Cellular prion protein (PrP^C) protects neuronal cells from the effect of huntingtin aggregation

Kyung-Jin Lee, Antony Panzera, David Rogawski, Lois E. Greene and Evan Eisenberg*

Laboratory of Cell Biology, NHBLI, NIH, Bethesda, MD 20892-0301, USA

*Author for correspondence (e-mail: eisenbee@nhlbi.nih.gov)

Accepted 17 May 2007
Journal of Cell Science 120, 2663-2671 Published by The Company of Biologists 2007
doi:10.1242/jcs.004598

Summary

The effect of normal cellular prion protein (PrP^C) on abnormal protein aggregation was examined by transfecting huntingtin fragments (Htt) into SN56 neuronal-derived cells depleted of PrP^C by RNA interference. PrP^C depletion caused an increase in both the number of cells containing granules and the number of apoptotic cells. Consistent with the increase in Htt aggregation, PrP^C depletion caused an decrease in proteasome activity and a decrease in the activities of cellular defense enzymes compared with control cells whereas reactive oxygen species (ROS) increased more than threefold. Therefore, PrP^C may protect against Htt toxicity in neuronal cells by increasing cellular defense proteins, decreasing ROS and increasing proteasome activity thereby increasing Htt degradation. Depletion of endogenous PrP^C in non-neuronal Caco-2 and HT-29 cells did not affect ROS levels or proteasome activity suggesting that only in neuronal cells does PrP^C confer protection against Htt toxicity. The protective effect of PrP^C was further evident in that overexpression of mouse PrP^C in SN56 cells transfected with Htt caused a decrease in both the number of cells with Htt granules and the number of apoptotic cells, whereas there was no effect of PrP^C expression in non-neuronal NIH3T3 or CHO cells. Finally, in chronically scrapie (PrP^{Sc})-infected cells, ROS increased more than twofold while proteasome activity was decreased compared to control cells. Although this could be a direct effect of PrP^{Sc}, it is also possible that, since PrP^C specifically prevents pathological protein aggregation in neuronal cells, partial loss of PrP^C itself increases PrP^{Sc} aggregation.

Key words: Neuroprotection, Prion, Huntingtin, Proteasome activity, Reactive oxygen species

Introduction

The normal cellular prion protein (PrP^C) is a glycosylphosphatidylinositol-anchored glycoprotein that is predominantly expressed in the brain (Prusiner, 1998; Weissmann and Flechsig, 2003). In prion diseases, the protease-resistant misfolded scrapie isoform of prion protein (PrP^{Sc}) is the causative agent of transmissible spongiform encephalopathies, which are neurodegenerative disorders that include scrapie in sheep and goats, bovine spongiform encephalopathies, chronic wasting disease in deer and elk and Creutzfeldt-Jakob disease in humans (Prusiner, 1998). In all of these disorders, exposure of nerve cells to PrP^{Sc} converts PrP^C to aggregated deposits of PrP^{Sc}. There have been numerous models proposed for the neuronal cell loss and spongiform changes in the brain that occur in scrapie, but it is still not clear whether this pathology is due to a loss of functional PrP^C or only to a gain of function by PrP^{Sc}. Clinical symptoms can occur without any obvious scrapie deposits (Collinge et al., 1990; Medori et al., 1992), which has led to the suggestion that the loss of normal PrP^C function, not formation of PrP^{Sc} deposits, causes prion disease (Aguzzi and Weissmann, 1997). Unfortunately, the normal function of PrP^C is unknown, although its conservation in many different species suggests that it plays a prominent role in a basic physiological process. It has been reported that PrP^C functions in cell survival, signal transduction, cell adhesion, copper-dependent antioxidant activity, and copper uptake and sequestration (Roucou and LeBlanc, 2005). Although PrP^C knockout mice are healthy, the brains of these mice were found to have reduced levels of cell defense enzymes activity, such as catalase, and increased levels of oxidative stress markers (Klamt et al., 2001; Brown and Besinger, 1998; Brown et al., 1997b; Sakudo et al., 2005; Wong et al., 2001; Wong et al., 2000; Wong et al., 1999). Similarly, tissue cultures of nerve cells derived from the PrP^C knockout mouse are less viable and more susceptible to oxidative damage and toxicity caused by agents such as copper and hydrogen peroxide than cells expressing wild-type PrP^C (Brown et al., 1997a; Kuwahara et al., 1999). PrP^C was hypothesized to act as an antioxidant (Brown and Besinger, 1998; Wong et al., 1999), but recent studies have established that PrP^C has no superoxide dismutase activity either in vivo or in vitro (Hutter et al., 2003; Jones et al., 2005). Since numerous studies suggest that, under stress conditions, PrP^C has a neuroprotective effect, this raises the question as to whether the neurodegenerative defects observed in scrapie-infected mice are aggravated by the loss of PrP^C, as well as the build up of PrP^{Sc} amyloid plaque. In fact, neurons from both PrP^C knockout mice and scrapie-infected animals show similar changes in neurophysiological function (Colling et al., 1996; Collinge et al., 1994; Jefferys et al., 1994; Johnston et al., 1997; Manson et al., 1995) and biochemical properties (Keshet et al., 1999; Ovadia et al., 1996). Furthermore, altered neuronal excitability can predispose individuals to neuronal damage and death (Leist and Nicotera, 1998) so it is possible that loss of

PrP^C function contributes to scrapie pathogenesis in this way. However, contrary to the idea that neuropathology is caused by loss of PrP^C function, Collinge and coworkers found that there was no effect on neuronal survival when PrP^C was knocked out from a 10-week-old mouse (Mallucci et al., 2002). Moreover, by disrupting the prion gene in a scrapie-infected mouse, they reversed the spongiosis, cognitive defects and neurological disfunction caused by scrapie (Mallucci et al., 2003; Mallucci et al., 2007).

In the present study, we have further examined whether knocking out PrP^C contributes to a loss of function under stress conditions by examining the effect of PrP^C depletion on protein aggregation. We used RNA interference (RNAi) to deplete endogenous PrP^C from neuronal-derived tissue culture cell lines that were also transfected with HttQ103. Our results show that there is an increase in HttQ103 aggregation in PrP^C-depleted cells. In addition, we found that PrP^C may protect against Htt-induced toxicity possibly by increasing cellular defense enzymes, decreasing reactive oxygen species (ROS) and thereby increasing proteasome activity. Interestingly these effects of PrP^C on ROS and proteasome activity are specific for nerve cells and do not occur in non-nerve cells even if these cells normally express PrP^C.

Results

Two different oligonucleotide sequences were used to knock down PrP^C in mouse neuronal cells. Sequence 1 is within the coding region and sequence 2 is in the 3′ UTR. Fig. 1 shows western blots against PrP^C of the cell lysates and lysates that were immunoprecipitated with the anti-prion before and after depleting PrP^C from SN56 cells. The immunoprecipitated lysate, which has a much higher concentration of PrP^C protein, shows multiple bands on the western blot as a result of the different glycosylated forms of PrP^C, which are not visible at lower concentrations. From the quantification of the western blots, both sequences reduced PrP^C by more than 90% following 2 days of transfection of SN56 cells with oligonucleotides. Similar levels of PrP^C depletion were measured 3 days following transfection with the siRNA oligonucleotides (data not shown). As expected, PrP^C levels were not affected by transfection of a scrambled sequence of oligonucleotide 1. Throughout this study, oligonucleotide sequences 1 and 2 produced the same phenotype. However, cells depleted of PrP^C with sequence 2 could be rescued by expressing PrP^C because unlike sequence 1, this sequence is in the UTR region of the message.

Since PrP^C has been reported to be neuroprotective (Kuwahara et al., 1999; Roucou et al., 2005; Roucou et al., 2003), we investigated whether PrP^C confers protection against Htt aggregation. Both control and PrP^C-depleted SN56 cells were transfected with GFP-Htt constructs. Routinely, the day after transfecting with oligonucleotides, the cells were transfected with the Htt constructs. The phenotype of the cells was analyzed 2 days later or 72 hours after transfection of the siRNA. We used both HttQ25, which normally does not form granules, and HttQ103, which forms granules and is toxic to the cell. As expected, there was no aggregation of HttQ25 either in the presence or absence of PrP^C (Fig. 2A). However, compared with cells only transfected with HttQ103 or scramble vector, cells depleted of PrP^C with sequence 2 caused a marked increase in the number of cells with granules of HttQ103 (Fig. 2A). This effect could be partially reversed by expressing mouse PrP^C.

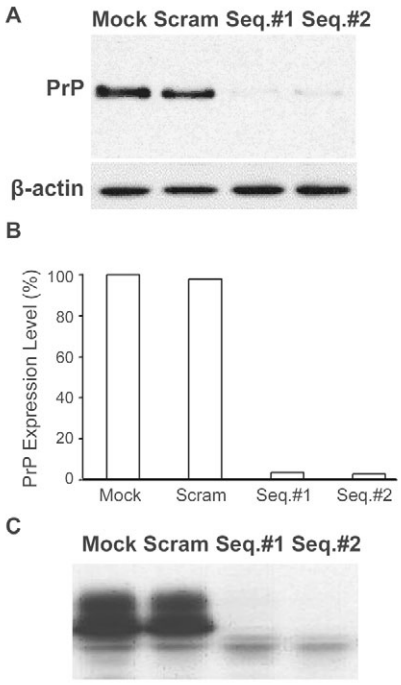

Fig. 1. Depletion of PrP^C by RNAi. (A) Western blot of PrP^C levels in control (Mock) SN56 cells and cells 48 hours after being transfected with the following oligonucleotides: scramble sequence (Scram), sequence 1, or sequence 2. In each lane, 100 μg of total protein was loaded. (B) Quantification of the western blot shown in A. The different intensities of the PrP^C bands were normalized to the mock-depleted value which was set at 100%. Quantification of the western blot showed that it was linear from 10 to 100 μg of cell lysate. (C) Immunoprecipitation of PrP^C from 500 μg cell lysates obtained from control cells and cells transfected with the scramble sequence, sequence 1, or sequence 2. The control cells were treated with Lipofectamine, the same as the PrP^C-depleted cells.

Quantification of the granules in the SN56 cells (Fig. 2A, open bars) shows that 48 hours after transfection with HttQ103, 60% of the PrP^C-depleted cells had HttQ103 granules whereas only 25% of the control cells had granules. This increase in the number of cells with granules was observed using both oligonucleotide 1 and 2. To insure that the observed phenotype was due to depletion of PrP^C, cells depleted of PrP^C with oligonucleotide 2 were partially rescued by transfecting with a plasmid expressing mouse PrP^C. As expected, we could not rescue the phenotype generated with oligonucleotide 1 because it is in the prion coding region and therefore it inhibits expression of the plasmid PrP^C along with the endogenous protein (data not shown). Remarkably, the extent of HttQ103 aggregation in the PrP^C-depleted cells was similar to that obtained when HttQ103-transfected SN56 cells were treated with the proteasome inhibitor, lactacystin (Fig. 2B, lane 6). Essentially, the same results were obtained with N2a cells (gray bars). Therefore, PrP^C depletion caused increase aggregation of HttQ103 in the neuronal cell lines, SN56 and N2a.

To ensure that the difference in the level of aggregation was not due to differences in expression levels of HttQ103, western

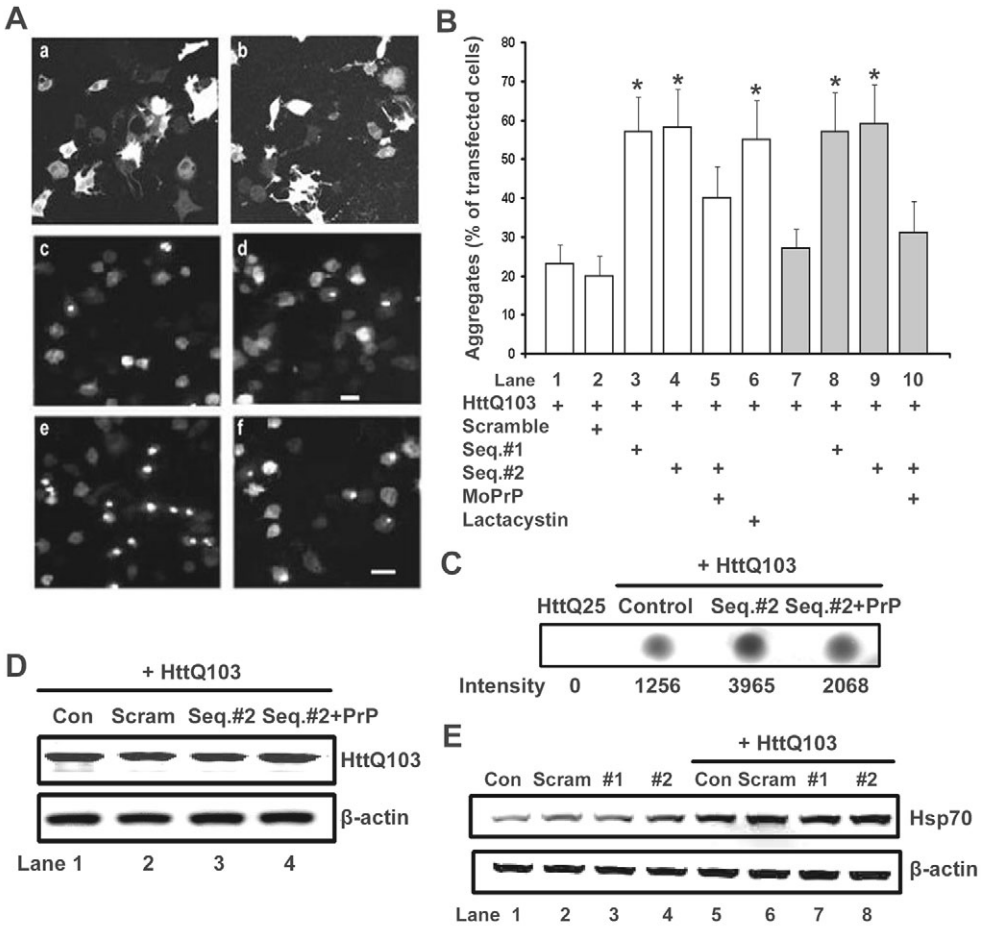

Fig. 2. Effect of PrP^C depletion on Htt aggregation in neuronal cells. (A) Immunofluorescence images of SN56 cells following 48 hours after transfection with GFP-HttQ25 (a,b) and HttQ103 (c-f) constructs. The cells were transfected with (a,c) GFP-Htt constructs only, (b,e) oligonucleotide 2 and GFP-Htt constructs, (d) scramble oligonucleotide and GFP-HttQ103 construct, (f) oligonucleotide 2, GFP-HttQ103 construct and mouse PrP^C expressing vector. (B) The number of aggregates was measured 48 hours after GFP-HttQ103 transfection in control neuronal cells (lane 1 and 7), and cells treated with scramble oligonucleotide (lane 2), oligonucleotide 1 (lanes 3 and 8), oligonucleotide 2 (lanes 4 and 9), oligonucleotide 2 and co-transfected with mouse PrP^C-expressing vector (lanes 5 and 10), and in transfected cells treated overnight with 10 μM lactacystin (lane 6). The open and gray bars are data obtained from SN56 and N2a cells, respectively. *P<0.05 and **P<0.01 compared with transfected control cells. (C) Filtration assay to measure aggregated protein lysates from SN56 cells transfected with HttQ25, HttQ103, oligonucleotides 2 followed by transfection of HttQ103 expressing vector, and oligonucleotides 2 followed by transfection of HttQ103 and mouse PrP^C expressing vectors. The intensity of the Htt retained on the membrane is quantified beneath the dot blot for each experimental condition. (D) The level of HttQ103 expression in cells transfected under varying conditions. Cell lysates (100 μg) from SN56 cells transfected with HttQ103, scrambled oligonucleotide followed by HttQ103, oligonucleotides 2 followed by HttQ103, and oligonucleotides 2 followed by HttQ103 and mouse PrP^C. The western blot was probed using anti-GFP and anti-actin antibodies. (E) The level of Hsp70 expression in SN56 cells under varying conditions in the presence and absence of HttQ103. Cells were transfected with either HttQ25 or HttQ103. Cells were either mock transfected or transfected with scramble sequence, sequence 1 and sequence 2 oligonucleotides.

blot analysis was performed on the cell lysates of the transfected cells. Similar levels of expression of HttQ103 were obtained in lysates from control cells, PrP^C-depleted cells, and the PrP^C-rescued cells (Fig. 2D). Moreover, PrP^C depletion did not significantly affect the expression of Hsp70, although HttQ103 expression did cause a significant increase in Hsp70 levels under all conditions (Fig. 2E).

Given that PrP^C depletion increased Htt aggregation, we examined whether depletion affected cell viability. Consistent with the results from the mouse PrP^C knockout studies, PrP^C depletion had no effect on either cell viability or apoptosis as measured by caspase-3 activity (Fig. 3). There was also no effect of HttQ25 expression on viability and caspase-3 activity in PrP^C-depleted SN56 cells. As expected, transfection with HttQ103 alone caused a marked decrease in viability and an increase in caspase-3 activity. Interestingly, in HttQ103-expressing cells, PrP^C depletion caused a further decrease in cell viability and increase in caspase-3 activity, which could be partially rescued by expression of mouse PrP^C. Therefore, PrP^C functions in neuronal cells to reduce HttQ103 aggregation and increase viability of the cells expressing HttQ103.

One possible mechanism for these observed phenotypes is that PrP^C depletion causes these effects indirectly by reducing proteasome activity. As shown in Fig. 4, this is indeed the case.

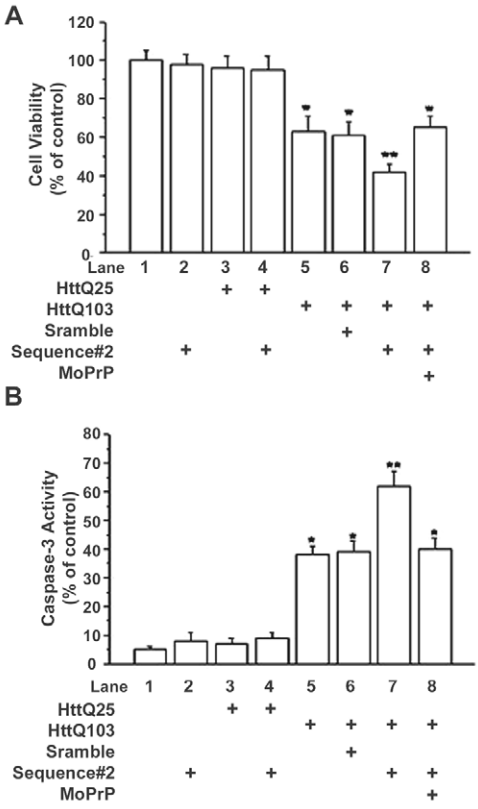

Fig. 3. Effects of PrP^C depletion on HttQ103 induced cytotoxicity and apoptosis in SN56 cells. The extent of cytotoxicity and apoptosis were measured in mock- and PrP^C-depleted cells 48 hours after transfection with either HttQ25 or HttQ103. (A) Cell viability was measured by MTT assay as described in Materials and Methods. (B) Apoptosis was measured by immunostaining for cleaved caspase-3 using Rhodamine-conjugated secondary antibody. The data represent an average of three experiments in which 500 transfected cells were analyzed to determine caspase-3 staining. $*P<0.05$ and $**P<0.01$ compared to control cultures.

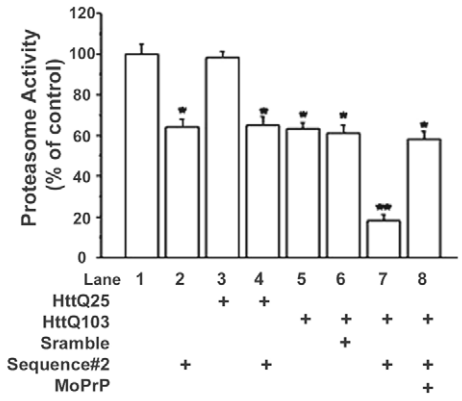

Fig. 4. Effects of PrP^C depletion on proteasome activity in SN56 cells. The activity of proteasome was measured in mock- and PrP^C-depleted cells 48 hours following transfection with either HttQ25 or HttQ103. $*P<0.05$ and $**P<0.01$ compared with control cultures.

PrP^C depletion causes a 40% decrease in proteasome activity. The expression of HttQ25 has no effect on proteasome activity, whereas expression of HttQ103 caused a 40% decrease in proteasome activity, in agreement with other studies (Jana et al., 2001; Nishitoh et al., 2002; Rangone et al., 2005). When HttQ103 was transfected in PrP^C-depleted cells, there was a further reduction in proteasome activity. Specifically, when HttQ103 was expressed in PrP^C-depleted cells, the proteasome activity was reduced to 15% of the control cells. Therefore, both HttQ103 expression and PrP^C depletion caused a reduction in proteasome activity and the two effects appear additive. The marked reduction in proteasome activity in PrP^C-depleted cells probably caused the marked increase in HttQ103 aggregation, similar to that observed when proteasome activity was inhibited with lactacystin.

Since oxidative stress causes a reduction in proteasome activity (Obin et al., 1998; Reinheckel et al., 2000), we measured whether PrP^C depletion causes an increase in ROS levels. First, using fluorescence imaging we determined whether the ROS level was higher in PrP^C-depleted cells. SiRNA transfections were performed using oligonucleotides conjugated to a fluorophore to enable us to visualize the transfected cells (Fig. 5Aa,c). Compared with transfection with scramble oligonucleotide, the ROS fluorescence intensity was much greater in cells transfected with oligonucleotide 1 than with the scramble oligonucleotide (Fig. 5Ab,d). The ROS fluorescence intensity of cells transfected with the scramble vector was not significantly different from that of the non-transfected cells (see cells with asterisks). Quantification of the ROS levels in the SN56 cells by FACs analysis showed that PrP^C depletion caused more than a threefold increase in ROS levels compared with control cells (Fig. 5B). Expression of HttQ103 also caused about a fourfold increase in ROS, in agreement with previous studies (Solans et al., 2006; Wyttenbach et al., 2002). PrP^C-depleted cells transfected with HttQ103 showed a sevenfold increase in ROS levels, so again, the effects of PrP^C depletion and HttQ103 appears additive.

Table 1 shows that the increase in ROS in PrP^C-depleted cells was due to a reduction in antioxidant enzyme activities. Compared to control cells, PrP^C-depleted cells showed a marked reduction in the activities of SOD, catalase and glutathione reductase. This reduction was partially rescued when the cells were transfected with mouse PrP^C vector. Therefore, PrP^C depletion caused a decrease in antioxidant activity, which in turn increased ROS levels thus causing decreased proteasome activity.

To determine whether PrP^C functions to protect other cell types that endogenously express PrP^C, Caco-2 and HT-29 cells, two human colonic adenocarcinoma cell lines, were depleted of PrP^C. As shown in Fig. 6, these cell lines endogenously express PrP^C, with HT-29 cells expressing much higher levels of PrP^C than the Caco-2 cells (Garmy et al., 2006). By using siRNA oligomers made against human PrP^C, we achieved at least a 90% reduction of PrP^C in both Caco-2 and HT-29 cells (Fig. 6A). In contrast to neuronal cells, depletion of PrP^C from both intestinal cell lines did not significantly affect either proteasome activity or ROS levels (Fig. 6B,C). These results suggest that PrP^C confers protection only on neuronal cells.

The protective effect of PrP^C in neuronal cells was further evident when mouse PrP^C was overexpressed in SN56 cells. Overexpression of PrP^C caused a reduction in the percentage of cells with HttQ103 granules and an increase in proteasome

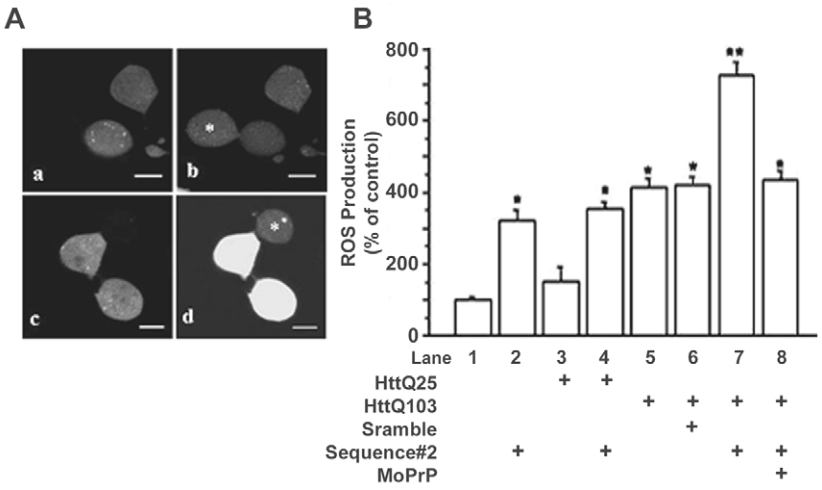

Fig. 5. Effects of PrP^C depletion on ROS levels in SN56 cells. (A) Immunofluorescence images of fixed cells that were transfected with either scramble (a,b) or sequence 1 (c,d) oligonucleotides and conjugated to Dye547-3′ as an indicator of transfection (a,c) or stained with DFA to show ROS levels (b,d). The asterisks indicate cells not transfected with oligonucleotides. The images were produced using identical scan-settings that allowed direct comparison of fluorescence intensities. Bar, 20 μm. (B) The level of ROS was measured 72 hours after siRNA oligomer transfection in the presence and absence of Htt. **P*<0.05 and ***P*<0.01 compared with control transfected cells.

activity (Fig. 7). Specifically, when mouse PrP^C was overexpressed in SN56 cells, HttQ103 granules decreased from 20% to 10%. Similarly, in SN56 cells overexpressing PrP^C showed that HttQ103 only caused a 10% decrease in proteasome activity compared to the 40% decrease that occurred in SN56 cells just transfected with HttQ103. Consistent with the lack of protection conferred by PrP^C on non-neuronal Caco-2 and HT-29 cells, there was no effect of PrP^C expression on the percentage of cells with either HttQ103 granules or proteasome activity in the non-neuronal cell lines NIH3T3 and CHO. Therefore, consistent with our finding that PrP^C only confers protection on neuronal cells, expression of PrP^C is not protective against Htt-induced toxicity in non-neuronal cells that do not express endogenous PrP^C.

Finally we examined whether the presence of the scrapie form of prion, PrP^{Sc} affected the aggregation properties of HttQ103. As shown in Fig. 8A, comparison of uninfected and scrapie-infected SN56 cells (ScSN56) showed that 40% of the infected cells had HttQ103 granules compared to 25% of the infected cells. ScSN56 and Sn56 cells had similar viability, probably due to the low levels of scrapie in most of the ScSN56 cells. However, HttQ103 had a different effect on the viability of SN56 and ScSN56 cells. Expression of HttQ103 caused a 40% reduction in the viability of the SN56 cells whereas it caused a 60% reduction in the viability of the ScSN56 cells. Although it had no effect on viability, PrP^{Sc} alone caused an increase in ROS and a corresponding decrease in proteasome activity, and HttQ103 caused further changes. Cells with both PrP^{Sc} and HttQ103 showed a sixfold increase in ROS activity compared to a twofold increase with PrP^{Sc} alone and a fourfold increase with HttQ103 alone (Fig. 8C). Similarly, cells with both PrP^{Sc} and HttQ103 showed a 60% decrease in proteasome activity compared to a 25% decrease with PrP^{Sc} alone and a 40% decrease in proteasome activity with HttQ103 alone (Fig. 8D). These data show that PrP^{Sc} further reduces proteasome activity beyond the reduction caused by HttQ103 expression alone, an effect that could explain the increase in the percentage of cells with HttQ103 granules and the decrease in cell viability in ScSN56 cells expressing HttQ103. PrP^{Sc} could cause these effects directly by contributing to the total amount of aggregated protein in the cell, or indirectly by decreasing the amount of active PrP^C, or both.

Discussion

A long-standing question in the prion field is whether the loss of PrP^C from scrapie-infected nerve cells contributes to the neuropathology of the disease. To investigate this question, we examined whether PrP^C depletion affects Htt aggregation. Our results showed that PrP^C depletion caused a marked increase in HttQ103 aggregation in both N2A and SN56 neuronal cell lines. The increase in the fraction of cells with Htt granules that occurred after PrP^C depletion was similar to the increase that occurred after treatment of the cells with the proteasome inhibitor, lactacystin. Consistent with this observation, the proteasome activity of PrP^C-depleted cells expressing HttQ103 was only 15% of that in control cells whereas it was 60% of the control activity in the absence of HttQ103 expression. Thus when the cells are stressed, there is a further decrease in proteasome function in PrP^C-depleted neuronal cells.

Table 1. Activities of antioxidant enzymes

	SOD (IU/mg protein)	Catalase (IU/mg protein)	Glutathione reductase (IU/mg protein)
Control	3.9±0.32	2.5±0.25	20.3±1.6
siRNA scramble	3.6±0.28	2.3±0.15	18.6±2.1
siRNAprion 1	2.4±0.19*	1.7±0.18*	8.1±0.8*
siRNAprion 2	2.1±0.18*	1.6±0.19*	7.3±0.9*
siRNAprion 2 + PrP^C	3.1±0.45	2.2±0.23	15.2±0.25

Each value is the mean ± s.d. from four independent determinations. All activities were determined in cell homogenates as described in Materials and Methods.
*Significantly different from control at *P*<0.01. SOD, superoxide dismutase.

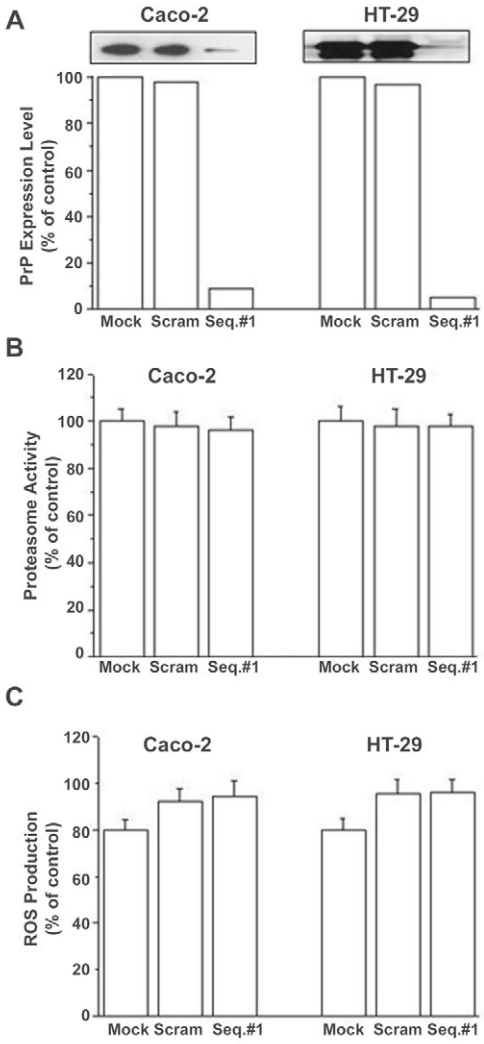

Fig. 6. Effects of PrP^C depletion on proteasome activity and cellular ROS in Caco-2 and HT-29 cells. (A) Western blot of PrP^C levels in siRNA-treated Caco-2 and HT-29 cells and quantification of this blot. (B) The activity of proteasome measured 72 hours after transfection with oligonucleotides in Caco-2 and HT-29 cells. (C) ROS levels measured 72 hours after transfection with oligonucleotides in Caco-2 and HT-29 cells.

Depletion of PrP^C from neuronal cells also caused a reduction in the activity of antioxidant enzymes. However, despite this reduction in antioxidant enzymes and proteasome activity, there is no obvious phenotype caused by PrP^C depletion in the absence of stress. Expression of HttQ25, had no effect on the PrP^C-depleted cells. However, when the cells were stressed by expression of HttQ103, the PrP^C-depleted cells showed a significant loss of viability and a marked increase in ROS levels. Our results are in agreement with the study of Klamt et al. (Klamt et al., 2001) in which an imbalance in antioxidant defense was found in PrP^C-knockout mice. Specifically, oxidative damage to lipids and proteins was much higher in the knockout mice, and the activities of SOD and catalase were reduced.

Interestingly, we found that scrapie-infected SN56 cells had properties similar to PrP^C-depleted cells. The scrapie infected SN56 cells showed increased HttQ103 aggregation, decreased proteasome activity, and increased ROS levels. In agreement with our results, scrapie-infected hypothalamic neuronal GT1 cells displayed a higher sensitivity to oxidative stress than non-infected cells, as well as a decrease in viability when subjected to stress (Milhavet et al., 2000). An increase in ROS levels was also found in scrapie-infected N2a cells (Fernaeus et al., 2005). It is not clear whether these effects of scrapie infection are due to the scrapie aggregation itself or whether it is also due to a reduction in the level of PrP^C. Recent results from the Collinge laboratory showed that disruption of the prion gene in scrapie-infected mice reversed any morphological, neurological or behavioral defects due to scrapie infection (Malluci et al., 2002; Malluci et al., 2007). This shows that scrapie pathology can be reversed by removing PrP^C from the cells. However, it is still possible that when scrapie aggregates are present, their effects are worsened by the absence of normal PrP^C function.

Although we found that PrP^C was protective in neuronal cells, it did not confer protection on non-neuronal cells. Depleting PrP^C from two human epithelial cell lines, Caco-2 and HT-29, which like neuronal cells express PrP^C endogenously, had no effect on proteasome activity and ROS levels. Furthermore, overexpression of PrP^C reduced Htt aggregation in neuronal cells, but had no effect in either HeLa or CHO cells. Other labs have found that expressing PrP^C in breast carcinoma MCF-7 cells inhibited the proapoptotic Bax conformational change (Roucou et al., 2005) and necrosis factor alpha-induced cell death (Diarra-Mehrpour et al., 2004), but there is no evidence that PrP^C expression affected either ROS levels or proteasome activity in these cells.

The neuro-specific protective effect of PrP^C suggests that the signaling pathway activated by PrP^C only occurs in neurons. Many proteins have been reported to bind to PrP^C, including Sti1, N-CAM, mNOS, APLP1, BL-2 and synapsin (Sakudo et al., 2006) and could be involved in the neuro-specific signaling pathway even if they are not only expressed in nerves. In addition, in a recent model proposed by the Harris laboratory to explain the toxic effects of the truncated PrP^C protein (Δ105-125) on mouse viability, they suggested that there is a receptor on the outer surface of nerve cells that normally binds intact PrP^C (Li et al., 2007). This putative receptor could be involved in the neuro-specific signaling pathway activated by PrP^C.

Whatever the nature of the neuro-specific receptor that interacts with PrP^C, it is clear that nerve cells respond to signaling triggered by PrP^C by increasing cellular defense enzymes. Several pathways implicated in PrP^C signaling are consistent with the increase in antioxidant enzymatic activities. A recent study showed that there is a reduction in AKT signaling in PrP^C knockout mice compared to control mice (Weise et al., 2006). Similarly, attachment of PrP^C-fusion proteins to monocytes caused an increase in AKT and ERK1 and ERK2 signaling (Krebs et al., 2006). Consistent with these observations there is an increase in phosphatidylinositol 3-kinase signaling in PrP^C-expressing N2a cells (Vassallo et al., 2005). These signaling pathways promote cell survival and are perhaps responsible for the neuroprotective effect of PrP^C on cell signaling. Ultimately, these neuroprotective pathways are not only regulated via phosphorylation but also by activation of the transcription factor, nuclear factor-κB, which is a central regulator of immunity, inflammation and cell survival.

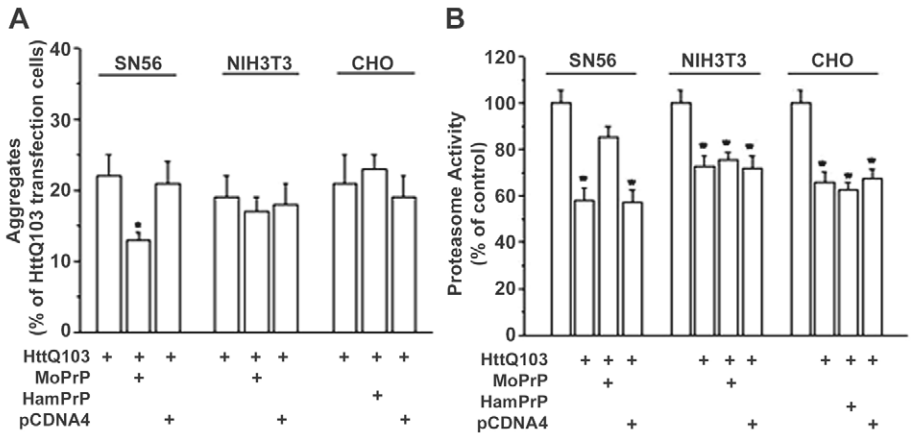

Fig. 7. Effects of expressing PrP^C on HttQ103 aggregation and proteasome activity in neuronal (SN56) and non-neuronal cells (HeLa and CHO). (A) Effect of overexpression of PrP^C on the extent of HttQ103 aggregation in different cell types. (B) Effect of overexpression of PrP^C on proteasome activity in different cell types. In addition to HttQ103, cells were co-transfected with the same concentration of either pcDNA4 or PrP^C expressing vector, as indicated. *P<0.01 compared with cells transfected with only HttQ103.

A diagram of the activation of PrP^C that we observed in neuronal cells is shown in Fig. 9. In this model, the deleterious effect of Htt aggregation caused by increasing ROS activity is mitigated by the action of PrP^C, which reduces ROS. This in turn increases proteasome activity and reduces Htt aggregation. Interestingly, in a transgenic mouse model of amyotrophic lateral sclerosis, the PrP^C protein was specifically repressed when the G85R SOD mutant was overexpressed, but overexpression of wild-type SOD had no effect on PrP^C (Dupuis et al., 2002). This suggests that there may be a feedback mechanism that actually downregulates PrP^C when cells are under stress, which in turn exacerbates the stress on the cells. There has been no parallel study in mouse models of Huntington disease to determine whether PrP^C levels are reduced in animals overexpressing Htt with expanded polyglutamine repeats.

In conclusion, PrP^C provides protection against protein aggregation and this protection is neuronal specific. It may be that neurons are particularly sensitive to damage from aggregated proteins and therefore have a specific regulatory pathway that protects against this damage. By maintaining ROS levels, PrP^C protects the cell from a reduction in proteasome activity, thereby helping to prevent protein aggregation. As decreased proteasome activity has been implicated in several neurodegenerative disorders and PrP^C specifically increases proteasome activity in neuronal cells, it will be of interest in the future to investigate the protective role that PrP^C plays in neurodegenerative diseases caused by protein aggregation.

Materials and Methods

Cell culture

The SN56 cells were a generous gift from Bruce Wainer (Department of Pathology, Emory University School of Medicine, Atlanta, GA). The chronically infected ScSN56 cell line infected with the Chandler strain of scrapie was a generous gift from Byron Caughey (RML, Hamilton, MT). SN56 and ScSN56 cells were cultured as described previously (Baron et al., 2006). N2A (mouse neuroblastama cell line),

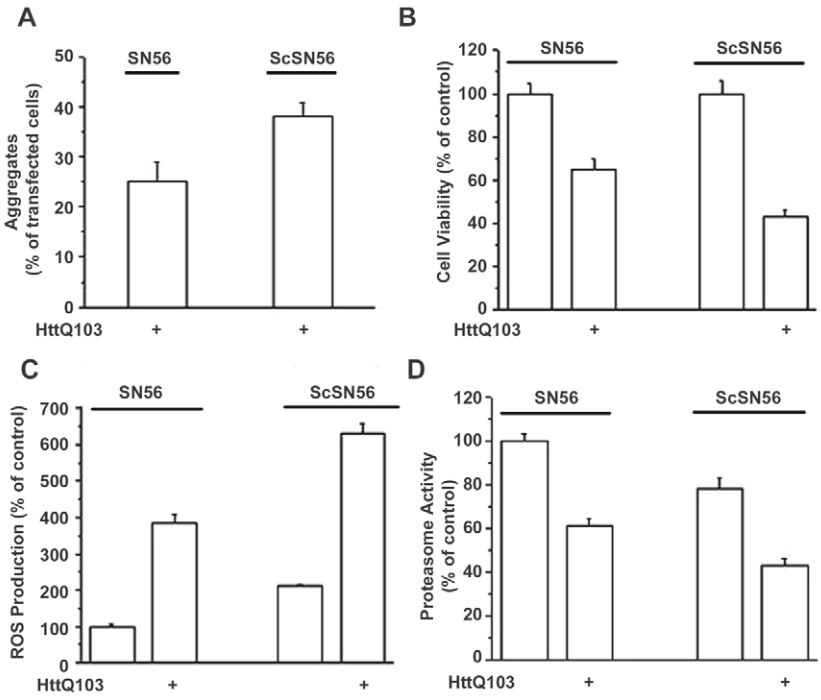

Fig. 8. Effects of scrapie on HttQ103 aggregation, viability, proteasome activity, and ROS level in SN56 cells. The extent of HttQ103 aggregation (A), neurotoxicity (B), ROS level (C) and proteasome activity (D) were measured 48 hours after SN56 and ScSN56 cells were transfected with Htt Q103.

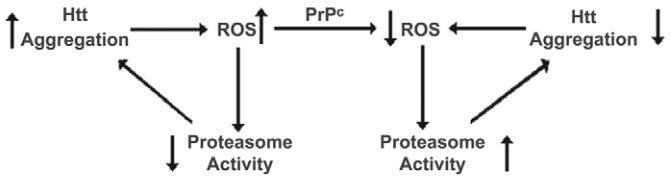

Fig. 9. Diagram showing the interrelationship between ROS, proteasome activity, huntingtin aggregation and PrP^C expression. At any given time in the nerve cell, there are competing pathways in which PrP^C expression reduces ROS and in turn increases proteasome activity, whereas Htt aggregation has the opposite effect.

NIH-3T3 (mouse fibroblast cell line) and Caco-2 and HT-29 (human intestinal cell lines) were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum, 2 mM glutamine, and 1% penicillin-streptomycin in 75 cm^2 culture bottles in a 5% CO_2 atmosphere at 37°C. CHO cells were cultured and seeded as described previously (Yim et al., 2005).

The following antibodies were used: anti-GFP polyclonal antibody (Abcam, Cambridge, UK), anti-Hsp70 monoclonal antibody (BD Transduction Laboratory, San Josa, CA), anti-β-actin polyclonal antibody (Abcam), anti-prion monoclonal antibodies D13 (InPro Corp., San Francisco, CA) and SAF70 (Cayman, Ann Arbor, MI). All secondary antibodies were from Jackson ImmunoResearch Laboratory (West Grove, PA).

RNAi, western blot, immunoprecipitation and filter retardation assays

The following oligonucleotides used in RNAi experiments were from Dharmacon (Chicago, IL): mouse prion 1 (nucleotides 299-320), mouse prion 2 (nucleotides 1278-1396), human prion 1(nucleotides 502-522) and human prion 2 (nucleotides 1034-1054), scrambled vectors of mouse and human prion sequence 1. Identical siRNA oligonucleotides were made that were conjugated to Dye547-3′ (Dharmacon, Chicago, IL) to indicate siRNA-transfected cells. These RNA olignucleotides were transiently transfected into SN56 and Caco-2 cell using Lipofectamine 2000 reagent (Invitrogen, San Diego, CA). Control cells were incubated with Lipofectamine under identical conditions as in the RNAi experiments.

To quantify protein, lysates were run on SDS-PAGE gels (Invitrogen) and then western blot analysis was performed. PrP^C was detected by immunoblotting using SAF-70 anti-prion antibody, Htt was detected using an anti-GFP antibody, and Hsp70 was detected using an anti-Hsp70 antibody. The protein bands were detected using chemiluminescent substrate (Pierce, Rockford, IL) and analyzed using the ChemiImager densitometer (Alpha Innotech Corp., San Leandro, CA). The linear range of the western blot analysis was determined by loading from 5 μg to 200 μg of cell lysates. Quantification of the western blots established linearity over a 10-fold range of protein concentration. PrP^C was immunoprecipitated by mixing the cell lysate (500 μg) with 4 μg of D13 anti-prion antibody followed by the addition of 50 μl of protein A-Sepharose 4 Fast Flow (GE Health Science, Piscataway, NJ). After incubating at 4°C, the Sepharose beads were collected by centrifugation at 14,000 ***g***, washed with PBS and resuspended in 50 μl of 2× Laemmli sample buffer. Protein concentration of the lysates was measured using the Bradford reagent (Bio-Rad, Hercules, CA). For the filter retardation assay, 20 μg of denatured protein samples were filtered through a 0.2 μm cellulose acetate membrane (Adventec MFS, Dublin, CA) using a dot-blot filtration unit (Bio-Rad).

Plasmids and transfection

The Htt polyglutamine constructs (HttQ25 and HttQ103) containing glutamine repeat expansion had an EGFP-tag on its C-terminal (Zeng et al., 2004). A mouse prion gene with the hamster epitope tag (a gift from S. Priola, Rocky Mountain Lab, MT) and a hamster prion gene (a gift from D. Ramanujan Hegde, NIH, Bethesda, MD) was subcloned using *Hin*dIII and *Xho*I restriction sites into pCDNA4 vector (Invitrogen). Cells were seeded and transfected with Htt plasmids as described previously (Zeng et al., 2004). At 48 hours after transfection, cells on coverslips were fixed with 4% paraformaldehyde and 3 μg/ml 4′,6-diamidino-2-phenylindole (Invitrogen) in 1× PBS at room temperature for 20 minutes and mounted.

Microscopy and immunostaining

Cells grown on two-well chamber slides (Labtek, NY) were imaged with a 40×, 1.4 NA objective using a Zeiss LSM 510 confocal microscope (Jena, Germany). The argon and helium lasers were used to excite at 488 nm and 543nm, respectively. Apoptosis was measured by fixing and staining the cells with cleaved caspase-3 antibody (Cell Signaling Technology, Danver, MA). Cells were counted as aggregate positive if one or several granules were visible within a cell. GFP-positive cells (500 per experiment) were counted in multiple random visual fields on each slide.

Measurement of proteasome activity, antioxidant activities, reactive oxygen species (ROS) and cell toxicity

Proteasome activity was measured from the fluorescence intensity of amino-methyl coumarin (AMC)-conjugated to the chymotrypsin peptide substrate LLVY, using a commercial activity assay kit (Chemicon, Temecula, CA). Cleavage products were measured using the Spectra max Gemini fluorescence plate reader (Molecular Devices Co., Sunyvale, CA). The activities of superoxide dismutase (SOD; Chemicon), catalase (Invitrogen) and glutathione reductase (Cayman) were measured in the cell homogenates. To measure ROS levels, cells were stained with DCF-DA or red CC-1 (Invitrogen). For live cells, FACS analysis was performed on the FACS Calibur instrument (Becton Dickinson, Franklin Lakes, NJ) and for fixed cells, images were obtained on the Zeiss LSM 510 confocal microscope. Cell viability was assessed using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)-based colorimetric assay kit (Invitrogen).

Data analysis

All data are an average of at least three independent experiments. Student's *t*-test was used to assess the statistical significance of differences.

References

Aguzzi, A. and Weissmann, C. (1997). Prion research: the next frontiers. *Nature* **389**, 795-797.

Baron, G. S., Magalhaes, A. C., Prado, M. A. M. and Caughey, B. (2006). Mouse-adapted scrapie infection of SN56 cells: greater efficiency with microsome-associated versus purified PrP-res. *J. Virol.* **80**, 2106-2117.

Brown, D. R. and Besinger, A. (1998). Prion protein expression and superoxide dismutase activity. *Biochem. J.* **334**, 423-429.

Brown, D. R., Qin, K., Herms, J. W., Madlung, A., Manson, J., Strome, R., Fraser, P. E., Kruck, T., von Bohlen, A., Schulz-Schaeffer, W. et al. (1997a). The cellular prion protein binds copper in vivo. *Nature* **390**, 684-687.

Brown, D. R., Schulz-Schaeffer, W. J., Schmidt, B. and Kretzschmar, H. A. (1997b). Prion protein-deficient cells show altered response to oxidative stress due to decreased SOD-1 activity. *Exp. Neurol.* **146**, 104-112.

Colling, S. B., Collinge, J. and Jefferys, J. G. R. (1996). Hippocampal slices from prion protein null mice: disrupted Ca2+-activated K+ currents. *Neurosci. Lett.* **209**, 49-52.

Collinge, J., Owen, F., Poulter, M., Leach, M., Crow, T. J., Rossor, M. N., Hardy, J., Mullan, M. J., Janota, I. and Lantos, P. L. (1990). Prion dementia without characteristic pathology. *Lancet* **336**, 7-9.

Collinge, J., Whittington, M. A., Sidle, K. C. L., Smith, C. J., Palmer, M. S., Clarke, A. R. and Jefferys, J. G. R. (1994). Prion protein is necessary for normal synaptic function. *Nature* **370**, 295-297.

Diarra-Mehrpour, M., Arrabal, S., Jalil, A., Pinson, X., Gaudin, C., Pietu, G., Pitaval, A., Ripoche, H., Eloit, M., Dormont, D. et al. (2004). Prion protein prevents human breast carcinoma cell line from tumor necrosis factor {alpha}-induced cell death. *Cancer Res.* **64**, 719-727.

Dupuis, L., Mbebi, C., Gonzalez de Aguilar, J. L., Rene, F., Muller, A., de Tapia, M. and Loeffler, J.-P. (2002). Loss of prion protein in a transgenic model of amyotrophic lateral sclerosis. *Mol. Cell. Neurosci.* **19**, 216-224.

Fernaeus, S., Reis, K., Bedecs, K. and Land, T. (2005). Increased susceptibility to oxidative stress in scrapie-infected neuroblastoma cells is associated with intracellular iron status. *Neurosci. Lett.* **389**, 133-136.

Garmy, N., Guo, X.-J., Taieb, N., Tourres, C., Tamalet, C., Fantini, J. and Yahi, N. (2006). Cellular isoform of the prion protein PrP^C in human intestinal cell lines: Genetic polymorphism at codon **129**, mRNA quantification and protein detection in lipid rafts. *Cell Biol. Int.* **30**, 559-567.

Hutter, G. G., Heppner, F. L. and Aguzzi, A. A. (2003). No superoxide dismutase activity of cellular prion protein in vivo. *Biol. Chem.* **384**, 1279-1285.

Jana, N. R., Zemskov, E. A., Wang, G.-h. and Nukina, N. (2001). Altered proteasomal function due to the expression of polyglutamine-expanded truncated N-terminal huntingtin induces apoptosis by caspase activation through mitochondrial cytochrome c release. *Hum. Mol. Genet.* **10**, 1049-1059.

Jefferys, J. G. R., Empson, R. M., Whittington, M. A. and Prusiner, S. B. (1994). Scrapie infection of transgenic mice leads to network and intrinsic dysfunction of cortical and hippocampal neurones. *Neurobiol. Dis.* **1**, 25-30.

Johnston, A. R., Black, C., Fraser, J. and MacLeod, N. (1997). Scrapie infection alters the membrane and synaptic properties of mouse hippocampal CA1 pyramidal neurones. *J. Physiol.* **500**, 1-15.

Jones, S., Batchelor, M., Bhelt, D., Clarke, A. R., Collinge, J. and Jackson, G. S. (2005). Recombinant prion protein does not possess SOD-1 activity. *Biochem. J.* **392**, 309-312.

Keshet, G. I., Ovadia, H., Taraboulos, A. and Gabizon, R. (1999). Scrapie-infected mice and PrP knockout mice share abnormal localization and activity of neuronal nitric oxide synthase. *J. Neurochem.* **72**, 1224-1231.

Klamt, F., Dal-Pizzol, F., Conte da Frota, M. L., Jr, Walz, R., Andrades, M. E., da Silva, E. G., Brentani, R. R., Izquierdo, I. and Fonseca Moreira, J. C. (2001). Imbalance of antioxidant defense in mice lacking cellular prion protein. *Free Rad. Biol. Med.* **30**, 1137-1144.

Krebs, B., Dorner-Ciossek, C., Schmalzbauer, R., Vassallo, N., Herms, J. and

Kretzschmar, H. A. (2006). Prion protein induced signaling cascades in monocytes. *Biochem. Biophys. Res. Commun.* **340**, 13-22.
Kuwahara, C., Takeuchi, A. M., Nishimura, T., Haraguchi, K., Kubosaki, A., Matsumoto, Y., Saeki, K., Matsumoto, Y., Yokoyama, T., Itohara, S. et al. (1999). Prions prevent neuronal cell-line death. *Nature* **400**, 225-226.
Leist, M. and Nicotera, P. (1998). Apoptosis versus necrosis: the shape of neuronal cell death. *Results Probl. Cell Differ.* **24**, 105-135.
Li, A., Christensnen, H. M., Stewart, L. R., Roth, K. A., Chiesa, R. and Harris, D. A. (2007). Neonatal lethality in transgenic mice expressing prion protein with a deletion of residues 105-125. *EMBO J.* **26**, 548-558.
Mallucci, G., Ratte, S. A., Asante, E. A., Lineham, J., Gowland, I., Jefferys, J. G. and Collinge, J. (2002). Post-natal knockout of prion portein alters hippocampal CA1 properties, but does not result in neurodegeneration. *EMBO J.* **21**, 202-210.
Mallucci, G., Dickinson, A., Linehan, J., Klohn, P.-C., Brandner, S. and Collinge, J. (2003). Depleting neuronal PrP in prion infection prevents disease and reverses spongiosis. *Science* **302**, 871-874.
Mallucci, G. R., White, M. D., Farmer, M., Dickinson, A., Khatun, H., Powell, A. D., Brandner, S., Jefferys, J. G. R. and Collinge, J. (2007). Targeting cellular prion protein reverses early cognitive deficits and neurophysiological dysfunction in prion-infected mice. *Neuron* **53**, 325-335.
Manson, J. C., Hope, J., Clarke, A. R., Johnston, A., Black, C. and MacLeod, N. (1995). PrP gene dosage and long term potentiation. *Neurodegeneration* **4**, 113-114.
Medori, R. M., Montagna, P. M., Tritschler, H. J. P., LeBlanc, A. P., Cortelli, P. M., Tinuper, P. M., Lugaresi, E. M. and Gambetti, P. M. (1992). Fatal familial insomnia: a second kindred with mutation of prion protein gene at codon 178. *Neurology* **42**, 669-672.
Milhavet, O., McMahon, H. E. M., Rachidi, W., Nishida, N., Katamine, S., Mange, A., Arlotto, M., Casanova, D., Riondel, J., Favier, A. et al. (2000). Prion infection impairs the cellular response to oxidative stress. *Proc. Natl. Acad. Sci. USA* **97**, 13937-13942.
Nishitoh, H., Matsuzawa, A., Tobiume, K., Saegusa, K., Takeda, K., Inoue, K., Hori, S., Kakizuka, A. and Ichijo, H. (2002). ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. *Genes Dev.* **16**, 1345-1355.
Obin, M., Shang, F., Gong, X., Handelman, G., Blumberg, J. and Taylor, A. (1998). Redox regulation of ubiquitin-conjugating enzymes: mechanistic insights using the thiol-specific oxidant diamide. *FASEB J.* **12**, 561-569.
Ovadia, H., Rosenmann, H., Shezen, E., Halimi, M., Ofran, I. and Gabizon, R. (1996). Effect of scrapie infection on the activity of neuronal nitric-oxide synthase in brain and neuroblastoma cells. *J. Biol. Chem.* **271**, 16856-16861.
Prusiner, S. B. (1998). Prions. *Proc. Natl. Acad. Sci. USA* **95**, 13363-13383.
Rangone, H., Pardo, R., Colin, E., Girault, J. A., Saudou, F. and Humbert, S. (2005). Phosphorylation of arfaptin 2 at Ser260 by AKT inhibits Poly-Q-huntingtin induced toxicity by rescuing proteasome impairment. *J. Biol. Chem.* **280**, 22021-22028.
Reinheckel, T., Ullrich, O., Sitte, N. and Grune, T. (2000). Differential impairment of 20S and 26S proteasome activities in human hematopoietic K562 cells during oxidative stress. *Arch. Biochem. Biophys.* **377**, 65-68.
Roucou, X. and LeBlanc, A. (2005). Cellular prion protein neuroprotective function: implications in prion diseases. *J. Mol. Med.* **83**, 3-11.
Roucou, X., Guo, Q., Zhang, Y., Goodyer, C. G. and LeBlanc, A. C. (2003). Cytosolic prion protein is not toxic and protects against Bax-mediated cell death in human primary neurons. *J. Biol. Chem.* **278**, 40877-40881.
Roucou, X., Giannopoulos, P. N., Zhang, Y., Jodoin, J., Goodyer, C. G. and LeBlanc, A. (2005). Cellular prion protein inhibits proapoptotic Bax conformational change in human neurons and in breast carcinoma MCF-7 cells. *Cell Death Differ.* **12**, 783-795.
Sakudo, A., Lee, D., Li, S., Nakamura, T., Matsumoto, Y., Saeki, K., Itohara, S., Ikuta, K. and Onodera, T. (2005). PrP cooperates with STI1 to regulate SOD activity in PrP-deficient neuronal cell line. *Biochem. Biophys. Res. Commun.* **328**, 14-19.
Sakudo, A., Onodera, T., Suganuma, Y., Kobayashi, T., Saeki, K. and Ikuta, K. (2006). Recent advances in clarifying prion protein functions using knockout mice and derived cell lines. *Mini Rev. Med. Chem.* **6**, 589-601.
Solans, A., Zambrano, A., Rodriguez, M. and Barrientos, A. (2006). Cytotoxicity of a mutant huntingtin fragment in yeast involves early alterations in mitochondrial OXPHOS complex II and III. *Hum. Mol. Genet.* **15**, 3063-3081.
Vassallo, N., Herms, J., Behrens, C., Krebs, B., Saeki, K., Onodera, T., Windl, O. and Kretzschmar, H. A. (2005). Activation of phosphatidylinositol 3-kinase by cellular prion protein and its role in cell survival. *Biochem. Biophys. Res. Commun.* **332**, 75-82.
Weise, J., Sandau, R., Schwarting, S., Crome, O., Wrede, A., Schulz-Schaeffer, W., Zerr, I. and Bahr, M. (2006). Deletion of cellular prion protein results in reduced Akt activation, enhanced postischemic Caspase-3 activation, and exacerbation of ischemic brain injury. *Stroke* **37**, 1296-1300.
Weissmann, C. and Flechsig, E. (2003). PrP knock-out and PrP transgenic mice in prion research. *Br. Med. Bull.* **66**, 43-60.
Wong, B.-S., Wang, H., Brown, D. R. and Jones, I. M. (1999). Selective oxidation of methionine residues in prion proteins. *Biochem. Biophys. Res. Commun.* **259**, 352-355.
Wong, B.-S., Liu, T., Li, R., Pan, T., Petersen, R. B., Smith, M. A., Gambetti, P., Perry, G., Manson, J. C., Brown, D. R. et al. (2001). Increased levels of oxidative stress markers detected in the brains of mice devoid of prion protein. *J. Neurochem.* **76**, 565-572.
Wong, B.-S., Venien-Bryan, C., Williamson, R. A., Burton, D. R., Gambetti, P., Sy, M.-S., Brown, D. R. and Jones, I. M. (2000). Copper refolding of prion protein. *Biochem. Biophys. Res. Commun.* **276**, 1217-1224.
Wyttenbach, A., Sauvageot, O., Carmichael, J., Diaz-Latoud, C., Arrigo, A.-P. and Rubinsztein, D. C. (2002). Heat shock protein 27 prevents cellular polyglutamine toxicity and suppresses the increase of reactive oxygen species caused by huntingtin. *Hum. Mol. Genet.* **11**, 1137-1151.
Yim, Y.-I., Scarselletta, S., Zang, F., Wu, X., Lee, D.-w., Kang, Y.-s., Eisenberg, E. and Greene, L. E. (2005). Exchange of clathrin, AP2 and epsin on clathrin-coated pits in permeabilized tissue culture cells. *J. Cell Sci.* **118**, 2405-2413.
Zeng, X.-C., Bhasin, S., Wu, X., Lee, J.-G., Maffi, S., Nichols, C. J., Lee, K. J., Taylor, J. P., Greene, L. E. and Eisenberg, E. (2004). Hsp70 dynamics in vivo: effect of heat shock and protein aggregation. *J. Cell Sci.* **117**, 4991-5000.

Journal of Cell Science