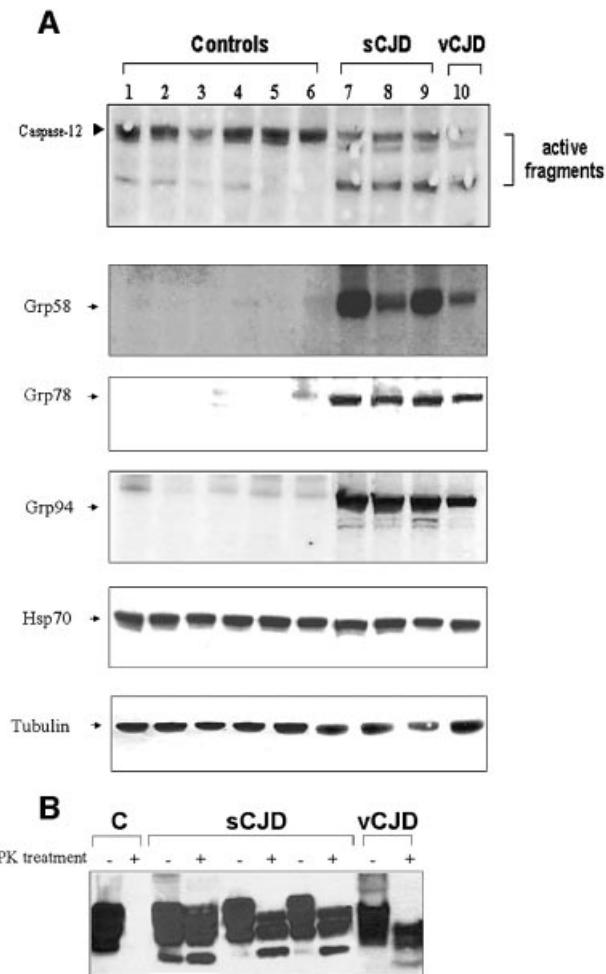
Hsp60 or Hsp70 (Figure 6C; and data not shown). The presence of PrP<sup>Sc</sup> was analyzed in the same brain areas by western blotting after PK treatment. As shown in Figure 6D, PrP<sup>Sc</sup> accumulation directly correlates with the expression levels of Grp58, consistent with the idea that the accumulation of PrP<sup>Sc</sup> triggers a neuroprotective response involving up-regulation of some ER chaperones.

### **ER-stress and caspase-12 activation in post mortem CJD brains**

The participation of ER-stress and caspase-12 activation was studied further in several human brain samples from patients affected with sCJD and vCJD. Western blot analysis of caspase-12 activation revealed that the active fragments could be detected in all samples analyzed from patients (three sCJD and one vCJD), whereas these fragments were not clearly detected in six control samples from healthy individuals (Figure 7A). Interestingly, we were able to detect higher levels of the ER chaperones Grp58, Grp78, and Grp94 in sCJD and vCJD brains, compared with control samples (Figure 7A). No significant changes were observed in the Hsp70 protein levels. The levels of PrP<sup>Sc</sup> replication were analyzed after PK treatment (Figure 7B) and the results show that all samples analyzed contained abundant levels of PK-resistant PrP. In agreement with these data, the up-regulation of Grp58 in human samples of patients with sCJD has been recently reported (Yoo *et al.*, 2002). However, the protein expression levels described by Yoo and co-workers were lower than the levels described here, possibly due to the fact that different brain areas (cerebellum instead of cortex) were examined in each case. Taken together, these data clearly implicate ER-stress and caspase-12 activation in neurodegeneration in humans affected by CJD.

### **Discussion**

In this paper we describe for the first time the signalling pathways involved in neuronal apoptosis induced by brain-derived PrP<sup>Sc</sup>. Our results indicate that nanomolar concentrations of purified PrP<sup>Sc</sup> are sufficient to trigger apoptosis of neuroblastoma cells. This effect appears to be very specific for the pathological conformation of the protein, since no toxicity was observed using recombinant PrP<sup>C</sup>, synthetic PrP peptides or PrP<sup>Sc</sup> treated with a peptide that disrupts  $\beta$ -sheet conformation. Apoptotic induction by PrP<sup>Sc</sup> was shown to be dependent on caspase-3 activation, evidenced by the detection of caspase-3 activity after incubation with PrP<sup>Sc</sup>, and the inhibition of toxicity by pre-treatment of N2A cells with Ac-DEVD-fmk, a molecule that preferentially inhibits caspase-3. Since neither substrates nor inhibitors are totally specific, it is not possible to rule out the participation of other executor caspases, such as caspase-7. However, previous reports have also shown the involvement of caspase-3 in PrP<sup>Sc</sup>-induced cell death (Hetz and Soto, 2003). Our results also show that upstream of caspase-3 activation there is a rapid and sustained release of calcium from the ER in response to PrP<sup>Sc</sup> treatment. Perturbation of calcium homeostasis and accumulation of misfolded proteins in the ER has been shown to elicit a cell-stress response that can lead to apoptosis (Nakagawa *et al.*, 2000). Consistent with this hypothesis, we found that PrP<sup>Sc</sup> treatment leads to the



**Fig. 7.** Caspase-12 activation and induction of ER-stress markers in sCJD and vCJD human brain samples. (A) Analysis of caspase-12 activation and expression levels of Grp58, Grp78, Grp94 and Hsp70 were performed in human samples from cortex of six normal individuals (lanes 1–6), three individuals affected with sCJD (lanes 7–9) and one with vCJD (lane 10). As internal control of total protein levels, a western blot using anti-tubulin antibody is shown in the bottom panel. (B) PrP<sup>Sc</sup> levels were quantified after PK treatment from human samples of a normal individual or patients affected with CJD.

induction of ER chaperones of the glucose regulated family proteins (Grps). Experiments with N2A cells chronically infected with scrapie prions further support the relationship between PrP<sup>Sc</sup> and ER-stress. Infected cells exhibit increased sensitivity to the ER-stress induced apoptosis mediated by several stimuli, including exogenous PrP<sup>Sc</sup> itself.

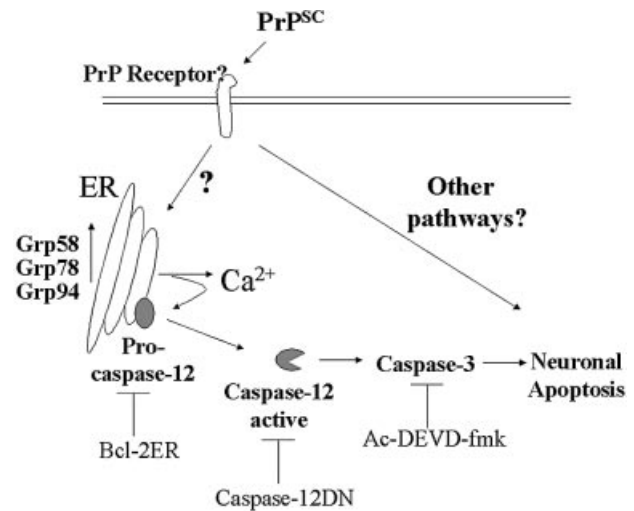
ER-stress has been associated with the pathogenesis of other neurodegenerative disorders, including Alzheimer disease, Parkinson's disease, Huntington disease and amyotrophic lateral sclerosis (Mattson, 2000; Sherman and Goldberg, 2001; Kouroku, 2002). These diseases are also characterized by accumulation of misfolded proteins in the brain (Soto, 2003). A molecular mechanism linking ER stress and apoptosis has been recently described, in which the ER-resident caspase-12 is activated, leading to caspase-3 activation and cellular death (Nakagawa *et al.*, 2000). Treatment of neurons with  $\beta$ -amyloid peptide, or the expression of polyglutamine aggregates associated with Huntington disease induces ER stress and activation



of caspase-12 *in vitro* (Nakagawa *et al.*, 2000; Kouroku, 2002). It has been proposed that the main function of caspase-12 is to facilitate apoptosis in cells irreversibly damaged by stress signals from the ER (Mehmet, 2000). In our experiments, exposure of the cells to PrP<sup>Sc</sup> leads to a significant activation of caspase-12, reflected by the decrease in the signal of procaspase-12.

Our *in vitro* results are supported by biochemical and histological studies in scrapie mouse brains, which show a good correlation between neuronal death and caspase-12 activation. In addition, we found a direct correlation between the up-regulation of ER-stress inducible protein Grp58 and the levels of PrP<sup>Sc</sup> in different brain areas of scrapie-infected mice. Grp58, also called ER-60 or Erp-57, is a disulfide isomerase that forms part of the ER chaperone family designated as glucose regulated proteins (Grps) (Mazzarella *et al.*, 1994). However, no good correlation was observed between PrP<sup>Sc</sup> deposition/Grp up-regulation and neuronal death/caspase-12 activation. The most likely explanation for these results is that PrP<sup>Sc</sup> replication and Grp58 expression are early events in the disease, whereas caspase-12 activation and neuronal loss occurs at late stages. Expression of Grp proteins may be down-regulated when the apoptotic programme is activated. An alternative explanation is that the extent of cellular loss might be associated with differential sensitivity to PrP<sup>Sc</sup> of neuronal cell types in each specific brain area. We are currently performing analyses at different time points after prion infection to address this issue. Preliminary results revealed that abundant PrP<sup>Sc</sup> accumulation and Grp58 up-regulation are observed in brain stem, thalamus and hippocampus early in the course of the disease and before extensive cell death or caspase-12 activation are observed (C.Hetz and C.Soto, unpublished data). As the disease progresses, caspase-12 is activated and this is followed by neuronal death. However, it is possible that the brain regions affected and the degree of ER-stress response may be variable in different species/strains. Indeed, in human brain from sCJD and vCJD a profound ER-stress response concomitant with caspase-12 activation was observed in cerebral cortex, a region with only mild effect in mice. Our findings, together with previous reports, suggest that caspase-12 may play a major role in neuronal apoptosis associated with protein misfolding and aggregation in the brain. The mechanism by which ER-stress and calcium release induces caspase-12 activation *in vivo* remains to be investigated.

Based on our findings we can propose a pathway for PrP<sup>Sc</sup>-induced apoptosis (Figure 8), in which the first step would be the interaction of PrP<sup>Sc</sup> with some as yet unknown cell surface receptor(s), which triggers a signal to the ER, resulting in release of intra-ER calcium (Figure 8). Our data do not rule out the possibility that PrP<sup>Sc</sup> migrates to the ER and by itself induces ER-stress and calcium release. ER-stress would then trigger the up-regulation of certain chaperone proteins with neuroprotective activities (such as Grp58, Grp78 or Grp94) and activation of the ER-resident caspase-12, leading to the degradation of cellular proteins and cell death (Figure 8). ER chaperones are probably up regulated in an attempt to correct misfolded PrP<sup>Sc</sup> or to remove it via the delivery of the proteins towards the proteasomal degradation pathway. Grp proteins are a family of molecular chaperones located



**Fig. 8.** A working hypothesis for PrP<sup>Sc</sup>-induced apoptosis in neuronal cells. Interaction of PrP<sup>Sc</sup> with an unknown receptor protein activates a signaling pathway, which induces the release of calcium from the ER and ER-stress, as evidenced by the up-regulation of ER chaperones (i.e. Grp58, Grp78 and/or Grp94). ER-stress leads to activation of caspase 12, which in turn cleaves and activates the executioner caspase-3, leading to apoptosis.

in the ER that have been shown to attenuate cellular death induced by increase of intracellular calcium and the accumulation of misfolded proteins in the ER (Mazzarella *et al.*, 1994; Liu *et al.*, 1997). However, no evidence is yet available on the interaction of PrP with Grps chaperones *in vivo*. Accumulation of PrP molecules in the ER has been shown to occur in cells expressing mutant forms of PrP linked to inherited forms of the disease (Singh *et al.*, 1997; Zanusso *et al.*, 1999; Jin *et al.*, 2000; Negro *et al.*, 2001). Interestingly, in neuroblastoma cells expressing mutant PrP, an association of the protein with Grp78 was detected, which appears to be important for proteasomal delivery and degradation of mutant PrP (Jin *et al.*, 2000). In addition, stimulation of retrograde transport of PrP towards the ER favors the formation of PrP<sup>Sc</sup> in prion-infected N2A neuroblastoma cells (Beranger *et al.*, 2002).

Our findings have clear implications for development of TSE treatments directed to prevent neurodegeneration. Since caspases are central to both normal programmed cell death and injury-dependent apoptosis, inhibition of these proteases usually results in serious adverse effects. However, caspase-12 appears not to be essential for normal development or physiological cell death, rather its activation seems confined to some specific pathological stress signals (Mehmet, 2000). Indeed, caspase-12-deficient mice have no noticeable developmental or behavioural defects (Nakagawa *et al.*, 2000). Therefore, inhibition of caspase-12 activation might provide a novel therapy for TSEs and other neurodegenerative diseases initiated by protein misfolding. However, until now the participation of caspase-12 in human pathologies has been controversial, since the functional human homologue has not been identified (Fischer *et al.*, 2002). Despite this, a protein detectable with diverse anti-murine caspase-12 antibodies is present in several human cell lines (Bitko and Barik, 2001; Rao *et al.*, 2001), and this putative human caspase-12 homologue exhibited similar involvement in

ER-stress mediated apoptosis. Our data suggest that targeting caspase-12 or other components of the ER-stress mediated apoptotic pathway may lead to a novel therapy for TSEs.

## Materials and methods

### Materials

The fluorogenic caspase substrates (Ac-DEVD-amc, Ac-YVAD-amc), caspase inhibitors (Ac-DEVD-cho, Ac-YVAD-cho, zVAD-fmk), staurosporine, tunicamycin, brefeldin A, A23187, thapsigargin, ALLN, calpain inhibitor III and calpeptin were purchased from Calbiochem (Darmstadt, Germany). The cell-permeable caspase-3 substrate FAM-DEVD-fmk was obtained from Promega (Madison, WI) and the cell-permeable caspase-8 substrate FAM-LETD-fmk and caspase-1 substrate FAM-YVAD-fmk were purchased from Intergen (New York, NY). Cell culture medium, fetal calf serum, and antibiotics were obtained from Life Technologies (Maryland, USA). PrP106–126 and iPrP13 peptide were purchased from Neosystem Inc.

### PrP<sup>Sc</sup> purification

PrP<sup>Sc</sup> was purified from mice infected with 139A scrapie as previously described (Soto *et al.*, 2000). Briefly, brain tissue was homogenized in PBS containing protease inhibitors and solubilized in 10% salkosyl. After centrifugation in a TL100 ultracentrifuge at 80 000 r.p.m. for 2 h at 4°C over a sucrose gradient, pellets were resuspended in PBS containing 0.1% SB-314. Thereafter, samples were treated with PK (30 µg/ml) for 2 h followed by another sucrose gradient centrifugation at 80 000 r.p.m. for 2 h. The pellet was washed twice with PBS and resuspended in the same buffer. After this step, purity was >95% as estimated by silver staining. PK treatment results in an N-terminally truncated version of PrP<sup>Sc</sup>, lacking the first 90 residues, usually termed PrP27–30, which has been shown to keep the major properties of PrP<sup>Sc</sup> including infectivity. PrP<sup>Sc</sup> concentration was estimated by immunoblot analysis, using known concentrations of the recombinant mouse PrP<sup>C</sup> (Prionics Inc, Zurich, Switzerland).

### Cell culture, viability and apoptosis assays

N2A cells were cultured in DMEM supplemented with 10% fetal calf serum and antibiotics (10 000 U/ml penicillin, 10 µg/ml streptomycin) at 37°C and 5% CO<sub>2</sub>. For cell viability analysis, cells were grown in collagen IV-coated 96-well plates for 24 h in cell culture medium containing 1% serum before addition of the sample (PrP<sup>Sc</sup> or other reagents). Cell viability was quantified using 3-(4,5-dimethylthazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) according to the recommendations of the supplier (Promega, CellTiter96<sup>®</sup> Aqueous, Madison, WI). *In situ* caspase-3/caspase-8/caspase-1 activity was detected using the fluorescent substrates following the manufacturer's instructions as previously described (Hetz *et al.*, 2002b). Quantification of phosphatidylserine exposure in the outer leaflet of the plasma membrane was detected by flow cytometry using FITC-coupled annexin V (Roche, Basel, Switzerland) as previously described (Hetz *et al.*, 2002b). For caspase inhibition experiments, cells were pre-incubated for 2 h with Ac-DEVD-fmk (100 µM), Ac-YVAD-fmk (100 µM) or zVAD-fmk (10 µM) before PrP<sup>Sc</sup> addition. For PrP106–126 toxicity, 1 mM peptide solution was incubated in PBS for 24 h at 37°C to induce aggregation, before addition to N2A cultures for cellular viability determinations at a final concentration of 100 µM or 100 nM.

### Generation of N2A cells chronically infected with scrapie

Stable chronically infected cells were established by subcloning the N2A neuroblastoma cells obtained from ATCC by limiting dilution, as described previously (Bosque and Prusiner, 2000). Individual subclones were tested for sensitivity to infection by the RML strain of mouse scrapie by adding 4 µl of a 10% late stage infected brain extract. Cells were serially passed thereafter in the absence of PrP<sup>Sc</sup>. After several passages individual cultures were tested for the presence of newly generated PrP<sup>Sc</sup> by western blotting.

### Measurement of intracellular calcium

The changes in intracellular calcium levels resulting from PrP<sup>Sc</sup> treatment were measured in a FLIPR1 machine (Molecular devices, Sunnyvale, CA) using the fluorescent dye Fluo-4, which shows increased fluorescence at 515–535 nm after calcium binding. Cells were grown on black

96-wells plates coated with collagen IV and loaded for 2 h with Fluo-4 at a final concentration of 10 µg/ml. The loaded cells were washed twice with FLIPR buffer (150 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 10 mM Hepes, 10 mM glucose) and fluorescence emission was quantified for a total time of 30 min. The basal fluorescence of the dye was usually determined before the addition of the samples and was assigned a value of zero. To determine the origin of intracellular calcium, cells were pre-treated with thapsigargin (5 µM) for 15 min before addition of the agonists.

### Cell transfection

Expression vectors encoding Bcl-2 lacking the transmembrane domain (Bcl-2/ΔTM), wild-type Bcl-2 (Bcl-2/WT) and Bcl-2 mutants targeted to the outer membrane of the ER (Bcl-2/ER) were kindly provided by B.Leber (Ontario, Canada) and C.Belka (Tubingen, Germany). ER-specific targeting of Bcl-2 was achieved by exchanging the C-terminal insertional sequence of Bcl-2 with equivalent sequence of the rat cytochrome b5. The distribution of ectopically expressed Bcl-2/ER, Bcl-2/WT and Bcl-2/ΔTM in the selected clones was analyzed by co-immunolocalization with the ER marker calnexin and nuclei were stained with Hoechst33342. Expression plasmid containing catalytically inactive caspase-12 mutant lacking its N-terminal pro-domain (amino acids 1–94, pC12DN) was kindly provided by R.Rao (California). Stably expressing N2a cells were produced by transfection using SuperFect (Qiagen, Valencia, CA) following the manufacturer's instructions. After 48 h, cells were selected using hygromycin (1.5 mg/ml) for 5 days, and individual clones were obtained by limiting dilution.

### SDS-PAGE and western blot analysis

Cells or brain tissue were homogenized on ice in RIPA buffer (20 mM Tris pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% DOC, 0.5% Triton X-100) containing a protease inhibitor cocktail (Roche, Basel, Switzerland). Protein concentration was determined by micro-BCA assay (Pierce, Rockford, IL). The equivalent of 30–50 µg of total protein was generally loaded onto 10% SDS-PAGE minigels (Novex NuPage, Invitrogen Life Technologies, Basel, Switzerland) and analyzed by western blotting as described (Saborio *et al.*, 2001). The following antibodies and dilutions were used: 6H4 anti-PrP, 1:10 000 (Prionics, Zurich, Switzerland), anti-Caspase-12, 1:2000 (Exalpa, Watertown); anti-GRP78(Bip), anti-Hsp60, anti-Hsp70, anti-Grp58 and anti-Grp94 1:2000 (StressGene, San Diego, CA); anti-actin, 1:2000 (Santa Cruz); anti-tubulin, 1:5000 (Oncogene EMD Bioscience Inc., Darmstadt, Germany).

### Animal samples

C57BL/6J mice were injected stereotaxically in the hippocampus with 1 µl of a 10% brain homogenate from mice infected with 139A scrapie. The onset of clinical disease was measured weekly by determination of muscle strength using a grid system as described previously (Soto *et al.*, 2000). Scrapie incubation times were defined from the date of injection to the time when the clinical symptoms persisted for three consecutive weeks, which for the conditions used in this study corresponded to 14 weeks after injection. For western blot analysis, sick animals were sacrificed 20 weeks after infection and different brain areas were dissected and analyzed separately.

### Human samples

Frozen samples from brain cortex of clinically and pathologically confirmed cases of sCJD and vCJD were kindly provided by Dr Maurizio Pocchiari (Istituto Superiore di Sanita, Rome, Italy) and Dr James Ironside (CJD Unit, Edinburgh, UK), respectively. Brain samples from normal individuals were generously provided by Dr Adam Golabek (New York State Institute for Basic Research, Staten Island, NY).

### Immunohistochemistry

Coronal 20 µm sections were taken from the mouse dorsal hippocampus. Sections were fixed overnight in a solution containing 4% paraformaldehyde and transferred to a 20% sucrose solution. Immunostaining was performed on free-floating sections. Anti-Neu-N antibody was used to specifically stain neurons and detected with the avidin–biotin peroxidase method using reagents from Vector Laboratories (Vectastatin Elite ABC, Burlingame, CA) and diaminobenzidine as the chromogen.

### Statistical analysis

Data were analyzed by parametric *t*-test (two-tailed) and significance was expressed as follow: \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.005. When more than two groups were analyzed, an ANOVA test was also used to estimate statistical significance.

**Supplementary data**

Supplementary data are available at *The EMBO Journal* Online.

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