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MODIFICATION OF γ-SECRETASE BY NITROSATIVE STRESS LINKS NEURONAL AGING TO SPORADIC ALZHEIMER'S DISEASE

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1st Editorial Decision 05 January 2012

Thank you for the submission of your manuscript to EMBO Molecular Medicine. Your study has now been seen by three referees and their comments are provided below.

As you can see, the referees find the study novel and of potential interest, but they also raise a certain number of issues that should be satisfactorily addressed in a major revision. Globally the referees recommend further experiments to strengthen the arguments put forth and provide additional mechanistic insight.

Given the overall interest in the study, and providing that you are able and willing to address all referees' comments, we would be happy to consider a revised version of the manuscript for publication in EMBO Molecular Medicine within 3 months (see below).

I would like to add that it is EMBO Molecular Medicine policy to allow only a single round of revision only and that acceptance or rejection of your manuscript will therefore depend on the completeness of your responses in the final revised version.

Revised manuscripts should be submitted within 3 months of a request for revision; they will otherwise be treated as new submissions, except under exceptional circumstances in which a short extension is obtained from the editor. Also, the length of the revised manuscript may not exceed 60,000 characters (including spaces) and, including figures, the paper must ultimately fit onto

optimally ten pages of the journal. You may consider including any peripheral data (but not methods in their entirety) in the form of Supplementary information.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1:

This study aims to address the role of aging on the regulation of gamma-secretase complex formation and activity using a hippocampal neuronal culture system. The authors found that total abeta secretaion and Abeta 42/40 ratio are increased during aging as well as enhanced formation of gamma-secretase complexes and nitrosative stress. They further showed that nitrosative stress modifies the gamma-secretase activity by possibly inducing its conformational change. Overall the central question being addressed in this study is very important to the field of Alzheimer's disease and will contribute to our understanding of the pathogenic mechanisms underlying the disease process. The results appear convincing. Therefore I recommend the manuscript for publication in EMBO Mol Med.

Referee #2 :

In this study, the laboratory of Bart De Strooper has investigated the potential role of nitrosative stress on the production of A 42 and A 40 species in model of isolated neuronal cultures.

The experiments are very well performed and strictly controlled, as one might expect from a highly professional and famous laboratory. If one were to nit-pick, however, one could point out that some of the assumptions of the study are rather speculative, and that the theory proposed is not fully supported by a gap-free chain of experimental evidence.

The first critical remark concerns the choice of the experimental model. The authors claim rather apodictically that the time points of two, three or four weeks represent terminal differentiation, early aging, and late aging, respectively. While I do not completely disagree with this statement, it seems a bit daring to make such a claim. On the positive side, however, the authors have gone through the trouble of testing AD brains. While the human findings do not represent a direct validation of the culture model, they are at least compatible with the results therefrom. Therefore, if one concedes that the experimental model is a realistic representation of what happens during real aging, then the experiments performed by De Strooper and colleagues are very convincing. The biochemistry is of high quality, including the detection of nitrosilation products - which is not always trivial.

The second issue is a mechanistic one. I find it entirely plausible, and well documented, that addition of peroxynitrite distorts the A 42/A 40 ratio. However, we are offered very little insight into how this is supposed to happen. If I would have had to guess the potential outcome of this experiment ahead of time, I would have - perhaps naively - surmised that nitrosylation would act as a kind of a non-specific -secretase inhibitor by damaging the catalytic site of the enzyme . However, De Strooper proposes that nitrosylation acts as a kind of "negative -secretase modulator" which enhances the production of the more deleterious A 42 molecular species to the detriment of A 40. How that is supposed to happen, however, is not really convincingly explained. The authors speculate that nitrosylation of the tyrosine residues in the neighborhood of the active site may bring about the enhanced 42 cleavage. However, this was not experimentally verified and is certainly not intuitive. Therefore, I still miss a mechanism that would explain structurally what might be going on there. I am not an expert in the PS field, and cannot suggest great experiments - but maybe the combination of peroxynitrate with secretase inhibitors/modulators, and/or the use of tyrosine-deleted PS mutants, might shed some light here.

The third point of my critique relates to the crossing with SOD hemizygous mice. Here the contention is that reduction of the SOD pool enhances the susceptibility of mice to peroxynitrate. I have to honestly say that these experiments seem a bit superficial, and do not fully do justice to the stringent biochemical arguments for which the De Strooper Laboratory is known. I understand that homozygous ablation of SOD2 in mice leads to perinatal letality, but this should not prevent the authors from deriving primary neurons and determine the presenilin-related parameters from such perinatally affected mice. Failing that (or in addition to that), one could think of knocking down SOD1 and SOD2 from neuronal cultures using siRNA as an alternative approach. Therefore, there are many experiments that could be done to drive home this point, and it is a bit disappointing that the authors have not chosen to do more in this respect.

In summary, this is a very interesting study which posits a provocative hypothesis, namely that reactive oxygen species-induced nitrosylation stress is directly responsible for creating the conditions that lead to accumulation of AB aggregates in the brain of patients with age-related Alzheimer's disease. The hypothesis is exciting and very much worth pursuing. I am just not entirely sure that the experimental evidence presented here is really sufficient to corroborate said hypothesis. My advice is that the authors (1) try to test, or at least to exclude, some of the possible specific mechanism by which Tyr nitrosylation may change the cutting sites of presenilins, and (2) amuse us with more details on the impact of SOD depletion onto their system.

Referee #3 (Comments on Novelty/Model System):

A major improvement could have been made by showing that the protein modifications are specifically causing the effects observed, e.g., by doing in vitro gamma-secretase tests with and without nitrotyrosination of PS-NTF/ -CTF.

Referee #3 (Other Remarks):

The study tried to identify molecular links between nitrosative stress and
neuronal aging leading to sporadic alzheimer's disease. This is an important subject but the story suffers from several setbacks.
Although it might well be that the observations shown fit together on the molecular level, the authors should provide better evidences to convince the potential readers.
Main point of criticism is that the statements made are not justified by the data presented and that the conclusions are based on rather independent experimental observations. Possible coincidences must be excluded.
Major
1. 'Age-associated impairment of SOD2 activity triggers conformational changes in the gamma-sec complex'. The evidence for a specific effect exerted by SOD2 is absent. Other stress factors could be involved, such as SOD1. Additional experiments need to be done to prove the specificity of an involvement of SOD2 compared to many other explanations.
2. Fig. 1: How was total Abeta measured? N=3 and $p<0.05$ is not sufficient.
3. Fig 2A: PS-CTF increase is higher than PS-NTF. There is an inconsistency between blot and bar diagram data.
4. SIN could have an effect on Abeta clearance which perfectly would explain the lacking effect on AICD but the increase of 42/40 ratio.
5. Is the effect of Abeta Tyr10 Nitration additive to PS-NTF/-CTF modification of independent?
Minor
AD is not necessarily an age-dep. pathology (see Introduction).

1st Revision - Authors' Response 14 March 2012

Referee #1:

This study aims to address the role of aging on the regulation of gamma-secretase complex formation and activity using a hippocampal neuronal culture system. The authors found that total abeta secretaion and Abeta 42/40 ratio are increased during aging as well as enhanced formation of gamma-secretase complexes and nitrosative stress. They further showed that nitrosative stress modifies the gamma-secretase activity by possibly inducing its conformational change. Overall the central question being addressed in this study is very important to the field of Alzheimer's disease and will contribute to our understanding of the pathogenic mechanisms underlying the disease process. The results appear convincing. Therefore I recommend the manuscript for publication in EMBO Mol Med.

We are very pleased with these positive comments and recommendation

Referee #2:

In this study, the laboratory of Bart De Strooper has investigated the potential role of nitrosative stress on the production of Aβ42 and Aβ40 species in model of isolated neuronal cultures.
The experiments are very well performed and strictly controlled, as one might expect from a highly professional and famous laboratory. If one were to nit-pick, however, one could point out that some of the assumptions of the study are rather speculative, and that the theory proposed is not fully supported by a gap-free chain of experimental evidence.

We thank the referee for the positive comment. We have, as we will describe below, addressed the criticism of the referee with extensive new experiments which should fill this "gap" now.

The first critical remark concerns the choice of the experimental model. The authors claim rather apodictically that the time points of two, three or four weeks represent terminal differentiation, early aging, and late aging, respectively. While I do not completely disagree with this statement, it seems a bit daring to make such a claim. On the positive side, however, the authors have gone through the trouble of testing AD brains. While the human findings do not represent a direct validation of the culture model, they are at least compatible with the results therefrom. Therefore, if one concedes that the experimental model is a realistic representation of what happens during real aging, then the experiments performed by De Strooper and colleagues are very convincing. The biochemistry is of high quality, including the detection of nitrosilation products - which is not always trivial.

We agree with the referee that neuronal differentiation and aging are a continuous process. The choices of 2, 3 and 4 weeks in vitro are therefore to be considered as representative time-points of a continuous process. This choice is based on extensive experimental evidence of our laboratory which has documented the aging process in those cultures. Moreover published scientific literature has shown how, once seeded in vitro, hippocampal neurons undergo a continuous series of morphological and functional maturation and aging processes which model the changes observed in

neurons in the brain i*n vivo* (Sodero et al, 2011a; Martin et al, 2008; Aksenova et al, 1999; Porter et al, 1997). More concretely, neurons aged in vitro, when compared to 2 weeks old neurons, show increase of protein oxidation, JNK phosphorylation and the activation of DNA repair p51/P21 pathways (Sodero et al, 2011a; Martin et al, 2008). All these changes are gradual (P21 expression and protein oxidation increases already at 3 weeks in vitro) and matches the changes occurring in an old brain (Aksenova et a, 1999; Suh 2001). Our experimental evidences (Supporting information Fig. S4) and scientific literature supports the notion that especially the 4 week time point shows dramatic, age related changes. Thus, we agree with the referee that the choice of the time points is somewhat arbitrary, but the 2 week cultures certainly reflect neurons which do not display the molecular signs of aging that guided us in this choice, while at 4 weeks all these changes are well documented to occur. We took 3 weeks as an intermediary step between these two extremes.

The second issue is a mechanistic one. I find it entirely plausible, and well documented, that addition of peroxynitrite distorts the Aβ42/Aβ40 ratio. However, we are offered very little insight into how this is supposed to happen. If I would have had to guess the potential outcome of this experiment ahead of time, I would have - perhaps naively - surmised that nitrosylation would act as a kind of a non-specific γ-secretase inhibitor by damaging the catalytic site of the enzyme . However, De Strooper proposes that nitrosylation acts as a kind of "negative γ-secretase modulator" which enhances the production of the more deleterious Aβ42 molecular species to the detriment of Aβ40. How that is supposed to happen, however, is not really convincingly explained. The authors speculate that nitrosylation of the tyrosine residues in the neighborhood of the active site may bring about the enhanced 42 cleavage. However, this was not experimentally verified and is certainly not intuitive. Therefore, I still miss a mechanism that would explain structurally what might be going on there. I am not an expert in the PS field, and cannot suggest great experiments - but maybe the combination of peroxynitrate with secretase inhibitors/modulators, and/or the use of tyrosine-deleted PS mutants, might shed some light here.

We agree with the referee that our previous interpretation of these experiments was speculative. We have now added a series of additional experiments to clarify the mechanism of action.

<u>1. Effect of nitrosylation on γ-secretase using CHAPSO solubilized microsomal membranes from HEK cells</u>.

Microsomal fractions were incubated for 24h at 25°C in the presence or the absence of 10 μM of the nitrating reagent SIN-1. After protein extraction with detergent, kinetic experiments were carried out using purified APP-C99-3xFlag as a substrate. Total AICD production (reflecting ε-cleavage) was only changed to a limited extent (Supporting information Fig. S4C), but we observed an increment in Aβ42 and no change in Aβ40 levels, in agreement with the Aβ42/Aβ40 ratio changes observed in the cell culture experiments (Supporting information Fig. S4A, B). This experiment shows that the alterations observed in cell culture also occur in cell free conditions, and therefore indicates a biochemical, and not a functional cell biological mechanism of action. We confirm in this experiment that γ-secretase complex is indeed nitrated using SDS-PAGE/western blot with an anti-nitrotyrosination specific antibody (Supporting information Fig. S4D).

<u>2. Effect of nitrosylation on the conformation of the γ-secretase using biophysical experiments (FLIM analysis).</u>

We have collaborated with Dr. Arimon and Dr. Berezovska (Harvard) to investigate the effect of nitrosative stress on the conformation of presenilin in the γ-secretase complex in situ. The effects on FLIM suggests a 'closed' conformation of presenilin after nitrosylation, which is similar to the effects reported for FAD mutations, NSIADs and other conditions related to increased Aβ42/Aβ40(Lleo, Berezovska et al. 2004; Berezovska, Lleo et al. 2005; Tesco, Ginestroni et al. 2005; Serneels, Van Biervliet et al. 2009) previously (Fig. 4E, F and supporting information Fig. S3D). Thus, we conclude that nitrosative stress affects γ-secretase conformation directly.

3. Effect of nitrosylation on the purified γ-secretase complex.

We purified γ-secretase (tagged with GFP on Nicastrin) from insect cells overexpressing the complex and precipitated the complex using nanobodies against GFP protein coupled to beads. We exposed the complex to 10 μM SIN-1 and checked for nitrotyrosination of the individual subunits using western blot as before. We found that under these conditions the PS1-NTF presents already a basal levels of nitrotyrosination, pointing to the higher tendency for nitration of this component in cells with basal levels of free radicals, as occurs with other proteins susceptible of nitration. We have further investigated the specific nitration of tyrosines in the purified γ-secretase complex and it is, as the referee anticipated, clear that also modification at other positions in the complex are observedAlthough this does not rule out our hypothesis at this moment, it is indeed more correct to leave out this speculative interpretation for the time being. Further biochemical and structural work is needed to explore this question.

However the combination of the three experiments support the conclusion that the effect of nitrosylation is at least partly explained by a direct effect on γ-secretase causing a conformational change of the complex which mimicks to a certain extent the alterations caused by FAD mutations. This can account for the increase of Aβ42 observed in the different assays. These new data are included in the Results section of the new manuscript either as additional figures or as supplemental data. The discussion section of the new manuscript was adapted accordingly

The third point of my critique relates to the crossing with SOD hemizygous mice. Here the contention is that reduction of the SOD pool enhances the susceptibility of mice to peroxynitrate. I have to honestly say that these experiments seem a bit superficial, and do not fully do justice to the stringent biochemical arguments for which the De Strooper Laboratory is known. I understand that homozygous ablation of SOD2 in mice leads to perinatal letality, but this should not prevent the authors from deriving primary neurons and determine the presenilin-related parameters from such perinatally affected mice. Failing that (or in addition to that), one could think of knocking down SOD1 and SOD2 from neuronal cultures using siRNA as an alternative approach. Therefore, there are many experiments that could be done to drive home this point, and it is a bit disappointing that the authors have not chosen to do more in this respect.

The referee is right that we need more experimental evidence to connect the observations with the SOD deficient neurons to changes in peroxynitrite if we want to invoke this as the working mechanism explaining the increase in Aβ42/Aβ40 ratio. We addressed the issue by down regulating SOD2 in rat primary neuronal cultures and by determining the Aβ42/Aβ40 ratio in the presence or the absence of a peroxynitrite scavenger. The scavenger PTIO induced high levels of neuronal death, and therefore we decided to use uric acid (UA). UA has been reported to be a natural and powerful scavenger of peroxynitrite and prevents tyrosine nitration (Hooper et al, 1998; Hooper et

al, 2000; Tran et al, 2003; Scott et al, 2005). As shown in the new figure 7A, the Aβ42/Aβ40 ratio, measured by ELISA from the media of 10 days primary neurons, increased when SOD2 levels are decreased confirming our previous observations. However, the Aβ42/Aβ40 ratio is significantly recovered when UA is added to the media of these neurons (Fig. 6). These new data supports the notion that peroxynitrite mediates at least part of the increase of the Aβ42/Aβ40 ratio triggered by SOD2 depletion. We discuss this in the new manuscript.

In summary, this is a very interesting study which posits a provocative hypothesis, namely that reactive oxygen species-induced nitrosylation stress is directly responsible for creating the conditions that lead to accumulation of AB aggregates in the brain of patients with age-related Alzheimer's disease. The hypothesis is exciting and very much worth pursuing. I am just not entirely sure that the experimental evidence presented here is really sufficient to corroborate said hypothesis. My advice is that the authors (1) try to test, or at least to exclude, some of the possible specific mechanism by which Tyr nitrosylation may change the cutting sites of presenilins, and (2) amuse us with more details on the impact of SOD depletion onto their system.

We thank the referee for the encouraging and constructive criticism. We hope that the referee is pleased with the additional work we have performed. We agree that further work is possible, and we intend to explore the modifications of γ-Secretase caused by nitrosylation in further molecular detail. We believe however that for EMBO mol med the physiological and pathophysiological insights from our work are highly interesting. We demonstrate here a mechanism that is occurring during aging and which links sporadic AD to mechanisms previously explored in FAD. This mechanism can be potentially explored for medical treatment and we cite here several papers that suggest indeed this possibility (Coffey et al, 2001; Chu and Pratico, 2011; Scott et al, 2005).

Referee #3 (Comments on Novelty/Model System):

A major improvement could have been made by showing that the protein modifications are specifically causing the effects observed, e.g., by doing in vitro gamma-secretase tests with and without nitrotyrosination of PS-NTF/ -CTF.

We refer to our answer to referee 2 and the new experiments we have added to the paper. We copy below the answer to ref 2 as it pertains to this criticism.

"We have now added a series of additional experiments to clarify the mechanism of action.
1. Effect of nitrosylation on γ-secretase using CHAPSO solubilized microsomal membranes from HEK cells.
Microsomal fractions were incubated for 24h at 25°C in the presence or the absence of 10 μM of the nitrating reagent SIN-1. After protein extraction with detergent, kinetic experiments were carried out using purified APP-C99-3xFlag as a substrate. Total AICD production (reflecting ε-cleavage) did not significantly change (Supporting information Fig. S4C), but we already observed an increment in Aβ42 and no change in Aβ40 levels, in agreement with the Aβ42/Aβ40 ratio changes observed in the cell culture experiments (Supporting information Fig. S4A, B). This experiment shows that the alterations observed in cell culture also occur in cell free conditions, and therefore indicates a biochemical, and not a functional cell biological mechanism of action. We confirm in this experiment that γ-secretase complex is indeed nitrated using SDS-PAGE/western blot with an

anti-nitrotyrosination specific antibody (Supporting information Fig. S4D). This suggests, but does not proof a direct effect of the nitrosative stress on the protease.

2. Effect of nitrosylation on the conformation of the γ-secretase using biophysical experiments (FLIM analysis).
We have collaborated with Dr. Arimon and Dr. Berezovska (Harvard) to investigate the effect of nitrosative stress on the conformation of presenilin in the γ-secretase complex in situ. The effects on FLIM suggests a 'closed' conformation of presenilin after nitrosylation, which is similar to the effects reported for FAD mutations, NSIADs and other conditions related to an increased Aβ42/Aβ40 (Lleo, Berezovska et al. 2004; Berezovska, Lleo et al. 2005; Tesco, Ginestroni et al. 2005; Serneels, Van Biervliet et al. 2009) previously (Fig. 4E, F and supporting information Fig. S3D). Thus, we conclude that nitrosative stress affects γ-secretase conformation directly.

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However the combination of the three experiments support the conclusion that the effect of nitrosylation is at least partly explained by a direct effect on γ-secretase causing a conformational change of the complex which mimicks to a certain extent the alterations caused by FAD mutations. This can account for the increase of Aβ42 observed in the different assays. These new data are included in the Results section and the Discussion section of the new manuscript was adapted accordingly"

Referee #3 (Other Remarks):
The study tried to identify molecular links between nitrosative stress and neuronal aging leading to sporadic alzheimer's disease. This is an important subject but the story suffers from several setbacks. Although it might well be that the observations shown fit together on the molecular level, the authors should provide better evidences to convince the potential readers. Main point of criticism is that the statements made are not justified by the data presented and that the conclusions are based on rather independent experimental observations. Possible coincidences must be excluded.

The referee points out that we tried to address an important question. He/she is less convinced that we provided sufficient evidence to support our claims. The concrete criticism is similar to the criticism of referee 2 who asked for additional evidence linking the observations more tightly to each other. We have now added several experiments to further strengthen the claims and we address the specific criticism of the referee point by point below.

Major

1. 'Age-associated impairment of SOD2 activity triggers conformational changes in the gamma-sec complex'. The evidence for a specific effect exerted by SOD2 is absent. Other stress factors could be involved, such as SOD1. Additional experiments need to be done to prove the specificity of an involvement of SOD2 compared to many other explanations.

We believe that this criticism is partly based on a miss-understanding of the aim of this particular experiment, and we apologize for lack of clarity from our side. The SOD KO experiment in the manuscript is indeed only one possible scenario of increased oxidative stress in aging brain. We certainly did not want to imply that other possible causes have to be not considered. We used the SOD2 KO mice and SOD2 down-regulation in essence to test our hypothesis in vivo, and found indeed that the absence of one of the enzymes crucial for anti-oxidant protection is already sufficient to alter gamma-secretase activity. Obviously other deficiencies causing increased oxidative stress are believed to increase similar effects. Hence, without ruling out other possible sources of nitrotyrosination during aging, our data provide proof of concept in vivo that lack of protection against nitrotyrosination stress alters gamma-secretase activity. As a matter of fact we never claim in the manuscript that increased nitrotyrosination in AD would derive exclusively from a lack of SOD2. We clarify this now in the text to avoid further misunderstanding by the readers.

We agree with this referee, and also with referee 2, that additional experiments are needed to address the question whether at least part of the SOD effects are indeed due to decreased protection against nitrosylation. We therefore now added an experiment using UA, a compound reported to be a natural and powerful scavenger of peroxynitrite and preventing tyrosine nitration (Hooper et al, 1998; Hooper et al, 2000; Tran et al, 2003). UA was able to recover the Aβ42/Aβ40 ratio in SOD+/- neurons (Fig. 6). indicating that at least part of the effect of SOD deficiency is due to increased peroxynitrite stress.

2. Fig. 1: How was total Abeta measured? N=3 and $p<0.05$ is not sufficient.

The measurements for the endogenous Aβ (Fig 1A) are carried out with ELISA. In this case 3 independent cultures, each one divided into 3 dishes to have the 3 time-point representation of the different aging states (2 weeks, 3 weeks and 4 weeks) were used. While we agree that n=3 is the lower limit for an experiment, it should be pointed out that the data are supported by the other experiments of the manuscript, such as the measurements of human Aβ from neurons infected with 3xFlag-huAPP-C99 (Fig. 1C-F). Therefore, an experiment reproduced three times is a sufficient high standard of experimentation, especially when similar data from other experiments strengthen the observations. The bar graph showing total Aβ (Fig 1E) produced by primary neurons infected with a 3xFlag-huAPP-C99 provides indeed independent proof (densitometry of western blot in panel D). The number of experiments is n=6 and the $p<0.001$ (see legend Fig. 1E) for this experiment.

3. Fig 2A: PS-CTF increase is higher than PS-NTF. There is an inconsistency between blot and bar diagram data.

The referee refers to the Fig 2A and 2B of the manuscript. PS1-NTF is increased comparing 2 to 4 weeks. Because the PS1-CTF levels are increased dramatically between 3 and 4 weeks in vitro and the signal of the antibody for PS1-CTF is stronger, it gives the impression that its increase is higher when compared to 2 weeks in vitro. We provide the quantifications for the referee.

4. SIN could have an effect on Abeta clearance which perfectly would explain the lacking effect on AICD but the increase of 42/40 ratio.

We think that the effect we observe at the level of the Aβ42/Aβ40 ratio is independent from the clearance, since we obtain the same results when we performed the in vitro assay with nitrated γ-secretase (new Supporting information Fig. S4). However we cannot rule out that peroxynitrite can also affect the clearance of the peptide which would hypothetically potentiate the effect we see. This aspect is now discussed, but does not take away from our conclusion that nitrative stress affects directly gamma-secretase activity.

5. Is the effect of Abeta Tyr10 Nitration additive to PS-NTF/-CTF modification of independent?

We think that the effect of Abeta Tyr 10 nitration is additive to the conformational change of γ-secretase discussed here. In fact one could imagine a scenario with increased nitration of γ-secretase, elevating the Aβ42/Aβ40 ratio, and that this would then result in higher aggregation of the peptide and therefore further increases in nitrosative stress. Nitration of Abeta would increase its aggregation, what would induce again more nitrosative stress. All this is potentially part of a complex system of feed forward loops occurring in the aging brain when AD becomes incipient.

Minor

*AD is not necessarily an age-dep. pathology (see Introduction***).**

We appreciate this comment and have now substituted the expression “it is an age-dependent pathology” by the more correct “it is an age-associated pathology”. It is true that aging is not sufficient to cause AD. However, some aging is necessary (even for the familial cases) to observe the pathology.

References

Aksenova MV, Aksenov MY, Markesbery WR, Butterfield DA (1999) Aging in a dish: age-dependent changes of neuronal survival, protein oxidation, and creatine kinase BB expression in long-term hippocampal cell culture. J Neurosci Res 58:308-17

Berezovska O, Lleo A, et al. (2005) Familial Alzheimer's disease presenilin 1 mutations cause alterations in the conformation of presenilin and interactions with amyloid precursor protein. J Neurosci 25:3009-17

Chu J, Praticò D. (2011) Pharmacologic blockade of 5-lipoxygenase improves the amyloidotic phenotype of an Alzheimer's disease transgenic mouse model involvement of γ-secretase. Am J Pathol 178:1762-9.

Coffey MJ, Phare SM, Peters-Golden M. (2001) Peroxynitrite-induced nitrotyrosination of proteins is blocked by direct 5-lipoxygenase inhibitor zileuton. J Pharmacol Exp Ther 299:198-203.

Hooper DC, Spitsin S, Kean RB, Champion JM, Dickson GM, Chaudhry I, Koprowski H (1998) Uric acid, a natural scavenger of peroxynitrite, in experimental allergic encephalomyelitis and multiple sclerosis. Proc Natl Acad Sci U S A 95:675-80

Hooper DC, Scott GS, Zborek A, Mikheeva T, Kean RB, Koprowski H, Spitsin SV (2000) Uric acid, a peroxynitrite scavenger, inhibits CNS inflammation, blood-CNS barrier permeability changes, and tissue damage in a mouse model of multiple sclerosis. FASEB J 14:691-8.

Lleo A, O. Berezovska, et al. (2004) Nonsteroidal anti-inflammatory drugs lower Abeta42 and change presenilin 1 conformation. Nat Med 10: 1065-6.

Scott GS, Cuzzocrea S, Genovese T, Koprowski H, Hooper DC. (2005) Uric acid protects against secondary damage after spinal cord injury. Proc Natl Acad Sci U S A. 102:3483-8

Serneels L, J. Van Biervliet, et al. (2009) {gamma}-Secretase Heterogeneity in the Aph1 Subunit: Relevance for Alzheimer's Disease. Science 324: 639-642.

Suh Y (2001) Age-specific changes in expression, activity, and activation of the c-Jun NH(2)-terminal kinase and p38 mitogen-activated protein kinases by methyl methanesulfonate in rats. Mech Ageing Dev. 122:1797-811

Tesco G, A. Ginestroni, et al. (2005) APP substitutions V715F and L720P alter PS1 conformation and differentially affect Abeta and AICD generation. J Neurochem 95: 446-56.

Tran MH., Yamada K., Nakajima A., Mizuno M., He J., Kamei H., Nabeshima T. (2003) Tyrosine nitration of a synaptic protein synaptophysin contributes to amyloid beta-peptide-induced cholinergic dysfunction. Mol Psychiatry 8 407-12.

Quantifications for Referee Three:

PS1-CTF							
		Intensity	actin		Normal	mean	**%**
	2w	23928	26432		0.905266344	1.111299273	81.46017598
	2w	28261	23623		1.196334081		107.6518369
	2w	28906	23457		1.232297395		110.8879871
	3w	26032	20363		1.278397093		115.0362574
	3w	26363	21835		1.207373483		108.6452148
	3w	29103	21511		1.352935707		121.7435969
	4w	45458	20178		2.252849638		202.7221372
	4w	44350	17533		2.52951577		227.6178731
	4w	36362	17962		2.024384812		182.1637844
PS1-NTF							
			actin			mean	%
	2w	4149.785714	26432		0.156998552	0.163246105	96.17292315
	2w	4116	23623		0.174236972		106.7326978
	2w	3718	23457		0.158502792		97.09437901
	3w	6506.285714	20363		0.319515087		195.7260089
	3w	8422.285714	21835		0.3857241		236.283799
	3w	7388.5	21511		0.343475431		210.4034459
	4w	8861	20178		0.439141639		269.0058904
	4w	8381.142857	17533		0.478021038		292.8223228
	4w	7420.5	17962		0.413122147		253.0670766
PS2-CTF							
			actin			mean	%
	2w	5720.5	26432		0.216423275	0.240784926	89.88240175
	2w	4961.5	23623		0.210028362		87.22654088
	2w	6941	23457		0.295903142		122.8910574
	3w	5346	20363		0.26253499		109.0329839
	3w	5981.5	21835		0.273940921		113.7699626
	3w	4169.5	21511		0.193831063		80.49966671
	4w	8309	20178		0.411785112		171.017812
	4w	7189	17533		0.410026807		170.2875728
	4w	4483	17962		0.249582452		103.6536862

2nd Editorial Decision 27 February 2012

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referees that were asked to re-assess it. As you will see the reviewers are now supportive and I am pleased to inform you that we will be able to accept your manuscript pending the final editorial amendment.

- As you know, we publish both reviewers' comments and authors responses as part of our Review Process File (see below for more details). We noted that you have provided data for the referee #3. Do you wish this data to be published as part of the RPF or would you prefer not to?

Please submit your revised manuscript within two weeks. I look forward to seeing a revised form of your manuscript as soon as possible.

I look forward to reading a new revised version of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #2:

I have now read the revised version and the authors' response. I am satisfied with the enhancements, and I now recommend publication as is.

Referee #3:

The authors have addressed most of the criticism although the strong statement given in the title is still not fully supported by the data shown.