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The cellular prion protein mediates neurotoxic signaling of -sheet-rich conformers independent of prion replication

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1st Editorial Decision 24 September 2010

Thank you for submitting your manuscript to the EMBO Journal. Your manuscript has now been seen by three referees and their comments are provided below.

As you can see the referees find the study interesting and important. However, they also raise some concerns with the analysis that have to be addressed in order for further consideration here. The referees find that further support for that endogenous PrPc mediates toxic signaling is needed and that the analysis should be extend to other cell models. Should you be able to address the concerns raised then we would consider a revised manuscript. Acceptance of your paper will be dependent upon persuading the referees that you have provided a sufficient amount of new data to answer all their criticisms. I should also add that it is EMBO Journal policy to allow a single round of revision only and that it is therefore important to address the raised concerns at this stage. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,
Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Resenberger et al explore the role of cellular PrP in toxicity from a range of misfolded proteins. They report the PrP-Sc, A-beta oligomers and yeast prions all mediated apoptosis in neuroplastoma cells via PrP-C. This signaling requires the N-terminal domain and the GPI anchor of PrP-C. It appears to be mediated by recognition of a common "oligomer" epitope detected with A11 antibody. The cell death is blocked by NMDA antagonists. While there had been suggestions that PrP-C plays a role in PrP-Sc induced degeneration and more recently in A-beta oligomer toxicity, these data provides a unifying and clear role for PrP-C in this process, and map the PrP sequence required. These findings will be a great interest and importance to the field. I have some technical comments.

1. Cell death is assessed only by active caspase immunostaining. It would be advantageous for the authors to use a second marker of apoptotic cells. More importantly, they should assess surviving cell number in parallel, at least in one experiment for each ligand.

2. The authors use neuroblastoma cells for their assays in a nice coverslip/overlay assay. However, they should assess function in primary neurons and its sensitivity to reducing endogenous PrP by knockdown, knockout or anti-PrP blocking. At least one cell death assay with each ligand this should be tested with such primary neurons.

3. The similarity of PrP-C dependence for these different ligands is striking. It would be of interest to assess their synergy in cell death mediated by PrP-C. Do low non-toxic doses of PrP-Sc and low non-toxic doses of A-beta synergize to produce cell death?

4. In the Methods, the description of peptide production provides nucleic acid sequence for a peptide.

Referee #2 (Remarks to the Author):

This paper builds on a previous study showing that PrP overexpression in SH-SY5Y human neuroblastoma cells sensitizes the cells to apoptosis induced by co-culture with scrapie infected N2a cells (ScN2a) (Rambold et al. 2008). Herein, the authors show that PrP overexpression in SH-SY5Y cells sensitizes the cells to apoptosis induced by ScN2a cells, toxic Aβ peptide oligomers, yeast NM and a β peptide, thereby addressing the important question of the role of PrP in neuronal toxicity occurring in protein misfolding neurodegenerative diseases other than prion diseases.

The main weakness of the paper comes from the fact that the findings are restricted to one single cell model using transient PrP overexpression. The basal level of PrP expression in SH-SY5Y cells is not shown (we can learn by going back to Rambold et al, 2006, that "SH-SY5Y cells do not express significant levels of endogenous PrPc (unpublished data)", which is not very informative). Therefore the reader does not know to which extent the PrP signals detected by western blot after transfection are due to endogenous PrP or to PrP resulting from transfection. It would be useful to show the levels of endogenous PrP in SH-SY5Y and N2a cells in comparison with the levels of expression of the various PrP constructs.

ScN2a cells that express PrP, do not undergo overt apoptosis, thus it is questionable whether physiological levels of PrP mediate ScN2a cells induced toxicity. It may be that the effect observed with SH-SY5Y cells is due to overexpression of PrP and would not occur when PrP is expressed at "physiological" levels. Moreover, the high rate of apoptosis in non-transfected cells exposed to lipofectamine treatment (10-15%) suggests that the lipofectamine transfection procedure itself causes cell death and in addition may sensitize cells to the various peptides and oligomers. Question: What is the frequency of apoptosis of SH-SY5Y cells not subjected to lipofectamine treatment? Why do the authors not use SH-SY5Y cells stably transformed with a PrP expression vector, which would allow more reproducible experiments and avoid exposure to lipofectamine? The conclusions would be considerably strengthened if the authors would use PrP-/- cells and their permanently PrP "reconstituted" counterparts. It would be even better to transfect such PrP-/- cells with a vector allowing inducible expression of PrP, as in the experiments of Vilette D et al (PNAS, 2000) and Paquet et al (J.Virol. 2007).

The small amplitude of the observed toxicity is of concern. The percentage of apoptotic cells increases by 30% above background after PrP expression, to reach 20% or less. Statistical analyses show that these increases are significant. However, the low level of apoptosis reached overall raises the question of the relevance of the findings to neurodegeneration. Does this low level come about because transfection is at a low level? What is the fraction of cells expressing PrP? What is the variability of PrP expression (which could be monitored by FACS?) Exposure to ScN2a cells is done only during 16 hours. What happens when exposure time is increased? Does PrP expression still have a sensitizing effect? A time course study over at least 48 hours would seem necessary to address this question.

Figures 3A suggests that the sensitizing effect of PrP expression does not entirely depend on the N-terminus. Consistent with this observation, Figure 5E shows that a secreted version of the C-terminal part of PrP diminishes the effect of PrP expression. It is not clear if the difference between the toxicity induced by co-culture with CHO-7PA2 in the presence of PrPC/Fc versus the presence of PrPN/Fc is statistically significant. This should be discussed.

The fact that NMDA receptor antagonists such as MK801, memantine, and flupirtine rescue cells from PrPsc and PrP 106-126 induced toxicity has already been published (Müller et al., 1993; Perovic et al.,1995, 1997). It is not clear how the data presented in Figure 6 help to demonstrate the model proposed by the authors in the present paper.

In summary, the data presented in this paper are suggestive of a role of PrP in sensitizing cells to a variety of peptides, but they were marginal effects, obtained only with a single cell model under non physiological conditions, and therefore do not warrant generalization.

Referee #3 (Remarks to the Author):

In this submission the authors provide evidence that the cellular form of the prion protein is able to bind a variety of misfolded beta-sheet-rich oligomers (beta-conformers), presumably through recognition of a similar conformation epitope present in all amyloid proteins. Moreover, binding of cellular PrP (PrPc) to various beta-conformers appears to mediate toxic signaling that results in a modest, albeit consistent, increase in apoptosis. The authors also provide evidence that this toxic signaling involves NMDA receptors because their inhibition attenuates the increased apoptosis.

Overall, this is an interesting and significant study with good rationale and, for the most part, conclusions that are in line with the data provided. However, clarifications are needed and certain conclusions need better justification.

Major concerns:
1) Figure 1. The authors state that their results occur "independent of prion replication" but they do not demonstrate this. Because there is often limited transmission among species due to differences in PrP sequence homology, the authors conclude that PrPc-Scrapie interactions causing cellular toxicity are not caused by conversion of PrPc into the toxic PrPSc form. However, Vorberg and colleagues have shown that acute heterologous PrPSc formation can be readily induced in cell culture, even if it does no lead to persistent prion infection. Even if conversion of heterologous PrP molecules is inefficient, it still may be significant in the results observed. The authors make no attempt to verify the amount of PrPSc present in their ScN2a cultures and in the ScN2a SH-SY5Y co-cultures. If the authors' assumptions are correct, no significant amounts of PrPSc will be generated. In any case, this issue substantially weakens the conviction with which one can make such statements as "independent of prion replication". Having said that, it is clear that no scrapie replication can be involved in the effects observed with the other types of beta oligomers, so the prion replication issue should not be a pervasive problem with the paper.

2) P.9, Figure 1B.
The authors state that levels of transfected heterologous PrPc expression are "comparable". However, this statement is difficult to evaluate based on the data shown here. More explicit explanations of the Western blot data need to be provided, including: the antibodies used in each blot provided, the respective antibody specificities and sensitivities, and whether the lanes shown are taken from the same blots. One assumes, after some scrutiny, that the PrP bands detected are

supposed to be solely from the SH-SY5Y cells, but this point is not explained directly or justified experimentally. If the heterologous PrPc is actually expressed in comparable amounts, then the authors should demonstrate this quantitatively. But if, in fact, PrPc levels are not comparable, as actually appears to be the case visually, the authors should address why they stimulate similar levels of apoptosis. If their hypothesis that PrPc mediates neurotoxic signaling is correct, then one might expect higher PrPc levels to correlate with greater amounts of apoptosis. For two of the 4 PrP constructs shown in this panel, and also for moPrP in Panel A, there seems to be more PrP in the N2a cocultures than the ScN2a cultures. Why?

Relatively minor issues:

3) State the species of origin and levels of basal PrP expression in SH-SY5Y cells at the beginning of the results. The reader should not have to dig through previous papers (as I did) to get this essential information.

4) Figure 1A. In the graph in Figure 1A, it should be indicated whether the difference seen for moPrP with and without co-culture with Scrapie is statistically significant. Also, is the blot supposed to be showing moPrP in both of the cell types in the cultures? Why is there more moPrP in the cultures containing the uninfectect N2a cells?

5) Since nearly every figure in this paper relies on quantification of apoptotic cells using caspase-3 antibody fluorescence, the authors should include in this figure (or in supplementary material) images of appropriate positive and negative controls. In addition, please indicate whether cell counting was done by someone who was blinded to sample identity.

6) In the first paragraph of the results section, the authors' state that apoptosis was induced "only in cells expressing mouse PrPc". This appears to be incorrect since all cultures shown appear to have at least 10% apoptotic cells.

7) Figure 5D. The PrPC/Fc and PrPN/Fc blots are messy and blotchy and without any controls that would allow a quantitative comparison. Is there not a more convincing gel for this figure (with standards that allow quantitation)?

8) Figure 5E. Please address why there is a difference in the GFP controls. This difference is not seen for the GFP controls in Figure 1A and 2A.

1st Revision - authors' response 28 January 2011

Referee #1 (Remarks to the Author):

Resenberger et al explore the role of cellular PrP in toxicity from a range of misfolded proteins. They report the PrP-Sc, A-beta oligomers and yeast prions all mediated apoptosis in neuroplastoma cells via PrP-C. This signaling requires the N-terminal domain and the GPI anchor of PrP-C. It appears to be mediated by recognition of a common "oligomer" epitope detected with A11 antibody. The cell death is blocked by NMDA antagonists. While there had been suggestions that PrP-C plays a role in PrP-Sc induced degeneration and more recently in A-beta oligomer toxicity, these data provides a unifying and clear role for PrP-C in this process, and map the PrP sequence required. These findings will be a great interest and importance to the field. I have some technical comments.

1. Cell death is assessed only by active caspase immunostaining. It would be advantageous for the authors to use a second marker of apoptotic cells. More importantly, they should assess surviving cell number in parallel, at least in one experiment for each ligand.
Reply: As suggested, we employed an additional assay to analyze PrP^C-mediated cell death. By quantifying fragmented nuclei we obtained cell death rates similar to those determined by the caspase assay (new Figure 1B).

2. The authors use neuroblastoma cells for their assays in a nice coverslip/overlay assay. However, they should assess function in primary neurons and its sensitivity to reducing endogenous PrP by

knockdown, knockout or anti-PrP blocking. At least one cell death assay with each ligand this should be tested with such primary neurons.
Reply: The referee raised a very important point. To provide experimental data for the role of PrP^{C} in primary neurons, we employed primary cortical neurons prepared from mice with a tageted disruption of the PrP gene ($PrP^{0/0}$) and from the corresponding wild-type line expressing PrP^{C}.

As illustrated in Figure 7, these approach revealed that:
1. PrP^{Sc} decreases viability of primary neurons expressing PrP^{C} (new Figure 7B)
2. PrP^{Sc} significantly reduces dendritic length in primary neurons expressing PrP^{C} but not in PrP-deficient neurons (new Figure 7C)
3. PrP^{Sc} induces abnormal perinuclear clustering of mitochondria in primary neurons expressing PrP^{C} but not in PrP-deficient neurons (new Figure 7D)
4. the toxic activity of beta peptides is increased in primary neurons expressing PrP^{C} (new Figure 7E).

3. The similarity of PrP-C dependence for these different ligands is striking. It would be of interest to assess their synergy in cell death mediated by PrP-C. Do low non-toxic doses of PrP-Sc and low non-toxic doses of A-beta synergize to produce cell death?
Reply: Based on the interesting suggestion of the referee we have established a 'co-co-cultivation' assay. SH-SY5Y cells expressing PrP^{C} were co-cultivated with both ScN2a and 7PA2-cells. However, we did not observe an additive effect (new Figure 2C).

4. In the Methods ,the description of peptide production provides nucleic acid sequence for a peptide.
Reply: In the revised version we have provided the amino acid sequence of the peptides in addition.

Referee #2 (Remarks to the Author):

This paper builds on a previous study showing that PrP overexpression in SH-SY5Y human neuroblastoma cells sensitizes the cells to apoptosis induced by co-culture with scrapie infected N2a cells (ScN2a) (Rambold et al. 2008). Herein, the authors show that PrP overexpression in SH-SY5Y cells sensitizes the cells to apoptosis induced by ScN2a cells, toxic Aβ peptide oligomers, yeast NM and a β peptide, thereby addressing the important question of the role of PrP in neuronal toxicity occurring in protein misfolding neurodegenerative diseases other than prion diseases.

The main weakness of the paper comes from the fact that the findings are restricted to one single cell model using transient PrP overexpression. The basal level of PrP expression in SH-SY5Y cells is not shown (we can learn by going back to Rambold et al, 2006, that "SH-SY5Y cells do not express significant levels of endogenous PrPc (unpublished data)", which is not very informative). Therefore the reader does not know to which extent the PrP signals detected by western blot after transfection are due to endogenous PrP or to PrP resulting from transfection. It would be useful to show the levels of endogenous PrP in SH-SY5Y and N2a cells in comparison with the levels of expression of the various PrP constructs.
Reply: We regret that we have not been more precise. In the revised manuscript, we show Western blot data to illustrate 1. the low expression of endogenous PrP^{C} in SH-SY5Y cells (new Figure 1A), 2. that the expression levels of the different PrP constructs are comparable (new Suppl. Figure 1C) and 3. the expression levels of transfected PrP in SH-SY5Y cells in comparison to endogenous PrP^{C} in primary neurons (new Suppl. Figure 3B). These data revealed that endogenous PrP^{C} in untransfected SH-SY5Y cells is not detectable. In addition, the amount of PrP in transfected SH-SY5Y cells is not significantly higher compared to the amount of endogenous PrP^{C} in primary neurons.

ScN2a cells that express PrP, do not undergo overt apoptosis, thus it is questionable whether physiological levels of PrP mediate ScN2a cells induced toxicity. It may be that the effect observed with SH-SY5Y cells is due to overexpression of PrP and would not occur when PrP is expressed at "physiological" levels. Moreover, the high rate of apoptosis in non-transfected cells exposed to lipofectamine treatment (10-15%) suggests that the lipofectamine transfection procedure itself causes cell death and in addition may sensitize cells to the various peptides and oligomers.

Reply: Indeed, the observation that ScN2a cells are obviously resistant to the toxic effect of PrP^{Sc} is still puzzling. However, it is unlikely that this phenomenon is linked to low expression levels of endogenous PrP^{C}. In this context it is interesting to note that ScN2a cell lines exist stably expressing 3F4-tagged PrP^{C} to increase PrP^{C} levels. Yet, these cell lines are still resistant to PrP^{Sc}-induced cell death. More likely, during the generation of ScN2a cells those subclones have been selected which can tolerate PrP^{Sc} expression. In line with this scenario, we previously observed that ScN2a cells are characterzied by a defective stress response (Tatzelt et al., PNAS 1996, Winklhofer et al., JBC 2001).

To overcome possible problems caused by transfection procedures and/or by using specific cell lines, we have now analyzed the toxic effects of PrP^{Sc} and beta peptides in primary cortical neurons prepared from $PrP^{0/0}$ mice and the corresponding PrP^{C}-expressing wild-type mouse line.
As illustrated in Figure 7, these new experiments revealed that:
1. PrP^{Sc} decreases the viability of primary neurons expressing PrP^{C} (new Figure 7B)
2. PrP^{Sc} significantly reduces dendritic length in primary neurons expressing PrP^{C} but not in PrP-deficient neurons (new Figure 7C)
3. PrP^{Sc} induces abnormal perinuclear clustering of mitochondria in primary neurons expressing PrP^{C} but not in PrP-deficient neurons (new Figure 7D)
4. the toxic activity of beta peptides is increased in primary neurons expressing PrP^{C} (new Figure 7E).

Question: What is the frequency of apoptosis of SH-SY5Y cells not subjected to lipofectamine treatment? Why do the authors not use SH-SY5Y cells stably transformed with a PrP expression vector, which would allow more reproducible experiments and avoid exposure to lipofectamine? The conclusions would be considerably strengthened if the authors would use PrP-/- cells and their permanently PrP "reconstituted" counterparts. It would be even better to transfect such PrP-/- cells with a vector allowing inducible expression of PrP, as in the experiments of Vilette D et al (PNAS, 2000) and Paquet et al (J.Virol. 2007).
Reply: As indicated above, we have now employed primary cortical neurons to avoid problems possibly linked to transfection procedures and/or the use of cultured cell lines.

The small amplitude of the observed toxicity is of concern. The percentage of apoptotic cells increases by 30% above background after PrP expression, to reach 20% or less. Statistical analyses show that these increases are significant. However, the low level of apoptosis reached overall raises the question of the relevance of the findings to neurodegeneration. Does this low level come about because transfection is at a low level? What is the fraction of cells expressing PrP? What is the variability of PrP expression (which could be monitored by FACS?) Exposure to ScN2a cells is done only during 16 hours. What happens when exposure time is increased? Does PrP expression still have a sensitizing effect? A time course study over at least 48 hours would seem necessary to address this question.
Reply:
With the assays employed we only analyze transfected cells. Conerning the seemingly low levels of apoptotic cell death, the following facts need to be considered: 1. activated caspase 3 can only be detected in a relatively short time frame and 2. apoptotic cells quickly detach. Thus, an increased exposure time (for example 48 h) is of limited help, since the fraction of apoptotic cells detected at 16 h would no longer be present after 48 h.

Figures 3A suggests that the sensitizing effect of PrP expression does not entirely depend on the N-terminus. Consistent with this observation, Figure 5E shows that a secreted version of the C-terminal part of PrP diminishes the effect of PrP expression. It is not clear if the difference between the toxicity induced by co-culture with CHO-7PA2 in the presence of PrPC/Fc versus the presence of PrPN/Fc is statistically significant. This should be discussed.
Reply:
This is an important point we forgot to mention. The differences are indeed significant and we have included this information in the new Figure 5E.

The fact that NMDA receptor antagonists such as MK801, memantine, and flupirtine rescue cells from PrPsc and PrP 106-126 induced toxicity has already been published (Müller et al., 1993; Perovic et al.,1995, 1997). It is not clear how the data presented in Figure 6 help to demonstrate the model proposed by the authors in the present paper.

Reply: We regret not having cited the work by Muller and colleagues (1993). This publication is now cited in the corresponding paragraph. Moreover, we incuded new data to show that memantine is also protective against β-peptide-induced toxicity (new Figure 6B). In addition, we think that our data are novel and important, since we showed a protective effect of memantine on the toxicity of three β-sheet-rich conformers of completely different origins in one model system.

In summary, the data presented in this paper are suggestive of a role of PrP in sensitizing cells to a variety of peptides, but they were marginal effects, obtained only with a single cell model under non physiological conditions, and therefore do not warrant generalization.
Reply: As indicated above, we have now employed primary cortical neurons to show that the described effects are not restricted to established cell lines.

Referee #3 (Remarks to the Author):

In this submission the authors provide evidence that the cellular form of the prion protein is able to bind a variety of misfolded beta-sheet-rich oligomers (beta-conformers), presumably through recognition of a similar conformation epitope present in all amyloid proteins. Moreover, binding of cellular PrP (PrPc) to various beta-conformers appears to mediate toxic signaling that results in a modest, albeit consistent, increase in apoptosis. The authors also provide evidence that this toxic signaling involves NMDA receptors because their inhibition attenuates the increased apoptosis.

Overall, this is an interesting and significant study with good rationale and, for the most part, conclusions that are in line with the data provided. However, clarifications are needed and certain conclusions need better justification.

Major concerns:
1) Figure 1. The authors state that their results occur "independent of prion replication" but they do not demonstrate this. Because there is often limited transmission among species due to differences in PrP sequence homology, the authors conclude that PrPc-Scrapie interactions causing cellular toxicity are not caused by conversion of PrPc into the toxic PrPSc form. However, Vorberg and colleagues have shown that acute heterologous PrPSc formation can be readily induced in cell culture, even if it does no lead to persistent prion infection. Even if conversion of heterologous PrP molecules is inefficient, it still may be significant in the results observed. The authors make no attempt to verify the amount of PrPSc present in their ScN2a cultures and in the ScN2a SH-SY5Y co-cultures. If the authors' assumptions are correct, no significant amounts of PrPSc will be generated. In any case, this issue substantially weakens the conviction with which one can make such statements as
"independent of prion replication". Having said that, it is clear that no scrapie replication can be involved in the effects observed with the other types of beta oligomers, so the prion replication issue should not be a pervasive problem with the paper.
Reply: The referee is absolutely right, we cannot exclude the possiblity that PrP^{Sc} induces a transient conversion of heterologous PrP^{C}, which does not lead to a sustained generation of PrP^{Sc}. Our statement is rather based on several animal and cell culture models indicating that an interaction of PrP^{Sc} with heterologous PrP^{C} is extremely inefficient in initating a persistent prion infection. The experiments described in the mentioned paper by Vorberg and colleagues (JBC, 2004, 279, pp 29218) analyze the transient fomation of PrP^{Sc} after the cells had been incubated with RML scrapie brain homogenate for 4 days. In our experimental setup the PrP^{C}-expressing SH-SY5Y cells are only exposed to PrP^{Sc} for 16h.
Moreover, in the revised manuscript we analyzed the formation of PK-resistant PrP^{Sc} in both ScN2a cells and transiently transfected SH-SY5Y cells (new Suppl. Figure 1B). Using the 4H11 antibody we could demonstrate that ScN2a cells propagate significant amounts of PK-resistant PrP^{Sc}. However, by employing the 3F4 antibody that is specific for the transfected PrP constructs expressed in SH-SY5Y cells, we were not able to detect PK-resistant PrP^{Sc} (new Suppl. Figure 1B). In addition, metabolic labeling experiments provided the same results (data not shown).

2) P.9, Figure 1B.
The authors state that levels of transfected heterologous PrPc expression are "comparable". However, this statement is difficult to evaluate based on the data shown here. More explicit explanations of the Western blot data need to be provided, including: the antibodies used in each blot provided, the respective antibody specificities and sensitivities, and whether the lanes shown

are taken from the same blots. One assumes, after some scrutiny, that the PrP bands detected are supposed to be solely from the SH-SY5Y cells, but this point is not explained directly or justified experimentally. If the heterologous PrPc is actually expressed in comparable amounts, then the authors should demonstrate this quantitatively. But if, in fact, PrPc levels are not comparable, as actually appears to be the case visually, the authors should address why they stimulate similar levels of apoptosis. If their hypothesis that PrPc mediates neurotoxic signaling is correct, then one might expect higher PrPc levels to correlate with greater amounts of apoptosis. For two of the 4 PrP constructs shown in this panel, and also for moPrP in Panel A, there seems to be more PrP in the N2a cocultures than the ScN2a cultures. Why?
Reply: We regret that we did not provide the experimental data for our statements. In the revised manuscript we now show Western blot data to illustrate 1. the low expression level of endogenous PrP^C in SH-SY5Y cells (new Figure 1A), 2. that the expression levels of the different PrP constructs are comparable (new Suppl. Figure 1C) and 3. the expression levels of transfected PrP in SH-SY5Y cells in comparison to endogenous PrP^C in primary neurons (new Suppl. Figure 3).
However, we cannot make the statement that heterologous PrP^C constructs are expressed in comparable amounts (Figure 1D) since we have used different antibodies. In addition, the 4H11 antibody has variable affinities for PrP^C from the different species. Thus, we deleted this statement from the revised manuscript.
The apparantly higher expression levels of PrP^C in SH-SY5Y cells co-cultured with N2a cells can be explained by the loss of apoptotic PrP^C-expressing SH-SY5Y cells co-cultured with ScN2a cells.

Relatively minor issues:

3) State the species of origin and levels of basal PrP expression in SH-SY5Y cells at the beginning of the results. The reader should not have to dig through previous papers (as I did) to get this essential information.
Reply: We are sorry, this information is now provided in 'Material and Methods' and in Figure 1A.

4) Figure 1A. In the graph in Figure 1A, it should be indicated whether the difference seen for moPrP with and without co-culture with Scrapie is statistically significant. Also, is the blot supposed to be showing moPrP in both of the cell types in the cultures? Why is there more moPrP in the cultures containing the uninfectect N2a cells?
Reply: The differences in toxicity are statistically significant, as now indicated in the new Figure 1A. The higher amount of PrP^C in SH-SY5Y cells co-cultured with N2a cells can be explained by the loss of apoptotic PrP^C-expressing SH-SY5Y cells co-cultured with ScN2a cells.

5) Since nearly every figure in this paper relies on quantification of apoptotic cells using caspase-3 antibody fluorescence, the authors should include in this figure (or in supplementary material) images of appropriate positive and negative controls. In addition, please indicate whether cell counting was done by someone who was blinded to sample identity.
Reply: In the new Suppl. Figure 1A the caspase assay is now illustrated. In addition, we have indicated in 'Material and Methods' that the analysis was done in a blinded manner.

6) In the first paragraph of the results section, the authors' state that apoptosis was induced "only in cells expressing mouse PrPc". This appears to be incorrect since all cultures shown appear to have at least 10% apoptotic cells.
Reply: Correct, we wanted to say that apoptotic cell death is increased in PrP^C-expressing cells. In the revised manuscript we state:
'co-culture with ScN2a cells increased apoptotic cell death … only in cells expressing mouse PrP^C.

7) Figure 5D. The PrPC/Fc and PrPN/Fc blots are messy and blotchy and without any controls that would allow a quantitative comparison. Is there not a more convincing gel for this figure (with standards that allow quantitation)?
Reply: We apologize for the low quality of this blot. We repeated the experiments a couple of more times. The results are reproducible, however, the blots were not nicer. This is probably due to the low concentration of the secreted constructs.

8) Figure 5E. Please address why there is a difference in the GFP controls. This difference is not seen for the GFP controls in Figure 1A and 2A.

Reply: The labeling of the Figure 5E may have been confusing. The analyzed SH-SY5Y cells express GPI-anchored PrP^{C} and the indicated constructs, for example GFP. We have now modified the labeling to indiate this important detail (new Figure 5E).

2nd Editorial Decision 22 February 2011

Thank you for submitting your revised manuscript to the EMBO Journal. The three original referees have now seen the revised manuscript and their comments are provided below.

As you can see, the referees appreciate the introduced changes and support publication in the EMBO Journal. I am therefore very pleased to proceed with the acceptance of your paper for publication in the EMBO Journal. Before doing so, there is one minor issue (referee #2) to be resolved. As soon as we receive the revised version, we will proceed with its acceptance here. When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review.

Best regards

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

All of my concerns have been addressed. This is an important and timely manuscript that should be published in its current form.

Referee #2 (Remarks to the Author):

The authors responded to our criticism regarding the use of a cellular model where PrP is transiently overexpressed. They repeated their experiment in PrP-expressing and PrP-deficient primary neurons and confirmed their findings in this model (new figure 7).
Moreover, they now show the absence of detectable PrP in non-transfected SH-SY5Y cells. They also show that the transfected cells express PrPc amounts comparable to those found in primary neurons, addressing our concern that the results could be interpreted as cell sensitization to neurotoxicity by excessively high amounts of PrPc.
The paper is now acceptable for publication with one modification:
In figure 7 B and C, it is necessary to show the error bars of the cell viability or dendritic length signal for PrP0/0 neurons, similar to what is shown in part D of the figure or in other figures.

Referee #3 (Remarks to the Author):

The authors have addressed my concerns adequately.

2nd Revision - authors' response 02 March 2011

Thank you very much for the positive review of our revised manuscript " **The cellular prion protein mediates neurotoxic signaling of β-sheet-rich conformers independent of prion replication**". As requested by referee 2 we included error bars in figure 7 B and C for $PrP^{0/0}$ neurons, similar to what is shown in part D of the figure and in other figures.

