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Inactive variants of death receptor p75^{NTR} reduce Alzheimer's neuropathology by interfering with APP internalization

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1st Editorial Decision 24th Feb 2020

Dear Carlos,

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and I am afraid that their overall recommendation is not very positive.

The referees find the topic interesting, but also raise concerns with the analysis that I am afraid preclude publication here. They raise technical concerns as well as level of insight. In particular, the referees find that we would need a better understanding for why there is a differential effect on APP internalization with the inactive p75NTR variant. I have had further follow-up discussions with the referees regarding this point and they are in agreement on this issue.

Given this input from good experts in the field, I am afraid that I can't offer to consider publication here.

I thank you for the opportunity to consider this manuscript. I am sorry that I cannot be more positive on this occasion, but I hope nevertheless that you will find our referees' comments helpful.

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Referee #1:

Li et. al investigate the effects of p75NTR mutations (and a full KO of p75NTR) on neuropathology in 5XFAD mice. p75NTR is a member of the death receptor family and can induce cell death pathways. Amyloid beta can induce rapid cell death in cultured neurons through direct interaction with p75NTR, as shown in cultured neurons. Here the investigators look at the effects of mutating or inactivating p75NTR signaling in 5XFAD mice.
Indeed mutating or inactivating p75NTR ameliorated the pathology and behavior seen in 5XFAD mice. Interestingly however, mutating p75NTR had a greater protective effect than knocking out p75NTR. The authors propose a mechanism where mutant p75NTR tethers APP to the cell surface thereby inhibiting APP internalization and preventing APP from interacting with BACE1 in intracellular compartments.
In general the idea is interesting, and the data from electrophysiology are particularly convincing. However there are several issues with the mechanistic experiments done, the lack of further evidence supporting their claims, data presentation, and handling of previous literature.

Main points:
1) The proximity ligation assay is an important part of the paper, where the authors look at the interaction of APP and p75NTR on the surface of neurons and inside cells. First of all, the data from

this assay should be shown in the main figure. Second, schematics should be added to clearly indicate that there are two sets of experiments - one looking at interactions on the surface of cells, and the other intracellular. The figure panels themselves should be clearly marked to show this. More importantly, controls are missing in this assay. It is essential that the authors show control where the APP used in this assay as a deletion or mutation that prevents it from binding to the receptor. The present controls with no APP or no receptor is not sufficient, because nonspecific interactions could still happen.

2) Biochemical interaction between APP and the receptor needs to be shown by immunoprecipitation.

3) Also, in all the internalization/surface-staining assays shown, it is difficult to be certain that the authors are looking at our internalized molecules, because there are no controls to show something that is on the surface or inside the cell is disrupted by these procedures. Thus these experiments are not done with the highest rigor. Was total surface staining similar in all cases? These data need to be shown somewhere.

4) The 5XFAD mouse is well-characterized and the figure panels in Fig. 1A do not add any new information and should be provided as supplementary material. In the place of those images should be representative images of the 6E10 staining in 5XFAD, 5XFAD/KO, 5xFAD/DD and 5XFAD/C259A mice (at either 9 or 12 months) so that the differences in plaques can be appreciated. Additionally, it's unclear if the reduction of plaque area reported in Figure 1B is due to decreased plaque number, plaque size or both. Representative images from each experimental group are needed in this and all other figures where relevant.

5) The biochemistry done is not adequate, and in particular, ELISA assays are needed to make firm conclusions. Details in points #6 and 7 below.

6) For instance the authors reference Kwart et. al 2019 in the figure legend to aid in the description of the Western blot. However, in the Kwart manuscript the bands obtained using B-CTF antibodies are distinct from one another but in the present figure the bands are smeared (and individual bands cannot be identified). Moreover, combining C99 & pC99 as a description of the top band is confusing (in Kwart et al, the top band labeled as pC99 is clearly distinct for the band labeled C99). While It does appear that the p75NTR mutations and KO have a clear difference in B-CTFs, the description of the bands is inadequate and individual bands cannot be identified as they were in the referenced manuscript.

7) Figure 4B shows that sAPPa levels are increased in p75NTR mutants and that the difference was detected in hippocampal lysates using the 6E10 antibody. 6E10 recognizes an epitope within amino acids 3-8 of (human) amyloid beta which is present on both sAPPa and full-length human APP. Because 5XFAD brain tissue homogenates would contain both full length APP and sAPPa, it's unclear whether the bands from the 6E10 probe are full-length APP or sAPPa. The authors do note that total APP levels (assed using the APP Y188 antibody which recognizes both human and mouse APP) were equal between groups, however some uncertainty whether or not the 6E10 bands are indeed sAPPA remains. The sAPPa needs to be analyzed by CSF sampling/ELISAs.

8) There is no statistical analysis in figure 5A-C, even though that's a key conclusion. Figures 5D-F have so many caveats (considering total volume of the cell, dendrites versus axons, etc.) that it is not possible to make much of these data and they should be excluded from the paper.

9) The authors need to temper their language as its not clear that they have nailed down the mechanism. For instance, the effects of the p75NTR-KO on APP internalization (and amyloid pathology) cannot be due to the mechanism suggested in figure 5 (i.e. p75NTR tethering APP to the cell surface). All the authors see is that there is slightly more APP internalized when they have the mutations v/s total KO of the receptor. Please be more careful in the words being used, throughout the manuscript.

Other important issues:
- "However, the molecular pathways that regulate APP internalization and intracellular trafficking in neurons are unknown." I don't how anyone can say that, given the hundreds of publications and extensive knowledge of APP internalization - including specific domains and associating partners. The authors should be more careful in presenting the literature correctly.

- Last paragraph, under "Reduced Aβ content and histopathology in the hippocampus of 5xFAD mice carrying inactive p75NTR variants" subheading: This is incorrect, it cannot be concluded that the in vitro results are contrasted by the in vivo data. In Figure 1 it is demonstrated that the p75NTR mutants have less Abeta/Abeta plaques than controls. Therefore, since Abeta levels vary between groups, no conclusions on whether or not the p75NTR mutant mice are better protected from Abeta insult can be drawn (i.e. the differences in behavior, neuroinflammation etc are likely the result of less Abeta, not greater neuroprotection against Abeta toxicity).

- Introduction: "Cleavage by the gamma-secretase complex liberates a soluble CTFβ and a small N-terminal fragment of 40 or 42 amino acids in length known as the amyloid beta peptide or Aβ". As stated, this is incorrect as "CTFB" describes the specific molecule that is released when APP is processed by BACE1. Cleavage of the CTFB by gamma secretase releases amyloid beta and an APP intracellular fragment (AICD).
- Figure 2: The representative image of MitoSox staining in Figure 2G for wild type mice shows virtually no signal which raises the question whether or not MitoSox was detectable in those slices. Moreover, Figure 2H shows that the MitoSox signal in 5xFAD mice is ~4X that of Wt mice however the images in 2G suggest a far greater difference. A more representative image of the Wt slices is needed. Note that in panels 2B and 2D that the "area %" is area of hippocampal tissue while "area %" in 2F is area of Ab plaques co-localized with RTN3 (according to the figure legend). Using "area %" for both of these situations is confusing and the Y-axis for 2F should be revised.

Misc. points:
Discussion:
A) "Interestingly, the levels of CTFβ and Aβ in the hippocampus of 5xFAD mice carrying different p75NTR alleles also correlated with the extent of APP internalization."
This is incorrect as no correlative analysis was done ("correlate" is a specific statistical term). It can be stated that the p75NTR alleles reduce Ab/CTFB in vivo and decrease APP internalization in vitro, however "correlated" is an over interpretation.
B) "The 5xFAD mouse model of AD displays enhanced and accelerated AD-like neuropathology and is perhaps one of the most aggressive AD models in mice"
Enhanced and accelerated compared to what? Moreover, "most aggressive AD models" sounds like the mice themselves are aggressive. Please restate this in terms of the early and aggressive neuropathology observed in 5xFAD mice.
C) "We find quite striking that changing a single amino acid in the mouse genome can have such dramatic effects on the course of this disease."
Though this is indeed interesting, there are other examples where single mutations drastically

affect AD pathology, particularly mutations in the YENPTY domain.
D) "Importantly, however, 5xFAD mice do not display any abnormality that is not found in the AD patient population."
The 5xFAD mouse does however overexpress APP which is not characteristic of the human condition. Therefore, it is incorrect to suggest that the 5XFAD mouse is representative of human AD.

• Abbreviations for presenilin-1 are inconsistent in the manuscript, both PS1 and PS-1 are used.
• This is also true for "BACE" and "BACE1"
• While the manuscript is generally well-written, additional proofreading is needed (at one point in the results "Fig. 3bB" is described and "specially" is used)
• In the "Materials and Methods", the genetics of the mice are not described. Without any other information, I'm assuming that the 5xFAD mice are homozygous for transgenes and the p75NTR mice are homozygous for the mutations (but it's impossible to know with the information provided).
• Mark boundaries of tissue and cells in images so reader can make out the anatomy
• Fig. 4A: sequence of bars does not match sequence of legends

Referee #2:

This manuscript investigates the effects of manipulating the signaling of p75 neurotrophin receptor (p75NTR) on APP internalization, cognitive and synaptic function, and amyloid pathology in the 5XFAD mouse model. The Authors utilize immunostaining to examine plaque deposition, microglial activation, astrocyte activity, and mitochondrial impairment. They also extract amyloid fractions, and analyze concentrations/transcripts of the APP processing pathway. The Authors also examine behavioral and synaptic plasticity. Lastly the authors image altered APP internalization dynamics. Variations to p75NTR all lowered AD associated pathophysiology, synaptic deficits, and improved cognitive function. The KIs were typically more advantageous than the KO. Using culture dynamics to examine the differences in APP processing, authors demonstrated that variants slowed internalization of APP, allowing less processing by BACE1 in endosomes, thus alleviating AD burden.

This manuscript does a good job of including broad analysis of AD-associated deficiencies in 5XFAD mice, by examining both functional, network, and behavioral alterations in different p75NTR variants. The authors also nicely control for internalization dynamics of p75NTR in culture, as well as APP interactions with the variant itself. The slowing of APP intracellular trafficking accounts for the decreased amyloid burden and shift to α-secretase metabolism in variant mice, and thus improved memory, synaptic function, and immune activation. The novel findings presented here provide important evidence of mechanisms regulating APP trafficking and open new targets for pharmaceutical targeting. In its present form, however, this manuscript raises a number of concerns, as outlined below, which should be addressed.

Figure 1A should include representative images from all 5 conditions (WT, 5XFAD, and all three mutant lines) so the results can be visualized. The same applies for 2A, 2C, 2E, 2G.

RTN3 is used as a marker of dystrophic neurites, however RTN3 modulates BACE1 activity and localization, which is a measured outcome in the paper. A different or additional marker of neurites should be examined. Discussion of the relationship between BACE1 and RTN3 should be added.

In Figure 2 the four markers should be examined at the same ages. MitoSox was only shown at 2 and 6 months, and RTN3 at 9 months only, whereas Iba1 and GFAP are examined at 6, 9, and 12 months.

Were there any effects on survival, cell proliferation, apoptosis, or axonal organization in the different mutant mice?

Were other forms of synaptic plasticity altered? Were there differences in Paired-Pulse Facilitation or I/O curves? The KO vs KI effects may be more apparent with a less intense stimulation or an LTD protocol, the slow decay (120 mins post stim) is a slower decay than expected indicating a potential over stimulation.

The behavioral and synaptic plasticity examinations were done at an earlier time point than the APP processing analysis. Do the results from Fig 4 replicate in the earlier age points? Or even precede the behavioral phenotype? Additionally, S4 should be at the same ages shown in the main figure. Fl APP seems visually decreased (especially compared to the elevated GAPDH) in the KO mice.
Would using an antagonist to the p75NTR receptor also slow APP internalization dynamics in 5XFAD mice? Rescue LTP? Conversely does application of neurotrophins hinder the dynamics?

In Figure 5D, the shape of the neuron suggests the super resolution image is centered on the nucleus. If so, why is there APP and BACE1 positive puncta in the nucleus, an organelle that is not expected to have either of these proteins?

With decreased amyloid, IBA1, and GFAP, are levels of neuroinflammation restored to WT levels in the variant mice?

A discussion of the effects of the mutations on other cell types involved in AD and APP processing should be included (Microglia, astrocytes, etc.).

The Methods section should describe the genetic background of the 5XFAD mice.

Minor Concerns:
- Figure S1 stats need clarification
- Results section refers to wrong supplemental figures
- GAPDH in S2B is unconvincing. B3 Tubulin may be cleaner.
- The same GAPDH is used twice in S4
- Statistics should be shown in Figure 4 (if significant, as implied by the text).

Referee #3:

In their manuscript, Yi and co-workers have investigated the role of p75NTR in the context of AD neuropathology and in particular APP processing. Previous work has demonstrated that the expression levels of this death receptor are upregulated in AD brain AD mouse models, and

knocking-out the gene in such models resulted in a mild improvement. The authors explored further the role herein, by studying signaling deficient p75NTR mutants (two, a Cys259 and deltaDD mutant) and generating the respective KI models that they crossed with the 5xFAD mice, one of the more severe AD models displaying pathology already at 2 months. These models recapitulated the earlier observations of increased levels of p75NTR, but in contrast to the KO, the signaling mutants had a more significant beneficial effect on pathology, as well as on behavior. They conclude that the downstream signaling function of p75NTR is therefore not involved in alleviating the neuropathology and that other mechanisms are responsible. They focused therefore on APP processing and noticed that the signaling mutants shift the processing to the non-amyloidogenic route. This is not caused by alterations in expression of secretases nor interactions with APP, but rather to a decreased internalization of APP, allowing more sAPPalpha production. Overall, the authors strengthen the importance of p75NTR expression in regulating APP proteolysis thereby contributing to a better knowledge on disease development. However, several aspects are confirming earlier studies and besides a decreased internalization, we don't learn that much on the mechanism behind this, ie why a signaling deficient p75NTR is less capable of internalizing APP. The data suggest that signaling might be inducing the recruitment of endocytic machineries or, alternatively, clustering to facilitate endocytosis. My appreciation is that the mechanistics behind this are not addressed and that a more comprehensive insight should be included to consider it for publication in the EMBO J. The story is original but maybe premature in its current form. In the following paragraphs, I highlight my major concerns and make some suggestions that the authors could consider to strengthen their story.
The whole study is conducted with the 5xFAD as the genetic background, which is a very severe AD model starting already on the age of 2 months. It is increasingly preferred to consider as well APP KI models with a later onset of pathology; maybe in this case, a much stronger beneficial effect could be observed.
I have an important critique on the interaction data sets. Firstly, the interaction of p75NTR with APP has been shown already in total brain extracts, at endogenous levels (Fombonne et al 2008). In this manuscript, the authors essentially confirm these data in primary neurons with the different genotypes. Also the link of p75NTR expression and alpha-shedding has been documented already: in the same paper (Fombonne et al., 208) it is demonstrated that overexpression of p75NTR reduces alpha-shedding of APP and that this can be restored by adding NGF or Abeta itself. The interesting point is here that overexpressed signaling-competent p75/NTR decreases alpha-processing while signaling-defective (this study) increases it. But the authors do not provide data that could shed light on the underlying mechanism.
Surprisingly, they don't find differences between the wt and signaling mutants, although one would expect that the signaling mutants would result in higher PLA signals compared to wt p75NTR, as internalization is delayed. How is this explained? I would also propose independent approaches, for instance, using cell surface biotinylation, and this can be done at endogenous levels.
More problematic is the next experiment where the authors study the co-internalization of p75NTR with (OE) APP. They do this by performing first PLA and next follow the internalization of the PLA signal. However, with this approach one artificially crosslinks both molecules and hence logic they co-internalize. In theory, one should be able to also show interaction and co-internalization with endogenous APP. Furthermore, as they use 6E10, they cannot conclude that full-length APP is (co-)internalizing: their data do not rule out that APP needs to be shedded first, prior to interaction and internalization. The authors should look for independent approaches that complement these data and try to show as well which fragment interacts and co-internalizes, as this could point to more mechanistic insights why the signaling mutants internalize less. Alternatively, are the signaling mutants failing to recruit adaptors and endocytic machinery or does p75NTR requires APP for getting internalized? In this case, endocytic deficient APP mutants might be checked for their ability to co-internalize with wt p75-NTR.

In their final set of data, they show that less APP is reaching BACE1-positive endosomes. Firstly, the quality of the imaging is not convincing. The authors describe this is super-resolution but actually rather obtained through deconvolution. The pictures have a patterned appearance (often seen in SIM images as well, and the result of an artefactual image rendering). Secondly, there is no clear co-localization visible and better examples should be provide (eg double labeling with as well endosomal markers).

Minor compulsory comments:

In general the writing could be improved. Particularly the introduction is verbose and could be much shortened to the essential information. The authors should also avoid to only refer to a small number of the same reviews and cite as much as possible also original papers. For instance the preferred endosomal processing of APP by Bace1 has been originally described in Rajendran et al. and Sannerud et al. 2011.

In general, the authors often mention that the C259A mutant is particularly more effective in reducing pathology but this is not statistically demonstrated. In fact, in many cases, like in fig 2, fig 3, fig 4D-E and fig 5, there is likely no difference between both signaling mutants. Only in the memory test (fig 3E) some trend might be spotted, but overall the data do not justify to differentiate between both mutants.

For figures 4 and 5 and suppl fig 5, only the time courses of control images are shown, next to the quantified data. This doesn't make much sense. It would be better to show for each primary culture and genotype one time point so that at least the difference can be appreciated from the images as well.

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Responses to reviewers

We thank the reviewers for the time invested in reading and commenting on our manuscript. We would like to start this rebuttal by noting that, as the epidemic began taking over the world this past February, we have had great difficulties in proceeding with our studies at a normal pace. Or at any pace. Our laboratories were shut for several months and mouse breeding was suspended, causing significant delays and disruptions. Nevertheless, we are happy to have been able to complete several studies that considerably support and advance the main notions of our manuscript. This has been a herculean effort given the present circumstances.

A summary of the main changes and additions made to the revised manuscript follows here:

1) We have incorporated several of the data previously shown in supplementary figures into the main figures of the manuscript, as requested by several referees.

2) We have added examples of micrographs showing A-beta accumulation for all genotypes and at different ages (Fig. 2D) as requested.

3) We have added new data showing that A-beta plaques greater than 30μm in diameter are less abundant in p75NTR mutant mice compared to wild type mice as requested (Fig. 2F).

4) We have added examples of micrographs showing GFAP, Iba1, RTN3 and MitoSox staining for all genotypes at 6 months of age as requested (Fig. 3).

5) We have added examples of micrographs showing internalization of APP, p75NTR and their co-internalization for several genotypes at one time-point as requested (Fig. 6 and 7).

6) As requested, we have included statistical analysis of internalization kinetics by 2-way ANOVA (Fig. 6 and 7).

7) We have added data showing total, steady-state cell surface levels of APP, p75NTR and APP/p75NTR complexes (as detected by PLA), used as 100% in internalization assays, for mutant and wild type p75NTR genotypes as requested (Fig. S3A, S3C and 7D).

8) We have added a new study assessing the effects of NGF treatment on the kinetics of internalization of APP in all p75NTR genotypes (Fig. 6D, E and S4A-F). These data show that NGF increases APP internalization in hippocampal neurons in a p75NTR-dependent manner. NGF did not affect APP internalization in KO, ΔDD or C259A neurons, further confirming that p75NTR signaling regulates APP internalization in neurons.

9) We have added a new study using co-immunoprecipitation showing a deficit in the association of p75NTR variants ΔDD and C259A with beta-adaptin (Fig. 6I), a key component of the AP-2 complex that mediates receptor endocytosis from clathrin-coated vesicles, providing a mechanistic rationale for their inefficient internalization.

10) We have added a new study showing interaction between APP and p75NTR by co-immunoprecipitation from hippocampus extracts, as requested by some reviewers (Fig. 7A). Mutant ΔDD and C259A molecules were found to associate with APP at levels comparable to those of wild type p75NTR. Importantly, no co-immunoprecipitation was found in extracts from KO mice or mice lacking the 5xFAD transgene.

11) We have totally re-made the imaging and analysis of co-localization between internalized APP and BACE1, and present improved images, including high-magnification insets, across the different genotypes (Fig. 8A). In the new analysis, we found that the fraction of internalized APP that co-localizes with BACE1 is lower in the ΔDD and C259A mutants compared to wild type neurons, confirming a trend that we observed earlier, but which was not statistically significant. This suggested the possibility of altered intracellular trafficking of APP in the mutant neurons, in addition to reduced/slower internalization, leading to the Rab11 study mentioned next.

12) We have added a new study assessing co-localization of either internalized APP or internalized p75NTR with the small GTPase Rab11, which marks recycling endosomes (Fig. 8D-I). We find that a greater proportion of internalized APP, roughly corresponding to the reduction in co-localization with BACE1, co-localizes with Rab11 in ΔDD and C259A mutant neurons compared to wild type (Fig. 8I). Importantly, we also find that internalized ΔDD and C259A p75NTR molecules themselves associate with the recycling pathway to a greater extent than wild type p75NTR (Fig. 8F). The significance of this finding is explained in our rebuttal below and in the revised manuscript.

These additional data support the notion that p75NTR molecules that are deficient in signaling, by virtue of their interaction with APP and altered internalization and trafficking, reduce APP internalization and divert a fraction of the internalized APP to recycling endosomes, in detriment to BACE1-containing endosomes and amyloidogenic cleavage.

Detailed responses to the reviewers' comments are presented below.

Referee #1

Main points:
1) The proximity ligation assay is an important part of the paper, where the authors look at the interaction of APP and p75NTR on the surface of neurons and inside cells. First of all, the data from this assay should be shown in the main figure. Second, schematics should be added to clearly indicate that there are two sets of experiments - one looking at interactions on the surface of cells, and the other intracellular. The figure panels themselves should be clearly marked to show this. More importantly, controls are missing in this assay. It is essential that the authors show control where the APP used in this assay as a deletion or mutation that prevents it from binding to the receptor. The present controls with no APP or no receptor is not sufficient, because nonspecific interactions could still happen.

We have clarified in the revised manuscript several of the points raised here

by the reviewer. Total and cell surface interaction measured between p75NTR and APP by PLA are now shown in the main figure of the manuscript. The only difference between this and the internalization studies is that, in the latter, antibodies are added to live cells which then internalize the label from the cell surface. It is a very conventional internalization assay, except that, for co-internalization studies, we have developed it through PLA, rather than using standard secondary antibodies.

Regarding the comment about controls, we note that PLA is about detecting interaction of protein A with protein B in situ. This manifest itself as tiny spots on an immunofluorescence image, which is always a concern with this assay. The two essential controls are omission of either protein: the spots should disappear or be drastically reduced in each case. Those are the controls we have provided, and in fact Reviewer #2 congratulates us for the thorough controls used in our PLA assay. We are of the opinion that the type of “control” studies mentioned by this reviewer are not suitable, at least as controls. First, it is unclear which APP deletion or mutation could be used to achieve this. Additional (and lengthy) research will be required to precisely identify the residues that mediate the contact between the two receptors. More importantly, such deletions and mutations are by themselves likely to affect expression and/or trafficking of APP. That kind of studies will be themselves pronged to require numerous controls to rule out other effects, and quite likely turn out to be a whole new line of enquiry on its own right.

2) Biochemical interaction between APP and the receptor needs to be shown by immunoprecipitation.

We politely disagree with the reviewer on this. We are convinced that PLA is much superior to co-immunoprecipitation, as it detects interactions in situ, respecting the organization, compartmentalization and three-dimensional structure of cells. In contrast, co-IP studies are done in a complex cell lysate or tissue extract where transmembrane proteins are now soluble and all protein content from all organelles come in contact with each other. To satisfy the reviewer on this point, we have performed a co-immunoprecipitation study using hippocampal extracts from wild type and mutant mice showing specific and comparable levels of co-immunoprecipitation between wild type and mutant p75NTR variants with APP, using appropriate controls (Fig. 7A).

3) Also, in all the internalization/surface-staining assays shown, it is difficult to be certain that the authors are looking at our internalized molecules, because there are no controls to show something that is on the surface or inside the cell is disrupted by these procedures. Thus these experiments are not done with the highest rigor. Was total surface staining similar in all cases? These data need to be shown somewhere.

It has been a bit difficult for us to grasp what the reviewer meant here. One important step that it is customary in this type of internalization assays is the acid wash. This is done after internalization to make sure that we are only looking at molecules that went inside the cell; all remaining cell surface antibody, if any, would be removed by the acid wash. If one does this at time=0 (i.e. prior to internalization) all labeling is removed, and that is what we see. After the acid wash, any signal that remains has to be inside the cell. We also note that all constructs started from a comparable level of total surface

staining (labeled “total” in Supplementary Fig. 3B, formerly Fig. 4), which was used as 100% to normalize the kinetics data. Quantification of the total surface staining is now shown in Supplementary Fig. 3A. We note that steady-state levels at the cell surface are not only affected by internalization, but also recycling as well as membrane insertion of *de novo* synthesized molecules. Normal steady-state levels at the cell surface despite slower internalization would suggest an overall slower turnover of these molecules in the mutant neurons.

4) The 5XFAD mouse is well-characterized and the figure panels in Fig. 1A do not add any new information and should be provided as supplementary material. In the place of those images should be representative images of the 6E10 staining in 5XFAD, 5XFAD/KO, 5xFAD/DD and 5XFAD/C259A mice (at either 9 or 12 months) so that the differences in plaques can be appreciated. Additionally, it's unclear if the reduction of plaque area reported in Figure 1B is due to decreased plaque number, plaque size or both. Representative images from each experimental group are needed in this and all other figures where relevant.

As requested by the reviewer, we are now showing 6E10 staining in all genotypes at 6, 9 and 12 months in the revised version of Figure 2. We have also included an analysis of plaque number (larger than 30μm in diameter) in Fig. 2F as requested. Indeed, all mutants show significantly reduced number of large Aβ plaques.

5) The biochemistry done is not adequate, and in particular, ELISA assays are needed to make firm conclusions. Details in points #6 and 7 below.

ELISA was indeed used to assess monomers, oligomers and fibrils of A-beta at different ages and in all genotypes. We kindly refer the reviewer to Figs. 2F-H.

6) For instance the authors reference Kwart et. al 2019 in the figure legend to aid in the description of the Western blot. However, in the Kwart manuscript the bands obtained using B-CTF antibodies are distinct from one another but in the present figure the bands are smeared (and individual bands cannot be identified). Moreover, combining C99 & pC99 as a description of the top band is confusing (in Kwart et al, the top band labeled as pC99 is clearly distinct for the band labeled C99). While It does appear that the p75NTR mutations and KO have a clear difference in B-CTFs, the description of the bands is inadequate and individual bands cannot be identified as they were in the referenced manuscript.

We are glad to hear that the reviewer acknowledges that “*mutations and KO have a clear difference”* in the production of C99 fragment. At the same time, the reviewer does not seem to be content with the way the bands were labeled in the figure. We have now clarified/changed the description of the bands following the nomenclature used the Kwart paper as suggested by the referee. Specifically, we note that we do in fact see two distinct top bands running at about 15kDa, as the C99 fragment normally does, and these have now been labeled as C99 and pC99, respectively after Kwart et al. While we do see distinct bands in the lower range, we have refrained from assigning

specific descriptions to them as they are less clearly separated. Nevertheless, the important thing here is that that there is clear difference in our mutants, a fact that is also acknowledged by the reviewer.

7) Figure 4B shows that sAPPa levels are increased in p75NTR mutants and that the difference was detected in hippocampal lysates using the 6E10 antibody. 6E10 recognizes an epitope within amino acids 3-8 of (human) amyloid beta which is present on both sAPPa and full-length human APP. Because 5XFAD brain tissue homogenates would contain both full length APP and sAPPa, it's unclear whether the bands from the 6E10 probe are full-length APP or sAPPa. The authors do note that total APP levels (assed using the APP Y188 antibody which recognizes both human and mouse APP) were equal between groups, however some uncertainty whether or not the 6E10 bands are indeed sAPPA remains. The sAPPa needs to be analyzed by CSF sampling/ELISAs.

We would like to clarify two things here. First, assessment of soluble APP-alpha fragment was not done in total brain tissue homogenates, but in the TBS fraction of our fractionation protocol (explained in the Methods section), which contains only soluble material (same protocol of extraction used to detect A-beta monomers for the ELISA shown in Figure 2F-H). Full length APP is not present in this fraction since it is associated with cell membranes. Second, soluble APP-alpha is of smaller molecular weight (≈90kDa) than full length APP (≈120kDa), so even if one were to run a total homogenate, the two bands could still be distinguished. We have provided MW guides in all the gels.

8) There is no statistical analysis in figure 5A-C, even though that's a key conclusion. Figures 5D-F have so many caveats (considering total volume of the cell, dendrites versus axons, etc.) that it is not possible to make much of these data and they should be excluded from the paper.

We have now included statistical analysis in all internalization kinetics done by 2-way ANOVA comparing genotypes across the whole time span of the analyses. These are now indicated in Figs. 6B, D, G and 7D. In all cases, the differences were either highly significant ($P<0.01$) or significant ($P<0.05$).

We would like to clarify that the analysis of images showing co-localization of APP and BACE1 was done at the cell body of cultured neurons, not in dendrites or axons. Moreover, the data on co-localized pixels of BACE and APP were normalized to either total internalized APP staining or total BACE staining present in the same image, therefore those data are independent of cell volume or ROI size. We have now totally redone the imaging and analysis of APP/BACE1 co-localization, and also included additional studies using the same methodology looking at co-localization of either APP or p75NTR with the small GTPase Rab11, which marks recycling endosomes (shown in revised Fig. 8D-I). Also, we now provide representative images across all the relevant genotypes, with higher magnification insets. We believe these images are of higher quality and hope they will satisfy the reviewer. As we re-made the analysis using a better microscope at another facility, we noted that there is also a significant difference in the fraction of internalized APP that co-localizes with BACE1 (Fig. 8C). In the previous data set, we saw a trend, but

this was not statistically significant. A lower fraction of internalized APP that co-localizes with BACE1 suggests that, not only overall internalization but also, the intracellular trafficking of APP is altered in the mutants. This is now discussed at greater length in the manuscript and is in agreement with the increased co-localization between APP and Rab11 in the mutant neurons (Fig. 8I).

Other important issues: - "However, the molecular pathways that regulate APP internalization and intracellular trafficking in neurons are unknown." I don't how anyone can say that, given the hundreds of publications and extensive knowledge of APP internalization - including specific domains and associating partners. The authors should be more careful in presenting the literature correctly.

We regret this misunderstanding. We were referring to external pathways that **regulate** APP internalization, aside of the basic endocytic mechanism per se. We did not mean to imply that nothing was known about the mechanism. Our point was about its **regulation**, namely other molecules, besides the main components of the endocytic machinery, that can quantitatively regulate up or down APP internalization. We also note that, even on the basic internalization mechanism, there are still fundamental gaps. For example, there is still controversy whether dynamin is involved in neurons or not, whether internalization requires APP oligomerization, and whether it happens at both somatodendritic as well as axonal compartments or in just the former. At any rate, we have now rephrased this statement to avoid confusion.

- Last paragraph, under "Reduced Aβ content and histopathology in the hippocampus of 5xFAD mice carrying inactive p75NTR variants" subheading: This is incorrect, it cannot be concluded that the in vitro results are contrasted by the in vivo data. In Figure 1 it is demonstrated that the p75NTR mutants have less Abeta/Abeta plaques than controls. Therefore, since Abeta levels vary between groups, no conclusions on whether or not the p75NTR mutant mice are better protected from Abeta insult can be drawn (i.e. the differences in behavior, neuroinflammation etc are likely the result of less Abeta, not greater neuroprotection against Abeta toxicity).

We respectfully believe this is purely a semantic issue, and it can be interpreted in different ways. The point here is that, *in vivo*, the knock-in mutants are better protected than the knock-out. Here we meant **protection from the disease** as such, not specifically whether their response to A-beta toxicity was different. (It wasn't.) On the other hand, *in vitro*, all three strains are equally protected. We did not write or meant to imply that the mutants are better protected from the A-beta insult. They do indeed show better protection from developing Alzheimer's disease.

- Introduction: "Cleavage by the gamma-secretase complex liberates a soluble CTFβ and a small N-terminal fragment of 40 or 42 amino acids in length known as the amyloid beta peptide or Aβ". As stated, this is incorrect as "CTFB" describes the specific molecule that is released when APP is processed by BACE1. Cleavage of the CTFB by gamma secretase releases amyloid beta and an APP intracellular fragment (AICD).

We wrote "**soluble CTFb**", to distinguish it from the product of BACE (called simply CTFb) which is not soluble but still membrane attached. The soluble

CTFb fragment (abbreviated sCTFb) is indeed the product of gamma-secretase cleavage. The reviewer uses the term AICD for the soluble CTFb and some, but not all, authors use that nomenclature as well. However, there are many other reviews and papers where "soluble CTFb" is used instead. We have now clarified this in the revised version of the manuscript.

- Figure 2: The representative image of MitoSox staining in Figure 2G for wild type mice shows virtually no signal which raises the question whether or not MitoSox was detectable in those slices. Moreover, Figure 2H shows that the MitoSox signal in 5xFAD mice is ~4X that of Wt mice however the images in 2G suggest a far greater difference. A more representative image of the Wt slices is needed. Note that in panels 2B and 2D that the "area %" is area of hippocampal tissue while "area %" in 2F is area of Ab plaques co-localized with RTN3 (according to the figure legend). Using "area %" for both of these situations is confusing and the Y-axis for 2F should be revised.

MitoSox staining in a healthy brain is indeed very low. The new images provided do show a bit of staining (background?) at high magnification. The quantifications we have made are indeed correct. We note that there may also be an issue of dynamic range loss due to the increased contrast that appears when the images are printed or viewed in a monitor. What we quantified in WT brains is likely a mix of very low signal and background. We have not removed from the analysis any background signal that may have been present, as we have adopted a more conservative approach to quantification of these changes.

Misc. points: Discussion:
A) "Interestingly, the levels of CTFβ and Aβ in the hippocampus of 5xFAD mice carrying different p75NTR alleles also correlated with the extent of APP internalization."
This is incorrect as no correlative analysis was done ("correlate" is a specific statistical term). It can be stated that the p75NTR alleles reduce Ab/CTFB in vivo and decrease APP internalization in vitro, however "correlated" is an over interpretation.

We respectfully disagree with the reviewer that our statement was an "over-interpretation" of our data. The mutant brains produced less A-beta plaques and less CTFb and their neurons also internalized less APP. The genotypes that internalized less APP also showed lower levels of A-beta and CTFb. That is clearly a correlation, namely, a series of changes that consistently go in the same direction. We do not wish to engage in additional semantic discussions of whether the term correlation can only be used when it has been described mathematically or not. We are simply noting that the changes go in the same direction, and that is a useful thing to know.

B) "The 5xFAD mouse model of AD displays enhanced and accelerated AD-like neuropathology and is perhaps one of the most aggressive AD models in mice" Enhanced and accelerated compared to what? Moreover, "most aggressive AD models" sounds like the mice themselves are aggressive. Please restate this in terms of the early and aggressive neuropathology observed in 5xFAD mice.

We do not see any problem with this sentence. We are discussing mouse

models of AD, and the 5xFAD model clearly shows enhanced and accelerated pathology compared to most (or all) other mouse models. With all respect, neither do we think that, by saying that this model shows more aggressive pathology, anyone, perhaps with the exception of the reviewer, will believe that we meant to say that the mice themselves were actually more aggressive. Nevertheless, we have now replaced “aggressive” with “severe” in that sentence.

C) "We find quite striking that changing a single amino acid in the mouse genome can have such dramatic effects on the course of this disease."
Though this is indeed interesting, there are other examples where single mutations drastically affect AD pathology, particularly mutations in the YENPTY domain.

We thank the reviewer for this insight, but the fact that mutations in APP will have effects on APP should be an obvious fact and that is clearly not what we meant.

D) "Importantly, however, 5xFAD mice do not display any abnormality that is not found in the AD patient population."
The 5xFAD mouse does however overexpress APP which is not characteristic of the human condition. Therefore, it is incorrect to suggest that the 5XFAD mouse is representative of human AD.

We did not write that the 5xFAD model is “representative of human AD”. We simply pointed out the fact that, despite its severe pathology, the model does not show any pathological abnormality that is not found in the human disease. Obviously, the model expresses a transgene (two, in fact) because that is how it was made. We did not mean to imply that AD patients have transgenes in them, if that is what the reviewer was thinking.

Referee #2

Figure 1A should include representative images from all 5 conditions (WT, 5XFAD, and all three mutant lines) so the results can be visualized. The same applies for 2A, 2C, 2E, 2G.

This has now been included in the revised version of this Figure (Fig. 2D).

RTN3 is used as a marker of dystrophic neurites, however RTN3 modulates BACE1 activity and localization, which is a measured outcome in the paper. A different or additional marker of neurites should be examined. Discussion of the relationship between BACE1 and RTN3 should be added.

We thank the reviewer for bringing this up to our attention. Indeed, it has been shown that reticulon proteins, including RTN3, are negative regulators of BACE1 activity, blocking access of BACE1 to APP, thus reducing the beta-cleavage of this protein and generation of A-beta fragments (He et al., Nature Medicine 2004). However, we found increased levels of RTN3 aggregates in degenerating neurites of 5xFAD mice, in agreement with studies in other mouse models and AD patients, which clearly produce high amounts of amyloid beta, and hence elevated BACE1-mediated cleavage of APP. We

note that the relationship between RTN3 and BACE1 does not appear to be very straightforward, and most studies coincide that aggregates of RTN3 play roles in AD that are independent of regulation of BACE1 activity. At any rate, there are numerous studies showing RTN3-neuropathology in brains of AD mice and patients, and so we believe that it can be used as a legitimate neurodegeneration marker in our study. We have included a short discussion of these issues in the Results section of the revised manuscript as requested by the reviewer.

In Figure 2 the four markers should be examined at the same ages. MitoSox was only shown at 2 and 6 months, and RTN3 at 9 months only, whereas Iba1 and GFAP are examined at 6, 9, and 12 months.

MitoSox is an early change in mouse models of AD and was examined early. RTN3 is a late change and was examined late. Astro and microgliosis increase throughout the progression of the disease and so were examined throughout. We feel that the analyses that we made are appropriate.

Were there any effects on survival, cell proliferation, apoptosis, or axonal organization in the different mutant mice?

Survival vs. apoptosis and neurite length in neurons from mutant mice are shown in Figure 1 (formerly a supplementary figure). Most studies agree that mouse models of AD lack neuronal cell death. Increased neuronal death has been reported in some studies that used the 5xFAD model, but we have not been able to detect significant levels of apoptosis (as assessed by cleaved caspase 3) in our hands. There are clearly many more aspects that can be investigated in these mice. We strongly feel that the data presented regarding pathology are sufficiently comprehensive to support the main conclusions of the study. Our view is that investigations of additional pathologies should be the focus of separate studies.

Were other forms of synaptic plasticity altered? Were there differences in Paired-Pulse Facilitation or I/O curves? The KO vs KI effects may be more apparent with a less intense stimulation or an LTD protocol, the slow decay (120 mins post stim) is a slower decay than expected indicating a potential over stimulation.

It has previously been reported that short-term plasticity in hippocampal synapses is unaltered in 6 months old 5xFAD mice (Kimura et al., 2009; Crouzin et al., 2013). However, late long-term plasticity was found to be impaired in those studies, as in the present study. It has also been demonstrated previously that Paired-Pulse facilitation (PPF) is not affected in 5xFAD mice. However, basal synaptic transmission (I/O curves) was affected in 6 months old 5xFAD mice (Kimura et al., 2009; Crouzin et al., 2013). The TBS-LTP used in our study was not “over-stimulated” as we used a standard stimulation protocol (5Hz) that is considerably less intense than conventional strong stimulation (3x100Hz) used in most other TBS-LTP studies. Finally, to the best of our knowledge, there are no data on LTD in the 5xFAD background. LTD is opposite to LTP and has very different mechanisms.

The behavioral and synaptic plasticity examinations were done at an earlier time point than the APP processing analysis. Do the results from Fig 4 replicate in the earlier age points? Or even precede the behavioral phenotype? Additionally, S4 should be at the same ages shown in the main figure. Fl APP seems visually decreased (especially compared to the elevated GAPDH) in the KO mice.

LTP and behavior were assessed at 6 month of age. The APP processing shown in Fig. 5 (formerly Figure 4) was from 9 month old mice. On the other hand, analysis of accumulation of different A-beta species by ELISA, a direct consequence of amyloidogenic APP processing (as shown in Fig. 2G-I), was done at all ages, from 2 months to 12 months. This showed clear differences at 6 months, the same age of the LTP and behavior assays. We feel it is safe to say that abnormal APP processing leading to A-beta peptide accumulation precedes deficits in neuron function and behavior. This is also the main tenet of the amyloid hypothesis. The data shown in Supplementary Fig. S2 (formerly S4) was also done at 9 months as in the main figure, and in the same samples, so this was an error in that legend which we have now corrected. We thank the reviewer for this heads-up. Finally, no, we do not see any statistically significant difference in the total levels of full length APP in any of the genotypes (n=9).

Would using an antagonist to the p75NTR receptor also slow APP internalization dynamics in 5XFAD mice? Rescue LTP? Conversely does application of neurotrophins hinder the dynamics?

An antagonist to p75NTR would be very good to have, and we are working hard to develop one based on a novel drug discovery pipeline which we have recently published (Goh et al. Cell Chem. Bio. 2018). Alas, no specific antagonist of p75NTR exists at the moment. There are indeed papers claiming to have made molecules that “modulate” p75NTR activity, but it is unclear whether they act as antagonists, agonists or whether they are at all specific for the receptor, as none of them have been tested in mice lacking p75NTR. On the other hand, we have now added evidence to the revised manuscript showing that activation of p75NTR by stimulation with NGF enhances APP internalization (Fig. 6D, E). Importantly, NGF had no effect on the knock-in neurons (Supplementary Fig. S4A-F), which is in line with the notion that what it distinguishes the knock-in variants from the wild type is an inability to signal. We also show that the mutants are impaired in their ability to recruit components of the AP-2 complex that mediates clathrin-dependent endocytosis (Fig. 6I). Together, these data explain why the mutants internalize at a lower rate and display altered intracellular trafficking. As these mutants are able to interact with APP, this also explains why there is less APP internalization in these mutants.

In Figure 5D, the shape of the neuron suggests the super resolution image is centered on the nucleus. If so, why is there APP and BACE1 positive puncta in the nucleus, an organelle that is not expected to have either of these proteins?

The image that was provided represented the collapsed projection of several layers, some through the nucleus and some above the nucleus. The signals shown were on top, not inside, the cell nucleus. Nevertheless, we have now

totally redone the imaging and analysis of APP/BACE1 co-localization, and also included additional studies using the same methodology looking at co-localization of either APP or p75NTR with the small GTPase Rab11, which marks recycling endosomes (shown in revised Fig. 8D-I). Also, we now provide representative images across all the relevant genotypes. We believe these images are of higher quality.

With decreased amyloid, IBA1, and GFAP, are levels of neuroinflammation restored to WT levels in the variant mice?

Microglia and astrocytes are (or can be) neuroinflammatory, so the changes that we see are indeed indications of neuroinflammatory reactions in the AD brain which were ameliorated in the mutant mice. We have not assessed additional inflammation markers, such as lymphocyte infiltration, cytokines, etc. As mentioned earlier, we strongly feel that the data presented support the main conclusions of the study. Further investigation of additional pathologies, including inflammatory reactions, should be the focus of separate studies.

A discussion of the effects of the mutations on other cell types involved in AD and APP processing should be included (Microglia, astrocytes, etc.). The Methods section should describe the genetic background of the 5XFAD mice.

The effects of the mutations on specific cell types is the subject of ongoing investigations using conditional alleles. To the best of our knowledge, APP is mainly (or exclusively) expressed in neurons. Microglia and astrocytes, as well as immune cells, clearly play a variety of roles in AD, including clearance of plaques, synapse elimination, etc., but are not main contributors to production of A-beta per se. Having said that, they do, under some circumstances, express p75NTR, so investigations on the functional consequences of the p75NTR alleles described here in such cell types will be very interesting. We feel, however, that such studies should lie the focus of separate reports. As mentioned in the Methods section, all strains were back-crossed for at least 10 generations to a C57BL/6J background, including the 5xFAD mice.

Minor Concerns:
- *Figure S1 stats need clarification*
- *Results section refers to wrong supplemental figures*
- *GAPDH in S2B is unconvincing. B3 Tubulin may be cleaner.*
- *The same GAPDH is used twice in S4*
- *Statistics should be shown in Figure 4 (if significant, as implied by the text).*

We have addressed all these points. The images previously shown in Figures S4A and C (now Fig. S2A) were reprobings of the same blot and so share the same GAPDH. We now present these blots in the same panel to avoid this problem.

Referee #3

However, several aspects are confirming earlier studies and besides a decreased internalization, we don't learn that much on the mechanism behind this, i.e. why a signaling deficient p75NTR is less capable of internalizing APP.

We respectfully disagree with this statement. We feel that it is not appropriate to confront our study, that uses very precise gene targeted mutations in living mice and real neurons, combining biochemistry, cell biology, histology, electrophysiology, and behavior studies, with one that overexpresses p75NTR and APP in a cancer cell line to claim that alpha APP processing is changed. p75NTR overexpression leads to aberrant activation of many pathways, including apoptosis, and so results from p75NTR overexpression in cancer cell lines are very difficult to interpret (more on this below).

On the second issue, i.e. *"why a signaling deficient p75NTR is less capable of internalizing APP"*, the reason is that, by virtue of their interaction at the plasma membrane, APP internalization (at least a fraction of it) is linked to p75NTR internalization. And because p75NTR internalization and trafficking is dependent upon its activation and signaling —as shown in for example in *Bronfman et al. (2003)*, *Saxena et al. (2004, 2005)* and *Escudero et al (2014)*, APP internalization becomes affected by the ability of p75NTR to signal.

We suggest that the ability of p75NTR and APP to interact with each other can be seen as a kind of double-edged sword: active p75NTR enhances APP internalization and amyloidogenic processing, but, if inactive, it will reduce APP endocytosis and trafficking to BACE1+ endosomes. If we imagine that APP is a commuter and p75NTR is a bus, a commuter can walk to work when the bus is not there, but that takes longer time than riding the bus. When the bus is there, the commuter takes the bus. But if the bus has a defective motor, the commuter is now stuck on the bus.

The revised version of the manuscript now includes several additional studies that further support and expand on this notion. We now show that NGF stimulation enhances APP internalization in a p75NTR-dependent manner in wild type hippocampal neurons. NGF does not affect APP internalization in KO, ΔDD or C259A neurons, further confirming that p75NTR signaling regulates APP internalization in neurons. We also show that ΔDD and C259A variants are deficient in their association with beta-adaptin, a key component of the AP-2 complex that mediates clathrin-dependent endocytosis (Fig. 6I). In addition, we have re-made the analysis of APP/BACE1 co-localization using improved images and found that the fraction of internalized APP that co-localizes with BACE1 is lower in the ΔDD and C259A mutants than in wild type neurons, confirming a trend that we observed earlier, but which was not statistically significant. This suggested the possibility of altered intracellular trafficking of APP in the mutant neurons, in addition to reduced/slower internalization. Indeed, we find that, in mutant neurons, a greater proportion of internalized APP, roughly corresponding to the reduction in co-localization with BACE1, now co-localizes with Rab11, a marker of recycling endosomes. Importantly, we also find that internalized ΔDD and C259A mutant p75NTR molecules themselves associate with the recycling pathway to a greater extent than wild type p75NTR. This is in agreement with earlier evidence in

motor neurons indicating that activation by NGF can divert internalized p75NTR from the recycling to the endocytic pathway (*Deinhardt et al. 2007*). This explains why a larger fraction of signaling-deficient p75NTR molecules remain associated with recycling endosomes. We believe these additional data strongly support the notion that p75NTR molecules that are deficient in signaling, by virtue of their interaction with APP, delay APP internalization and divert a fraction of the internalized APP to recycling endosomes, in detriment of BACE1-containing endosomes. We note that, while BACE1 has been found in Rab11 endosomes for recycling to the plasma membrane, beta-cleavage of APP is not believed to occur in that compartment, given that Rab11+ endosomes are not acidic, as they lack functional vacuolar ATPase. Our results showing redirection of a fraction of internalized APP from BACE1+ to Rab11+ endosomes in p75NTR mutant neurons would suggest that BACE1 traffics more rapidly through recycling endosomes, or else APP and BACE1 transit through different pools of Rab11+ endosomes in those cells. At any rate, these data support the idea that inactive p75NTR variants can alter the intracellular trafficking of APP in detriment to the amyloidogenic cleavage pathway. These issues are now discussed in the revised manuscript.

The whole study is conducted with the 5xFAD as the genetic background, which is a very severe AD model starting already on the age of 2 months. It is increasingly preferred to consider as well APP KI models with a later onset of pathology; maybe in this case, a much stronger beneficial effect could be observed.

We agree with the reviewer and would go further to argue that the use of such a severe model put us in a more stringent position to show a positive outcome. The fact that our mutants had such dramatic effect in this so severe model makes our data even stronger. We feel that such an argument ought to be in our favor, rather than against us. Clearly, our findings pave the way to test mutations affecting p75NTR activity in progressively less severe models, including APP knock-in mice.

I have an important critique on the interaction data sets. Firstly, the interaction of p75NTR with APP has been shown already in total brain extracts, at endogenous levels (Fombonne et al 2008). In this manuscript, the authors essentially confirm these data in primary neurons with the different genotypes. Also the link of p75NTR expression and alpha-shedding has been documented already: in the same paper (Fombonne et al., 208) it is demonstrated that overexpression of p75NTR reduces alpha-shedding of APP and that this can be restored by adding NGF or Abeta itself. The interesting point is here that overexpressed signaling-competent p75/NTR decreases alpha-processing while signaling-defective (this study) increases it. But the authors do not provide data that could shed light on the underlying mechanism.

We find that the critique comparing our study with Fombonne et al. is unfair. First, the interaction that the reviewer mentions in that paper was done by co-immunoprecipitation in transfected cancer cell lines and total brain extracts. Total brain extracts contain all regions of the brain, all cells in the brain (neurons, glia, blood vessels, etc) and their organelles all lysed together. No knock-out tissue was used as control in that paper. In the absence of any other data, those results are poorly controlled and inconclusive. As requested

by another reviewer, we now provide properly controlled co-immunoprecipitation data in our revised paper (Fig. 7A). These data show comparable levels of association between APP and wild type as well as mutant p75NTR molecules, confirming the PLA results.

Second, and as indicated earlier, the studies on shedding of APP-alpha by Fombonne et al. were conducted in a cancer cell line transfected with plasmids overexpressing APP and $p75^{NTR}$, which causes all sorts of aberrant signaling, including apoptosis. In fact, the authors of that paper also reported a dramatic increase in cell death in the cells double transfected with APP and p75NTR plasmids (Fig. 7). We are not surprised. We would in fact question the validity of any observations made in cells that are dying. The authors of that paper reported that co-transfection of p75NTR along with APP appears to decrease soluble APP production compared to only APP overexpression in that cancer cell line (Fig. 5). This claim was based on one single blot, without any quantification, nor replication, nor any statistical analysis. Only the image of that one blot was provided. In fact, we would argue that their result is more likely due to the competition between the two expression plasmids introduced in the cells. In addition, as mentioned earlier, those cells were dying as shown by the authors themselves. In our view, the Fombonne paper reports effects that are at best supra-physiological, and more likely non-physiological.

We believe that the underlying mechanism of the differential effects pf $p75^{NTR}$ variants is explained by our studies looking at in vivo interaction, co-localization and internalization, as discussed above and in our manuscript Together with the new results added to the revised version, our data explain how the mutants affect APP internalization, intracellular trafficking, access to BACE and, ultimately, AD pathology in the mutant mice

Surprisingly, they don't find differences between the wt and signaling mutants, although one would expect that the signaling mutants would result in higher PLA signals compared to wt p75NTR, as internalization is delayed. How is this explained?

We reported comparable PLA signals over the whole cell in the data presented in the original version of the manuscript, as this was performed in fixed and permeabilised cells. These data are now shown in Fig. 7C. The new co-IP study added to the manuscript confirms these results. We have also added quantification of PLA signals at the cell surface, done by antibody-feeding of live cells, followed by wash, fixation and PLA reaction, representing the 100% level used in the internalization studies (as requested by another reviewer). This result also shows comparable levels of APP/p75NTR complexes at the cell surface for all genotypes (Fig. 7D). Similarly, we show that cell surface p75NTR levels are also comparable among wild type and mutant molecules in hippocampal neurons (Supplementary Fig. 3C).

We note that measurements of this type only reflect steady-state levels, which are not only affected by internalization, but also recycling as well as membrane insertion of *de novo* synthesized molecules. Our results show that 85-90% of cell surface APP is internalized within 30min at 37°C in wild type neurons, suggesting rapid turnover of plasma membrane APP. Normal

steady-state levels at the cell surface despite slower internalization would suggest an overall slower turnover of these molecules in the mutant neurons.

More problematic is the next experiment where the authors study the co-internalization of p75NTR with (OE) APP. They do this by performing first PLA and next follow the internalization of the PLA signal. However, with this approach one artificially crosslinks both molecules and hence logic they co-internalize. In theory, one should be able to also show interaction and co-internalization with endogenous APP. Furthermore, as they use 6E10, they cannot conclude that full-length APP is (co-)internalizing: their data do not rule out that APP needs to be shedded first, prior to interaction and internalization.

This is a misunderstanding, as PLA was performed **<u>after</u>** the internalization was completed. The PLA reaction requires cell permeabilization and fixation and can not be done in live cells. There was no crosslinking and the two molecules can internalize freely. We have now revised and clarified the description of this experiment in the Methods section. In light of this, the argument that the APP being co-internalized could be a soluble APP fragment is not relevant, as such fragment would have diffused away in the medium. We also note that the kinetics of internalization obtained after PLA in wild type neurons was very similar to that observed when we only assessed APP.

Are the signaling mutants failing to recruit adaptors and endocytic machinery or does p75NTR requires APP for getting internalized? In this case, endocytic deficient APP mutants might be checked for their ability to co-internalize with wt p75-NTR.

p75NTR has been shown to internalize in response to ligand in heterologous systems that lack APP expression. On the other hand, most (all?) neurons express some levels of APP, so it remains possible that p75NTR and APP mutually affect their internalization rates and trafficking in neurons. Mapping the precise sites of interaction between APP and p75NTR (we know already it can not be the death domain) will be interesting, but we consider this an extension of the present study, not essential for the argument being made here.

Signaling capacity is inextricably linked to receptor internalization. Thus, the reduced internalization of the mutants is due to their inability to signal. We now show that both ΔDD and C259A variants have a deficit in their ability to recruit beta-adaptin, a key component of the AP-2 complex that links receptors to the clathrin coat for clathrin-dependent endocytosis (Fig. 6I). This observation is in agreement with previous studies showing that only ligand-mediated internalization of p75NTR is clathrin dependent, while spontaneous, ligand-independent receptor internalization takes place in a clathrin-independent manner from lipid rafts and preferentially targets internalized receptors to recycling endosomes (see Discussion in revised manuscript).

As mentioned above, we have also extended our studies in several ways that shed light on specific properties of the internalization and intracellular trafficking of the mutant receptors by looking at the effects of NGF treatment and the association of receptor molecules with the recycling pathway. We believe these additional data considerably strengthen the mechanistic aspect

of our study.

In their final set of data, they show that less APP is reaching BACE1-positive endosomes. Firstly, the quality of the imaging is not convincing. The authors describe this is super-resolution but actually rather obtained through deconvolution. The pictures have a patterned appearance (often seen in SIM images as well, and the result of an artifactual image rendering).

This was done using a Leica TCS SP8 X microscope equipped with structured illumination, and with the assistance of an engineer from Leica. Deconvolution was done using the Leica HyVolution module, which can resolve down to 130nm, also together with a Leica engineer. Structured illumination microscopy is one of the several techniques that are used to achieve super-resolution microscopy. We are confident that the images we got were not artifacts.

As mentioned above, we have completely re-done this part of the study and now provide a larger set of higher quality images, higher magnification insets, and new quantitative analysis (Fig. 8). We note that the numbers we report have been obtained from specialized software designed to quantify co-localized labels.

In general the writing could be improved. Particularly the introduction is verbose and could be much shortened to the essential information. The authors should also avoid to only refer to a small number of the same reviews and cite as much as possible also original papers. For instance the preferred endosomal processing of APP by Bace1 has been originally described in Rajendran et al. and Sannerud et al. 2011.

Our Introduction is less than 2½ pages double-space font 12, and we don't find that particularly long. We described APP processing and p75NTR signaling, and it is difficult to see how avoiding either would improve the writing in the manuscript. More references can always be added, but that will only make the text longer, not shorter. The two references mentioned by the reviewer are interesting but not super "original" (one is indeed a review). The ones we cited in our text on APP internalization and processing are in fact much older (from 1994, 1996, 2005 and 2009). In any case, we are happy to oblige and have incorporated the two references mentioned into the revised manuscript.

In general, the authors often mention that the C259A mutant is particularly more effective in reducing pathology but this is not statistically demonstrated. In fact, in many cases, like in fig 2, fig 3, fig 4D-E and fig 5, there is likely no difference between both signaling mutants. Only in the memory test (fig 3E) some trend might be spotted, but overall the data do not justify to differentiate between both mutants.

There was an obvious trend in our data set and we deemed it was important to bring this to the attention of the reader. We have removed this from the revised manuscript.

For figures 4 and 5 and suppl fig 5, only the time courses of control images are shown, next to the quantified data. This doesn't make much sense. It would be better

to show for each primary culture and genotype one time point so that at least the difference can be appreciated from the images as well.

We now show examples of one time point for each genotype as requested. The time-course is now presented in a supplementary figure.

1st Revision - Editorial Decision 13th Oct 2020

Dear Carlos,

Thank you for submitting your manuscript to The EMBO Journal. This submission is a re-submission of EMBOJ-2020-104450 that was rejected post review back earlier this year. Your study has now been re-reviewed by the original referee #3 and the comments are provided below.

As you can see from the comments, the referee appreciates the introduced changes and is overall supportive of publication here. There are a few remaining points raised that I would like to ask you to resolve in a final revision.

When you submit the revised version please also take care of the following points:

- you can only have 5 keywords - at the moment there are 7

- We require a data availability section. As far as I can see no data is generated that needs to be deposited in a database. If this is correct please state: This study includes no data deposited in external repositories

- Please upload an author check list - see guide to authors

- Please upload figure files as individual figures

- The four supplementary figures should uploaded as an Appendix - please see https://www.embopress.org/page/journal/14602075/authorguide#expandedview. Make sure also to correct the figure callouts in the text and the appendix should have a table of content. You can also choose to change the supplementary figures to EV figures

- If you choose to have an appendix I would suggest to add the table to the appendix.

- 'Methods' needs correcting to 'Materials and Methods'.

- We encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. It would be great if you could provide me with a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files.

- We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

- We also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels). You can also use something from the figures if that is easier.

- I have attached a word document with editorial edits from our publisher. Please take a look at the figure legends and respond to their comments.

That should be all. You can use the link below to upload the revised version.

Best Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
https://bit.ly/EMBOPressFigurePreparationGuideline

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 11th Jan 2021.

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #3:

Ibanez and co-workers have revised their initial manuscript on the protective role of signaling-deficient p75 receptor on AD pathology using a 5xFAD model. Despite the manuscript was initially rejected, the authors did a very good job in addressing the criticisms of the reviewers. Many of the issues raised by the reviewers are in the revised manuscript now more convincingly addressed. Importantly, additional sets of data, in particular with respect to the characterization of the mice model at different time points, the interactions and internalization assays, have strongly improved the manuscript. In addition imaging quality improved.
With respect to my specific questions and how the authors addressed these, I have the following replies.
- As initially stated, the current manuscript confirms previous studies. I agree that these were not done as rigorous as the current manuscript using simple overexpression in cancer cells, but the 5xFAD is also far from a physiological relevant situation. Also, when looking at effects on APP processing, using either OE or endogenous usually doesn't make a lot of difference and rarely opposite effects occur (referring to the critique on the intereaction data). Nevertheless I support the fact that data always need to be confirmed and strengthened using endogenous levels of expression, as is done in this manuscript. Anyhow, despite the authors disagreed with my statements, they exactly did what was requested and provided more controls, and more qualitative data points to strengthen their paper. Of note, when looking into primary neurons and transducing them with mutant APP, they have to avoid to use the term '5xFAD APP' in these cultures: 5xFAD relates to the mutations in APP AND PSEN1, and this is not recapitulated in the primary neurons transduced with mutant APP.
- With respect to the choice of 5xFAD, and their arguments, I don't agree that this model puts them in a more stringent position. Because of the more subtle phenotype of KI models, it might be possible that such models provide full rescue over a longer period, strengthening the later claim of p75NTR as a therapeutic target.
- The authors now added data on the decreased recruitment of endocytic adaptors, specifically AP-2 complexes, that supports the underlying mechanisms of decreased internalization. This adds more novelty to the story.
- I am happy to notice that the authors agreed on the poor quality of imaging data, and re-did the imaging on the APP-BACE1 co-loc. The quality (also in deconvolution) of the new data sets is much better as compared to the original. Moreover, more detailed datasets are included on the APP-Rab11 and p75NTR-Rab11 co-loc, strengthening the author's conclusion of a derailed trafficking of a pool of APP.
- With respect to the writing, I found in particular the first page of the introduction too verbose: this is very general knowledge on the amyloidogenic vs non-amyloidogenic routes that can be easily and clearly summarized in half of the current length. I admit I made a mistake in referring to Rajendran et al. It should have been Schneider, Rajendran et al., 2008. The older papers the authors refer to demonstrated that endocytosis is needed for most amyloidogenic processing, whereas the latter more directly demonstrated processing within endosomes.
- When re-reading the manuscript and M&M I noticed that the authors define soluble, oligomeric and fibril states of Abeta as being extractable by TBS, RIPA and formic acid, respectively. For fibrillary Abeta this is OK. But one doesn't need to extract with RIPA to obtain oligomeric Abeta. Oligomeric Abeta forms are found in conditioned media and extracellular fluid (how should they otherwise find their way to the synapse and cause defects by interfering with synaptic receptors). The protocol, as originally described by the Walsh-lab ,confirms the use of merely physiological buffer to extract soluble and oligomeric Abeta:
"grey matter was dissected free of white matter/vasculature and homogenized in five volumes of ice-cold base artificial cerebrospinal fluid (aCSF-B; 124 mM NaCl, 2.8 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, pH 7.4) plus protease inhibitors (5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethyleneglycoltetraacetic acid (EGTA), 5 μg/mL leupeptin, 5 μg/mL aprotinin, 2 μg/mL pepstatin, 120

µg/mL Pefabloc and 5 mM NaF) with 25 strokes of a mechanical Teflon-glass Dounce homogenizer (Fisher, Ottawa, Canada). Resulting homogenates (20% w/v) were centrifuged at 200,000×g and 4 {degree sign}C for 110 min in a SW41 Ti rotor (Beckman Coulter, Fullerton, CA). The upper 80% of the supernatant was removed and dialyzed against 100-fold excess of fresh aCSF-B at 4 {degree sign}C using 2 kDa MWCO Slide-A-Lyzer cassettes (ThermoFisher, Waltham, MA)."
RIPA buffer is a strong extraction buffer, and solubilizes membranes: what the authors measure here could be equally more aggregated, higher order assemblies, not oligomers. They should re-consider this division in soluble/oligomeric/fibrillary Abeta.
Minor comments:
- The authors use the abbreviation 'TBS' for two totally different things, a buffer and theta-burst stimulation. This should be avoided.
- I agree with other reviewers to change s(oluble)CTFbeta to AICD. This is far more commonly used. In fact, this paper is for me the first time I see this definition for the AICD.

Dear Karin,

Thanks for your feedback on the revised version of our manuscript. Please find enclosed the hopefully final version of this paper. We have attended to all your remarks as well as those made the reviewer, and made the necessary changes. For the fractionation of different high-order states of APP, we followed the protocol described by Sherman and Lesne (2011) cited in the manuscript. There surely are different ways of doing this and we appreciate the additional protocol suggested by the reviewer. Regarding the nomenclature issue of whether to use soluble CTF beta (sCTFb) or APP intracellular domain (AICD) for the product of consecutive cleavages by BACE1 and gamma secretase, we have stated both names in the Introduction. This appears only once in the paper, so I think it should not be a problem. Personally, I find the name AICD too ambiguous. The product of gamma secretase after alpha secretase is also an “APP intracellular domain”, yet very different functionally. The alpha/beta nomenclature eliminates that ambiguity. We have also added the new reference indicated by the reviewer, and resolved the TBS abbreviation ambiguity. I think that is all.

I hope you will find this version satisfactory and suitable for publication.

Many thanks in advance.

Best regards,

Carlos Ibanez

2nd Revision - Editorial Decision 19th Oct 2020

Dear Carlos,

Thank you for submitting your revised manuscript to The EMBO Journal. I have now had a chance to take a look at the introduced changes and all looks good. I am therefore very pleased to accept the manuscript for publication here.

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Corresponding Author Name: Carlos Ibanez
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2020-104450

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was determined based on preliminary experiments and previous experience with biochemical (typically 5-8), cell biological (3 to 5), histological (3 to 5), electrophysiological (5-to8) and behavioral (8-12) studies
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	See above
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No animals were exlcuded from analysis
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No steps were taken to allocate animals to specific groups. On the other hand, researchers were unaware of the animals genotype during electrophysiological and behavioral studies
For animal studies, include a statement about randomization even if no randomization was used.	No randomization was used
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	See above
4.b. For animal studies, include a statement about blinding even if no blinding was done	See above
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Data met the assumtions of the test in terms of number of variables and distribution. This was assessed using standard statistical methods.
Is there an estimate of variation within each group of data?	SEM was used

Is the variance similar between the groups that are being statistically compared?	yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	This is provided in a table appened as supplementary information to the paper
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	No cell lines were used

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	All this was stated in the methods section of the paper
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Ethcial regualtion were complied upon. This is stated in the paper.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Noted

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	not applicable
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	not applicable
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	not applicable
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	not applicable
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	not applicable
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	not applicable
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	not applicable

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	This study includes no data deposited in external repositories.
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21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	not applicable

G- Dual use research of concern

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