

Altered expression of Presenilin2 impacts endolysosomal homeostasis and synapse function in Alzheimer's disease-relevant brain circuits

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Perdok et al., shows the molecular mechanism underlying Presenilin2 (Psen2) mediated AD pathogenesis. For this, they use both primary neuronal culture and two mouse models, Psen2KO and N141I KI mutant. Overall, the authors show in these models, that the mossy fiber-CA3 circuit is mainly impacted, causing synaptic defects, which results in endo-lysosomal dysfunction in AD. The manuscript is well-written, albeit with a few grammatical errors (Lines 150,154,248-249) with well-supported data.

Minor Comments:

- In Fig.2c, high mag panel for APPxPSEN2KO is switched. Also, is the fibrillar Ab increased compared to the oligomeric species in the KO?
- N141I mutation has been shown to induce neuro inflammation. Have the authors investigated the astrocyte and microglia in the PSEN2KO and PSEN2FAD model as NLGF mouse model also has been shown to have gliosis as early as 3 months?
- In Fig.2f, is the intensity /level of Lamp1 reduced in APPxFADPSEN2?
- In Fig.4g, authors indicate purple and green highlighted areas but there is no green area indicating PSDs.
- In Line 248-252, authors mention white arrow heads, but the corresponding figure panel does not show any arrow heads. Also, this line does not clearly explain the observed results.
- Authors do not describe in the method section how PSD length was measured. Also, the representative figure does not clearly show this difference.
- In Fig.5e, authors show the PSEN2+ Lamp1+ axons (MAP2 staining). MAP2 is primarily a dendritic marker (Caceres M, 1984, Dev. Brain Res). Can authors show another axonal marker (ex:RT-97) to indicate that PSEN2+Lamp1+ puncta are found on axons? Also, the boxed regions in three different genotypes in Fig.5e, are not magnified to show if there is overlap or not.
- In Fig. 5i FADPSEN2 panel, surface vGLUT1 considerably reduced even compared to the PSEN2KO but the quantification shows that there are comparable. A different figure supporting the quantification might be better.
- In Line 313, authors mention TRPML1 agonist but do not give a background or reference to the agonist MLSA1 in the text.
- In Fig.6b, IB panels are not labelled with the antibodies used.
- In Fig.6n, the co-localization of mcherry and GFP + puncta are found in different compartments in KO and FAD neurons. Does the presence or absence of PSEN2 alter the localization of the puncta?
- Do authors find similar expression patterns for synaptic markers and LE/Lysosomal markers in vivo?
- Some of the results in FADPSEN2 or KO are similar suggesting the presence or absence of PSEN2 does not influence these observations (Fig.4f,l,k, Fig.5j,k, Fig.6c,f,g). How do the authors explain the role of PSEN2-mediated impact on MF-CA3 circuit and synaptic defects?

Reviewer #2

(Remarks to the Author)

The knowledge gap explored pertains to the detailed mechanisms by which Presenilin2 (PSEN2) mutations can drive

Alzheimer's disease (AD) pathology. The authors propose that it is specifically through its impact on endolysosomal homeostasis and synaptic signaling in vulnerable brain circuits. Although it is known that mutations in the PSEN2 gene are sufficient to cause familial Alzheimer's disease, the specific cellular processes and pathophysiological changes resulting from these mutations, particularly in terms of synaptic function and neurodegeneration, are not fully understood. The study aims to elucidate how changes in PSEN2 expression affect neurobiological and cognitive functions, contributing to AD progression. The authors specifically investigate how PSEN2 contributes to AD. For that, the authors cross PSEN2 KO and FADPSEN2 KI mice with APP FAD KI mice (carrying three FAD mutations) and evaluate how the APP mice phenotype is altered by PSEN2 loss of function.

The authors conclude on an accelerated pathology when PSEN2 is KO or mutated compared to the APP mutant alone based on the following:

- Worse deficits in working memory
- Smaller presynaptic compartments
- Impaired LTP
- Aggravated Endolysosomal defects
- Reduced surface levels of synaptic proteins

The significance is related to the finding that the loss of function of PSEN2 is as important as its mutation in influencing AD pathology. Studying the contribution of PSEN2 alone is original, and the Annaert lab has been leading this research. However, the exciting results fail to reach the significance anticipated. The authors identify the need to understand how PSEN2 mutations cause familial AD. Still, a conclusion is precluded because the authors did not study the phenotype in PSEN2 KO or PSEN2 FAD KI (without APP FAD KI background) for most of the experiments. This leads to the major concern with the study:

- 1) One wonders if this is due to a lack of phenotype in the PSEN2 KO and KI. Did the authors do the experiments? If there is a lack of phenotype, this would indicate that using the mouse may not be the ideal experimental model for PSEN2-dependent AD. The authors should discuss this.
- 2) In most experiments, the authors do not include a wild-type mouse as a healthy control and only compare it to the APP FAD KI mouse model, an important control. The data would be more significant if compared to the wild-type. The second major concern is: Can the authors include the wild-type control in all experiments?

Overall, the data supports the conclusions and claims, but in several instances, the data is overinterpreted, as indicated below:

The data shows that PSENKO and FAD mutation alter amyloid pathology driven by APP FAD mutations.

- 1) The authors hypothesize that "altering PSEN2 expression in vivo, through KO or introducing a FAD mutation, would have opposing effects on AD pathogenesis." However, do not present direct evidence of it. The authors only present the Abeta levels in neurons cultured from APPxPSEN2KO and APPxFADPSEN2 but not from the PSENKO or FADPSEN2 KI that the authors prepared. In the supplementary figure 1c are presented crops of WB analysis of brain lysates from these mice (please indicate age in the figure legend) for APP, APP CTF, PSEN2, and Nicastrin. Still, the results section must include analyzing and interpreting the differences in APP CTFs. It seems that the APP CTFs decrease in both PSEN2 models. The authors must interpret and discuss these results. What does it mean if the APP CTFs do not accumulate in the knockout as when one inhibits gamma-secretase?
- 2) Why don't the authors prepare primary neurons from the PSEN2 KO and KI mice and analyze intracellular and secreted Abeta, as they have done for the APP KI mice in Figure 1a?
- 3) It would be essential to show the APP FL and APP-CTFs levels in Figure 1 A, as in FigS1c.

Worse deficits in working memory.

- 1) Figure 3f-j presents the evidence supporting this conclusion. However, the data analysis is not sufficient to support the conclusions. Assuming that the p-values in the figure represent significant differences from the first swim (if not, please clarify in the fig legend), it is necessary to compare the difference in time spent swimming between the mouse groups or at least to analyze the area under the curve or the slope, to conclude if there are worse deficits in working memory.

Smaller presynaptic compartments

- 1) The authors describe in Fig 4A that PSEN2 is increased in the mossy fibers of the CA3 region and the cortex (Fig. S1). The intensity of PSEN2 in the different brain regions, cortex, CA1, and CA3, should be quantified.
- 2) Interestingly, the size of presynaptic terminals in the CA3 regions is decreased in the PSEN2 models relative to the wt and the APP KI, indicating that loss of function and mutation affect the presynapse. Moreover, the PSEN FAD KI is different from the KO. In contrast, only PSEN2 KI increases the postsynaptic compartments compared to the APP KI. Can the authors comment on these results?
- 3) It is unclear if the APP KI has an effect postsynaptically, as reported in transgenic models. Could the authors include the wild-type control?

Impaired LTP

- 1) Given PSEN2 localization, LTP is investigated, and the authors report a decrease in the APP KI PSEN KO and PSEN2 KI. Moreover, the authors indicate that LTP was further reduced in APPxPSEN2KO and APPxFADPSEN2 mice. The authors need to present a statistical comparison between PSEN KO and FADPSEN2 of the amplitude in the late phase of LTP in panel 4f. Otherwise, this is an overinterpretation that needs to be corrected.

Aggravated Endolysosomal defects

- 1) The authors indicate that the Abeta and APP-CTFs increase in endolysosomes, endolysosomal mass increases, the

lysosomal calcium release decreases, lysosomal exocytosis reduces, and autophagosomes and autolysosomes accumulate with altered PSEN2 expression. LAMP1-positive organelles are referred to as lysosomes. At least the first time, it should be mentioned that LAMP1 identifies both late-endosomes and lysosomes.

Reduced surface levels of synaptic proteins

- 1) The authors provide evidence of a similar reduction of the surface levels of vGLUT1 and Glua1 in both PSEN2 models. How did the authors perform this labeling and its analysis? The method used needs to be indicated in the methods section.
- 2) Unexpectedly, could the authors explain that the vGLut1 and GluA1 labeling are not in puncta as if in a synapse but are linearly distributed? Is this a phenotype of APP KI neurons? Can the authors compare it to wild-type controls?
- 3) Could this presynaptic impairment be confirmed using synaptosomal preparations of neurons or from the affected CA3 region (and CA1 as a control) of the different mouse groups?

Although the results are generally convincing, the data analysis could be improved, as follows:

- 4) In Fig.6A, colocalization analysis should complement the representative figure, and the number of examples should be reduced to facilitate the perception of colocalization to 1 or 2 examples. We recommend using the Fiji plugin ComDET because it allows triple colocalization, which would enable quantitatively determining if the "Organelles positive for both antibodies identify APP β -CTF build up, whereas 82e1-only organelles point to A β " accumulation" differ with the genotypes.
- 5) Although the WB in 6B shows an increase in APP CTFs, no statistics are indicated in the quantification presented in panel 4D. The increase seems small and may not significantly differ from APP KI; thus, the interpretation needs to be toned down. "Finally, western blot analysis also showed higher levels of APP-CTFs in APPxPSEN2KO and APPxFADPSEN2 hippocampal neurons (Fig. 6b,d)." Moreover, as mentioned before, the authors could complement this data by analyzing APP CTFs in brain lysates.
- 6) Fig. 6C Colocalization should be presented in % of overlap over Lamp1 since LAMP1 labeling increases (fig. 6F).
- 7) Fig6 E-H, the analysis of % of the area to conclude on endosome enlargement or upregulation is indirect and not ideal. Instead it should be measured the size of LAMP1, EEA1, and VPS35 and the density (#/um²) similarly to what the authors did in Fig4C, D for PSD-95,

Other points:

- 8) line 301: "In conclusion, both APPxPSEN2KO and APPxFADPSEN2 neurons presented distinct accumulation of APP fragments that may impair endolysosomal homeostasis, as reported in other models (see above)." It should clearly state which APP fragments accumulate to clarify the conclusion instead of being referred to (see above).

The general concern with experiment design and data analysis relates to the indication that two or three independent experiments were performed using mouse neuronal cultures. There is an enormous concern with reproducibility in biomedical research, and performing only twice an experiment is a major concern regarding its reproducibility.

- 9) We assume that performing twice was the exception, but each statistical analysis must indicate the exact number of independent experiments.
- 10) Moreover, it is indicated that multiple comparisons were performed, but it is necessary to indicate what was compared for each comparison. The indication of the p values in the figures is essential. Still, it needs to be indicated the comparisons between the experimental models, APP KI Psen2 KO vs. APP Psen2 KI and vs. APP KI and vs. wt (ideally, this group should be included in all experiments).
- 11) Notably, a smaller p-value indicates that the reproducibility of that result is higher. However, it does not mean a bigger reduction or increase (PMID: 25161440). The magnitude of the decrease or increase must be significantly different.

Figure labeling and legends need improvement and review. For example:

- 12) The identification of the different groups is missing from Fig. S1, panels D, and f.
 - 13) Fig.2a Thioflavin labeling the brain slices needs to be indicated in the figure and indicate which Thioflavin was used.
 - 14) Fig.3d, G-j, statistical comparison between groups is missing; data should be plotted as in Fig. S3.
 - 15) Fig.3F-J number of animals analyzed is missing
 - 16) Fig5 Control WT is missing.
 - 17) Fig5A Requires quantification of PSD-95 density and PSEN2 colocalization with LAMP1 and PSD-95.
 - 18) Fig5b second panel with PSEN2 in APP NLGF KI misses a white square
 - 19) Fig5b magnified insets are saturated, precluding a clear assessment of colocalization.
 - 20) Fig 5i has a label floating on top of the figure.
 - 21) Fig6N should use the same lut colors as in other panels; red lut should not be used as color-blind people cannot assess colocalization.
 - 22) Fig6B is missing the label of proteins detected
 - 23) Fig6O-P p values labels should not be on top of the data
- Other minor points
- 24) Line 221 figS4a should be Fig.4a
 - 25) The APPNLGF mice could have been introduced in line 27, "APP (KI) knock-in mouse model."
 - 26) In the Title, "synaptic signaling" should be substituted for synapses as signaling often refers to regulating synaptic activity mediated by kinases, phosphatases, or other signals.
 - 27) The discussion and the introduction should be more concise.
- Generally, the methods provided enough detail for the work to be reproduced, except for the following:
- 28) The methodology used for image analysis is somewhat incomplete; for example, the % area of synaptic and endosomal

proteins is not clearly explained.

29) In the analysis of % surface area, what is it relative to?

Reviewer #3

(Remarks to the Author)

This is an outstanding and highly original manuscript describing how the most prevalent presenilin 2 (PSEN2) mutation linked to familial Alzheimer's disease (AD), and also the knockout of PSEN2, impact the neuron cell biology, pathology and phenotypes (behavior & physiology) of AD mouse models. Such a comprehensive and high-level mechanistic cell to in vivo studies is rare in the field of experimental AD research.

Specific points

In the Introduction, line 83, they write: "Post-embryonic full knock out of γ -secretase, obtained by conditionally knocking out all three APH1 isoforms, results in clear c-terminal fragment (CTF) accumulation of various substrates, neurodegeneration and neuroinflammation (25), underscoring the centralized role γ -secretase plays in AD pathogenesis". It is not clear why this is a model of AD pathogenesis if there are no plaques while AD patients with PSEN 1 & PSEN2 variants invariably develop amyloid plaques. Or are there cases of PSEN variants having neurodegeneration but no amyloid plaques? Can they later on then really speak of AD pathogenesis when it comes to PSEN2 KO?

On line 183 it is not clear what is meant by "and alike APP-FADPSEN2". Do they mean that the dystrophies were increased at 9 months in the KO mice similar to what is seen with the PSEN2 mutants? This could be rewritten for clarity.

Line 189, this heading would seem to read better as "Altering PSEN2 expression in APP NLG F mice impacts working memory"

Line 217 "significantly" instead of "significant". Also, at the end of this sentence, don't they mean APPxPSEN2KO and APPxFADPSEN2 here?

On line 252 they mention: "...white arrowheads, in Fig.4h,..." I was not able to see these arrowheads in Figure 4h.

On line 353 they write: "The lack of PSEN2 expression drives all APP metabolism towards PSEN1/ γ -secretase, which resides at the cell surface and in early/recycling endosomes." Is there a reference for this localization of PSEN1 specifically in the setting of PSEN2 KO?

Related to the above point, on line 358 they write "These findings provide in vivo support for PSEN2/ γ -secretase being the main generator of intracellular A β , which is significantly increased in an FAD setting whilst PSEN1 preferentially contributes to the secreted pool (23)." Ref 23 might be the reference asked for in the point above? They further suggest then that without PSEN2 increased plaques come from elevated Abeta secretion. Might it not be possible that the disrupted endolysosomal biology induced by PSEN2KO leads to an altered subcellular PSEN1 localization into the more abundant LE/Lys compartments and thereby aberrant/elevated iA β 42 in LE/Lys by PSEN1? However, they then nicely show elevated Abeta secretion in primary APP mutant neurons with PSENKO and importantly also show that Abeta42 in cell lysates is reduced, which argues against the possibility raised in my prior sentence. However, when one looks at the MSD results in Figure 1 it is intriguing that there are not only decreases in both 40 and 42 in lysates but also in 42 secretion in the primary APPxPSEN2KO compared with just the APP(NLF) neurons, while 40 shows a trend for a decrease but certainly not an increase. This does not really argue for just elevated PSEN1 secretion then causing increased plaques in the setting of PSEN2 KO.

They use the terms lysosome, endolysosomes and late endosomes/lysosomes somewhat interchangeably in the manuscript. There are somewhat different definitions for the term lysosome. Typically, lysosomes are considered to be predominantly perinuclear and in the cell bodies of neurons; it is e.g. considered one of the vulnerabilities of neurons that they need to traffic cargo back along their processes all the way to their cell bodies for degradation. Lysosomes by most definitions have lower pH than LEs and it would seem unlikely that what they at times call lysosomes in terminals have the required low pH of lysosomes. Alternatively, lysosomes were traditionally defined by their appearance on electron micrographs. This reviewer is fine with the term LE/Lys of endolysosomes but believes that using just 'lysosomes' when it comes to neuronal terminals as suboptimal. On line 437 for example they write: "At the post-synapse, lysosomal Ca²⁺ release and lysosomal exocytosis...". At the synapse these will more likely be LEs; in particular MVBs are common in or near to synaptic terminals from where they release their inner vesicles: exosomes. However, since this specific point is just one of semantics, this reviewer is fine with flexibility around these terms and is not demanding any changes.

Is it possible that some of the changes in plaque morphology with altered PSEN2 might even relate in some degree to microglia? This reviewer is not clear about the expression of PSEN2 in activated microglia and thereby a possible contribution. - This point actually is then addressed in their Discussion where they even describe a manuscript in preparation about this.

On line 385 and in several other places they have no space between the # and "months": "6months"

Page 467: "In support, in sporadic AD forms, only splice variants are found in PSEN2 but PSEN1 genes that result in truncated/lowered transcripts..." Please correct/rewrite for clarity.

It is not clear why APP labelling is so apparent in the APP but not in the APPxPSEN2KO and APPxFADPSEN2 mice in Figure 2f.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #5

(Remarks to the Author)

As requested by the editor, this review relates only to the generation of the new genetically modified (GM) mice, specifically the PSEN2 KI line (i.e., N141I), given that the APP KI and the PSEN2 KO lines have already been characterized and published.

- As per figure S1, there are only two silent mutations (not three), and they are located on the binding site, not on the PAM. This needs to be corrected (line 512).
- Which algorithm (to prevent off target) has been used to design the selected sgRNA? Please add this information in the Methods section.
- The provided genomic sequences on Figure S1 are not correct. Indeed, they contain the 3 expected mutations, however, when aligned to the WT (*Mus Musculus*) sequence, extra mismatches are present. This needs to be corrected.
- The primers used to genotype the Founders are to be presented by pairs (M&M section). It is currently impossible to check for the expected band sizes.
- The identification of potential founders by PCR is highly risky and insufficient because additional (unwanted) mutations often occur when using CRISPR. The authors mentioned that the genomic sequence of the selected Founder has been sequenced (line 519). Which sequencing was used (Sanger, NGS..)? The raw data for the sequencing should be provided.
- The doses of CRISPR reagents used for microinjection are extremely high (e.g., 1ug/ul). Such high concentrations are usually used for electroporation (not microinjection) of zygotes. Can this be reviewed and justified?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have adequately addressed the comments of the previous review.

Reviewer #2

(Remarks to the Author)

We acknowledge the improvement of the data now supporting better the results.

The significance of this paper is limited by the choice of studying the cumulative effect of two familial mutations of AD that, to my knowledge, have not been identified in AD mutations. Nevertheless, the field will benefit from the publication of these results. The mechanisms whereby mutations in PS2 alone are sufficient to drive AD pathology remain unclear.

The authors addressed many of the suggested revisions, but some critical points remain and should be revised:

1) We thank the authors for explaining the inconsistent use of WT mice as healthy controls. However, the manuscript must include the rationale provided in the rebuttal to clarify the experimental approach used for Nature Communication readers.

Respectfully, the problem the authors propose to solve in the abstract and introduction is understanding the mechanisms whereby a sole mutation of PSEN2 causes AD.

Example:

Abstract "Rare mutations in the gene encoding presenilin2 (PSEN2) cause familial Alzheimer's disease (FAD); however, the underlying molecular mechanisms remain elusive."

Introduction:

"Given its LE/Lys localization, we hypothesized that changes in PSEN2 function, through KO or FAD-linked mutations, may directly impact on LE/Lys homeostasis through regulating iA β and APP-CTF levels."

The authors do not use an experimental design that allows them to (dis)prove this hypothesis.

Instead, the authors investigated how PSEN2 changes amyloidosis, endolysosomal abnormalities, and synaptic function

caused by the knock-in of mutant APP. This choice must be better communicated (written) in the abstract and the introduction.

The authors can strengthen the manuscript by explaining why the APPKI background was essential for their conclusions. Ideally, the authors should mention that the PSEN2 mutation does not cause amyloidosis in mice, thus requiring a cross with the APP mice.

As it is, the results provide novel and exciting findings supporting that PSEN2 loss of function has a cumulative harmful effect on already diseased mice. However, they do not provide proof that PSEN2 loF-dependent mechanisms are sufficient to trigger AD in a mouse model, indicating that for PSEN2, other models may be more relevant. The authors need to discuss this limitation in the manuscript. An alternative model could be the Humanized Abeta KI mice (developed by model-AD), which is available and could be a promising alternative for future research.

2) Regarding supplementary Fig. S1D (former 1C), although one can perceive the appearance of a higher molecular weight band potentially being APP-bCTF (C99), the prominent one band detected in wild-type, that corresponds to both alpha (C83) and beta-CTF (C89) decreases in PSEN 2 KO and FAD neurons. In the manuscript reads:

Line 125 Furthermore, in PSEN2KO and FADPSEN2 mice, the APP-CTF profile included an additional lower mobility band, likely representing APP- β CTF,
127 not detected in wild-type mice (Fog/S1d).

The authors must revise the text (or data) to include a description of the observations concerning the decrease in APP CTFs and to interpret these results.

3) Regarding Figure 4 and its description in the results section:

Indication of p-values is missing from panels of figure 4e, 4f, 4i, 4k till 4O, as mentioned in the figure legend (All graphs are represented as mean $\pm$ SEM and include p-values.), is needed to support the description of the data:

Line 220-228: "We then explored short-term presynaptic plasticity using paired-pulse stimulations in the CA1 and CA3 regions. APPxPSEN2KO mice showed increased synaptic facilitation in the CA1, while in the CA3, PSEN2KO rescued the lowered response seen in APP KI to wild-type levels. In contrast, APPxFADPSEN2 mice displayed clear synaptic depression in the CA3 circuit, with normal responses in CA1 (Fig. 4h-k). To investigate the effects on presynaptic dynamics during periods of more demanding synaptic activity, we performed train stimulations in both hippocampal regions. In line with the paired pulse data, we observed increased synaptic facilitation in the APPxPSEN2KO predominantly in CA1 and partially in CA3, while APPxFADPSEN2 mice showed clear synaptic depression, exclusively in the CA3 circuit (Fig. 4l-o, Fig. S4h)."

Or the description of the data needs to be revised.

3) While in Figure 4, the number of animals and cells is indicated in the figure legends, it should be indicated for each experiment in the figure legends in the other figures. This is essential for transparency and reproducibility.

4) The magnified insets in Figures 6B and 7N are still saturated, preventing a clear assessment of colocalization. The authors should address the saturation issue to ensure the data can be accurately interpreted.

Reviewer #3

(Remarks to the Author)

This is a revised and now even further improved manuscript showing how PSEN2 deficiency compared to the familial Alzheimer's disease (AD)-associated PSEN2 N141I mutation affect AD pathogenesis when crossed into an AD APP knockin mouse model. I had viewed this work as outstanding already in my initial review due to the multiple excellent and diverse (cell biology, pathology, behaviour and physiology) methods used and the high level of scientific importance to this work. This reviewer believes that the authors extensively addressed the points of the original reviews. Reviewer 2 did a particularly thorough and excellent review, although I agree with the authors that the requests to extend all their work to wild-type and just PSEN2 KO mice (as the authors point out, they do look at these for many experiments) is requesting too much more work given that the central question is how PSEN2 deficiency differs from an AD mutation in PSEN2 in the setting of AD pathogenesis. Their work contains intriguing findings showing e.g. that either PSEN2 deficiency or AD mutation worsen AD pathogenesis. Although I don't fully agree with their interpretation of increased Abeta pathology with PSEN2 deficiency originating from elevated Abeta secretion, their data does point to this interpretation, and they certainly might be correct. One alternate possibility is that ageing might change the results they detected in their primary neurons, although I consider that requesting to explore such a complex possibility would be very unreasonable given not only the difficulty of the task but also the extensive work that they have already presented in this excellent manuscript. I have no further comments for this now even better manuscript.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #5

(Remarks to the Author)

All comments related to the generation of the mutant mice have now been satisfactorily addressed and could I have no other comments.

One important typo might still need correction though: the M& M section (mice) mentions that Exon 4 has been targeted using CRISPR, when in fact it was directed to Exon 6.

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NCOMMS-24-16020-T: point-to-point rebuttal

Reviewer #1 (Remarks to the Author):

Perdok et al., shows the molecular mechanism underlying Presenilin2 (Psen2) mediated AD pathogenesis. For this, they use both primary neuronal culture and two mouse models, Psen2KO and N141I KI mutant. Overall, the authors show in these models, that the mossy fiber-CA3 circuit is mainly impacted, causing synaptic defects, which results in endo-lysosomal dysfunction in AD. The manuscript is well-written, albeit with a few grammatical errors (Lines 150,154,248-249) with well-supported data.

We thank Reviewer#1 for the very positive assessment of our manuscript. We have fully addressed the minor comments, many of which are related to textual changes and figure layouts.

Minor Comments:

- In Fig.2.c, high mag panel for APPxPSEN2KO is switched. Also, is the fibrillar Ab increased compared to the oligomeric species in the KO?

The high magnification panels are in the correct order. Fibrillar species seem to be more intense in both KO and FAD-PSEN2 backgrounds, but the main morphological difference is that plaques in KO are larger and ‘more loose’ which we relate to the different Abeta profiles generated. This is as such explained in the result section.

- N141I mutation has been shown to induce neuro inflammation. Have the authors investigated the astrocyte and microglia in the PSEN2KO and PSEN2FAD model as NLGF mouse model also has been shown to have gliosis as early as 3 months?

We have indeed looked at microglia (iba staining) and astrocytes (GFAP). We did not observed changes in astroglia at different time points, but noticed a difference in microglial activation: as highlighted in the Discussion, this is part of a second manuscript in preparation. We decided to focus in this study on the neuronal hallmarks.

- In Fig.2f, is the intensity /level of Lamp1 reduced in APPxFADPSEN2?

There is no change in the intensity/level of Lamp1 between the genotypes, as shown by the insets. There is only a slightly lower background in the APPxFADPSEN2, likely caused by the higher abundance of dystrophic lamp1-positive neurites around plaques.

- In Fig.4g, authors indicate purple and green highlighted areas but there is no green area indicating PSDs.

The PSDs were indicated in green, however, we agree that this was not very visible. We changed the color to a more intense green and additionally added some zoomed insets to further highlight the PSDs. We have also split up the original figure 4 and moved all EM data to a new figure 5.

- In Line 248-252, authors mention white arrow heads, but the corresponding figure panel does not show any arrow heads. Also, this line does not clearly explain the observed results. We indeed forgot this, and white arrowheads are now added to the panel. This paragraph has been changed for clarity.

- Authors do not describe in the method section how PSD length was measured. Also, the representative figure does not clearly show this difference.

We have better highlighted the PSDs in our EM images, and provided representative zoomed insets to better highlight the differences. The method how PSD length was measured is now explained in the Mat& Meth.

- In Fig.5e, authors show the PSEN2+ Lamp1+ axons (MAP2 staining). MAP2 is primarily a dendritic marker (Caceres M, 1984, Dev. Brain Res). Can authors show another axonal marker (ex:RT-97) to indicate that PSEN2+Lamp1+ puncta are found on axons? Also, the boxed regions in three different genotypes in Fig.5e, are not magnified to show if there is overlap or not.

We agree with Reviewer#1, that only MAP2 staining is not conclusive. We have therefore included new immunostainings with AnkyrinG which is an axonal initial segment marker, and as such identifying the axon. Here we can show that PSEN2+LAMP+ vesicles are present within axons. Representative images and zoomed insets are now been added to the new figure 6f.

- In Fig. 5i FADPSEN2 panel, surface vGLUT1 considerably reduced even compared to the PSEN2KO but the quantification shows that there are comparable. A different figure supporting the quantification might be better.

We observe indeed for both FADPSEN2 and PSEN2KO significantly less surface vGLUT1 compared to APPNLGF, and which is evident from the quantifications (3 independent biological experiments). However, the reviewer might have been biased by the choice of the zoomed insets, which appear to be more intense for PSEN2KO compared to FADPSEN2. The overview panels however clearly show the overall, and equally decreased surface labeling in both genotypes. We have changed the zoomed insets of the PSEN2KO panels to more representative ones.

- In Line 313, authors mention TRPML1 agonist but do not give a background or reference to the agonist MLSA1 in the text.

We have now added references related to studies in which MLSA1 has been successfully used.

- In Fig.6b, IB panels are not labelled with the antibodies used.

This has been corrected and the antibodies used have been added to the panels.

- In Fig.6n, the co-localization of mcherry and GFP + puncta are found in different compartments in KO and FAD neurons. Does the presence or absence of PSEN2 alter the localization of the puncta?

We highlighted two zooms for each genotype, one from the soma one from the neurite. However, we did not find any obvious differences in localization between the genotypes.

- Do authors find similar expression patterns for synaptic markers and LE/Lysosomal markers in vivo?

We have used Airyscan imaging to look at the distribution of PSD95 (postsynaptic) vs synaptobrevinII (presynaptic), and did not really see significant differences. We have looked at LE/Lys markers, like LAMP1, as a readout for dystrophic neurites surrounding plaques and noticed clear increases (in dystrophic neurites) in the APPK1xFADPSEN2 background (Fig 2f).

- Some of the results in FADPSEN2 or KO are similar suggesting the presence or absence of PSEN2 does not influence these observations (Fig.4f,I,k, Fig.5j,k, Fig.6c,f,g). How do the authors explain the role of PSEN2-mediated impact on MF-CA3 circuit and synaptic defects?

Despite marked opposing altered synaptic vesicle clustering (more dense in PSEN2KO and more sparse in FADPSEN2) we see a similar impact on a decreased LTP in the CA3 region. For the revised manuscript, we have performed in addition train and paired pulse stimulations on acute brain slices and can now correlate these changes with the altered synaptic pool (Fig. 4 and 5). It shows that both can result ultimately in lowered LTP, underscoring that PSEN2 is required in homeostatic regulation of these synapses and any change, induced by either KO or FAD, will impact on LTP. Secondly, we indeed notice for both genotypes more APP-CTF accumulating in LE/Lys and an increased LE/Lys area as well as increased number of autophagosomes/autolysosomes. In both cases, we believe this is due to an overall decrease in substrate processivity specifically within LE/Lys. In case of PSEN2KO this is due to PSEN2 absence, in case of FADPSEN2 this is likely due to complex instability leading to reduced cleavage (and accumulation of APP-CTFs) and the release of longer products. For example, the longer more aggregate prone A β species which we see by ELISA (Fig. 1). In both cases we see a clear accumulation of APP-CTF (Fig. 7). We recently showed that accumulation of

APP-CTF causes LE/Lys dysfunction through decreasing lysosomal Ca²⁺ (via LE/Lys-MCSs, see Bretou et al., Dev Cell, 2024), which impacts on autophagy degradation, and which is explained as well in our Discussion section.

Reviewer #2 (Remarks to the Author):

The knowledge gap explored pertains to the detailed mechanisms by which Presenilin2 (PSEN2) mutations can drive Alzheimer's disease (AD) pathology. The authors propose that it is specifically through its impact on endolysosomal homeostasis and synaptic signaling in vulnerable brain circuits. Although it is known that mutations in the PSEN2 gene are sufficient to cause familial Alzheimer's disease, the specific cellular processes and pathophysiological changes resulting from these mutations, particularly in terms of synaptic function and neurodegeneration, are not fully understood. The study aims to elucidate how changes in PSEN2 expression affect neurobiological and cognitive functions, contributing to AD progression. The authors specifically investigate how PSEN2 contributes to AD. For that, the authors cross PSEN2 KO and FADPSEN2 KI mice with APP FAD KI mice (carrying three FAD mutations) and evaluate how the APP mice phenotype is altered by PSEN2 loss of function.

The authors conclude on an accelerated pathology when PSEN2 is KO or mutated compared to the APP mutant alone based on the following:

- Worse deficits in working memory
- Smaller presynaptic compartments
- Impaired LTP
- Aggravated Endolysosomal defects
- Reduced surface levels of synaptic proteins

The significance is related to the finding that the loss of function of PSEN2 is as important as its mutation in influencing AD pathology. Studying the contribution of PSEN2 alone is original, and the Annaert lab has been leading this research. However, the exciting results fail to reach the significance anticipated. The authors identify the need to understand how PSEN2 mutations cause familial AD. Still, a conclusion is precluded because the authors did not study the phenotype in PSEN2 KO or PSEN2 FAD KI (without APP FAD KI background) for most of the experiments. This leads to the major concern with the study:

1) One wonders if this is due to a lack of phenotype in the PSEN2 KO and KI. Did the authors do the experiments? If there is a lack of phenotype, this would indicate that using the mouse may not be the ideal experimental model for PSEN2-dependent AD. The authors should discuss this.

The major question of our study is to investigate the impact of altered expression of PSEN2 (KO and FAD, which is already rather unique) on amyloid pathology. We did not compare this with the single models (PSEN2KO and FADPSEN2), as these models do not develop AD-related pathology, including amyloid accumulation. Therefore, studying PSEN2 in these models would show the role of PSEN2 in a non-disease context. This could be relevant on its own, but our question here is very different: we aimed to study how altered PSEN2 expression impacts on a well-documented amyloid pathology, on behavioral changes and memory deficits in APPKI models: this allows to distinguish loss of function with gain of (toxic) function effects. To our knowledge, it is rarely done in one study to cover all genotypes. Moreover, the APPKI was chosen as we combine in vivo functional experiments with cell biology and want to avoid overexpression artefacts caused by the more broadly used transgene models of mutant APP and PSEN (and tau). The use of APPKI backgrounds provides a physiological more relevant background to study the contribution of PSEN2 as a

causal gene.

2) In most experiments, the authors do not include a wild-type mouse as a healthy control and only compare it to the APP FAD KI mouse model, an important control. The data would be more significant if compared to the wild-type. The second major concern is: Can the authors include the wild-type control in all experiments?

We chose not to include the WT in our IHC characterization of the models as it has been well established that WT mice do not develop AD-related pathologies due to the lack of ability of mouse amyloid to aggregate. This requires the mutation of three specific amino acids to the human sequence that is present within the APPNLGF model. Respectfully, we do not see the relevance to include therefore a WT control in IHC, merely to demonstrate no amyloid. However, in all other in vivo experiments, we did include the WT, notably in all our behavior analysis, in our electrophysiology experiments (LTP) (even more extensive in our revised version) and in our transmission EM analysis of MF synapses. In all those instances, we showed that the WT displayed no cognitive deficits nor pathologies that were present in the APPNLGF. As such, we feel that we have majorly addressed this concern through our in vivo data.

Overall, the data supports the conclusions and claims, but in several instances, the data is overinterpreted, as indicated below:

The data shows that PSENKO and FAD mutation alter amyloid pathology driven by APP FAD mutations.

1) The authors hypothesize that "altering PSEN2 expression in vivo, through KO or introducing a FAD mutation, would have opposing effects on AD pathogenesis." However, do not present direct evidence of it.

Respectfully, we think that reviewer#2 has misinterpreted this sentence. This was the initial hypothesis, that we aimed to test at the very beginning of our study. The hypothesis is based on our seminal finding that PSEN2/ γ -secretase is restricted to LE/Lys and is responsible for the major pool of intracellular Abeta (Sannerud et al., Cell, 2016). Also, within LE/Lys, processing by PSEN2/ γ -secretase tends to already produce more A β 42 whereas a FAD-PSEN2 (we investigated 5 FAD mutations) all fully switch production to A β 42. Given that intracellular A β 42 is demonstrated to be pathological relevant (and appears as well at very early pre-clinical stages in AD; for instance see the work of Gunnar Gouras' group), we postulated that aggravating the toxic intracellular pool of A β 42 would worsen amyloid pathology, whereas depleting this pool (by KO of PSEN2) would alleviate this. This prior knowledge was translated into a hypothesis to be tested next. The nature of a hypothesis is that it can be correct, or (often) give rise to unanticipated new findings. Indeed, to our surprise, we observed in both cases an aggravation of amyloid pathology probably due to the accumulation of CTF products within LE/Lys in case of PSEN2KO (Fig. 7). We further extended this to memory deficits and specific defects in the MF-CA3 circuit.

The authors only present the Abeta levels in neurons cultured from APPxPSEN2KO and APPxFADPSEN2 but not from the PSENKO or FADPSEN2 KI that the authors prepared. WT mice do not produce A β as we know it in the human context (the mouse and human A β sequence differs by 3 amino acids), and at endogenous level very, very little mouse A β is produced, that is barely detectable. However, in the context of our main question, how does altered PSEN2 expression impact on amyloid pathology, we respectfully do not see the added value of measuring mouse A β levels for this study.

In the supplementary figure 1c are presented crops of WB analysis of brain lysates from these mice (please indicate age in the figure legend) for APP, APP CTF, PSEN2, and Nicastrin. Still, the results section must include analyzing and interpreting the differences in APP CTFs. It seems that the APP CTFs decrease in both PSEN2 models. The authors must interpret and

discuss these results. What does it mean if the APP CTFs do not accumulate in the knockout as when one inhibits gamma-secretase?

The difference between both PSEN2 models vs wild-type is rather the shift in mobility of the APP-CTFs. While in wild-type essentially one band is found (likely α CTF), both PSEN2KO and FADPSEN2, present an additional band, likely β CTFs. It has been demonstrated that in AD mainly the production of neuronal APP- β CTFs are toxic and causing endolysosomal defects. Hence the major conclusion here is that these PSEN2 models have distinct APPCTF profiles. Therefore not the total amount but the specific upregulation of APP- β CTFs should be taken into account. This is similar as observed for A β , where it is rather the ratio of shorter of longer peptides (processivity) that is more important/relevant. We have added this in the revised text.

2) Why don't the authors prepare primary neurons from the PSEN2 KO and KI mice and analyze intracellular and secreted Abeta, as they have done for the APP KI mice in Figure 1a? We refer here to our answer to a similar comment on measuring mouse A β . This would additionally require antibodies for mouse A β , of which the levels are anyway very, very low falling within the undetectable range with ELISA. We doubt that intracellular A β is measurable in these models. Therefore, and respectfully, we feel this falls outside the scope of our research question.

3) It would be essential to show the APP FL and APP-CTFs levels in Figure 1 A, as in FigS1c.

These data are provided in Fig.7b and d, where we have analyzed the relative levels of APP-CTFs to FL-APP, and where we feel this fits better to the research question to what extent APP-CTFs accumulate in LE/Lys.

Worse deficits in working memory.

1) Figure 3f-j presents the evidence supporting this conclusion. However, the data analysis is not sufficient to support the conclusions. Assuming that the p-values in the figure represent significant differences from the first swim (if not, please clarify in the fig legend), it is necessary to compare the difference in time spent swimming between the mouse groups or at least to analyze the area under the curve or the slope, to conclude if there are worse deficits in working memory.

We appreciate this remark and have clarified the statistical comparison within the figure legend. However, we noticed swimming speed differences between the mouse genotypes, as shown in supplementary figure 3h. Therefore we cannot use time as a readout for cognition. We have explained this in the text.

Smaller presynaptic compartments

1) The authors describe in Fig 4A that PSEN2 is increased in the mossy fibers of the CA3 region and the cortex (Fig. S1). The intensity of PSEN2 in the different brain regions, cortex, CA1, and CA3, should be quantified.

We thank the reviewer for this suggestion. We have quantified PSEN2 intensities within the CA3 and CA1 region and added it to the figure (Fig. 4a-b). Herein, we opted to show the PSEN2 intensity in CA3 over CA1 to show an increased intensity in the CA3 region. In addition, we included additional immunostaining for a CA3 specific marker, ZNT3 (new Fig.4c).

2) Interestingly, the size of presynaptic terminals in the CA3 regions is decreased in the PSEN2 models relative to the wt and the APP KI, indicating that loss of function and mutation affect the presynapse. Moreover, the PSEN FAD KI is different from the KO. In contrast, only PSEN2 KI increases the postsynaptic compartments compared to the APP KI. Can the authors comment on these results?

The changes in the PSDs *in vivo* are more subtle: while in the KO background they are shorter (and a trend to be more abundant/synapse), in the FAD, they are clearly lower in number, but no difference in length. *In vitro*, using airyscan imaging (not EM), we noticed indeed a significant increase in the number of PSD95 punctae, however this reflects more the number of (post)synapses and not the PSDs themselves. Additionally, synapses *in vitro* will probably never recapitulate completely the *in vivo* morphology and functioning, especially for synapses as complex as the mossy fibers. Therefore, these two sets of results cannot be directly correlated.

3) It is unclear if the APP KI has an effect postsynaptically, as reported in transgenic models. Could the authors include the wild-type control?

We have included immunostainings of wild type hippocampal neurons for PSD95, PSEN2 and LAMP1 (Fig. S5d). Additionally, we quantified PSD95 puncta and saw that, indeed, APPNLGF neurons shows a postsynaptic defect. This is, surprisingly, rescued by PSEN2KO and FADPSEN2 (Fig. 6d-e).

Impaired LTP

1) Given PSEN2 localization, LTP is investigated, and the authors report a decrease in the APP KI PSEN KO and PSEN2 Ki. Moreover, the authors indicate that LTP was further reduced in APPxPSEN2KO and APPxFADPSEN2 mice. The authors need to present a statistical comparison between PSEN KO and FADPSEN2 of the amplitude in the late phase of LTP in panel 4f. Otherwise, this is an overinterpretation that needs to be corrected

In Fig. 4e-g we show LTP recordings in the CA3 where we see a slight, but not significant, decrease in LTP maintenance in the APPNLGF compared to the wild-type. This decrease is indeed exacerbated in APPxPSEN2KO and wild-Type and APPxFADPSEN2, as these differences become significant. However, we did not mean to imply that there was a worsening between APPxPSEN2KO and APPxFADPSEN2. We have now included statistics to show that APPxPSEN2KO and APPxFADPSEN2 are not significantly different. We have clarified this in the text.

Aggravated Endolysosomal defects

1) The authors indicate that the Aβ and APP-CTFs increase in endolysosomes, endolysosomal mass increases, the lysosomal calcium release decreases, lysosomal exocytosis reduces, and autophagosomes and autolysosomes accumulate with altered PSEN2 expression. LAMP1-positive organelles are referred to as lysosomes. At least the first time, it should be mentioned that LAMP1 identifies both late-endosomes and lysosomes.

Thank you for this remark and this is now corrected.

Reduced surface levels of synaptic proteins

1) The authors provide evidence of a similar reduction of the surface levels of vGLUT1 and GluA1 in both PSEN2 models. How did the authors perform this labeling and its analysis? The method used needs to be indicated in the methods section.

We apologize to have this overlooked in the initial version. This has now been included in the materials and methods.

2) Unexpectedly, could the authors explain that the vGluT1 and GluA1 labeling are not in puncta as if in a synapse but are linearly distributed? Is this a phenotype of APP KI neurons? Can the authors compare it to wild-type controls?

We, respectfully, do not fully agree with this statement. We often see a more punctate distribution, as can be seen in the zooms, and in cases where the staining looks linear, in fact they constitute clusters of focal hot spots. But these details may not always be obvious given that they are not fully resolved if one aims to both visualize the more intense clustered spots vs more individual spots. We have included an example of staining on WT neurons in Fig.

S5e which shows an identical immunostaining pattern.

3) Could this presynaptic impairment be confirmed using synaptosomal preparations of neurons or from the affected CA3 region (and CA1 as a control) of the different mouse groups?

This is an interesting suggestion, but for several reasons outlined below, we believe it would not sufficiently scrutinize our current data. Isolating synaptosomes from distinct brain areas requires individually optimized protocols, as the MF synapse is exceptionally large and requires specific steps in affinity enrichments to provide an, at best, highly enriched but not pure, fraction. Synaptosome isolations from only small hippocampal areas (CA3 vs CA1), requires as well a high amount of starting material. Further, given that in such protocols, material is lost in each subsequent purification step, it cannot be considered a true quantitative approach; also, it can be questioned whether it will allow to distinguish biochemically the 25% differences we observe between genotypes at EM level. At best it may provide redundant biochemical support, but with lower resolution and accuracy.

We used transmission EM to confidently identify presynaptic defects in the MF-C13 region with the highest available resolution: the quantified data clearly show significant differences in both the size of the presynapse and the number of small synaptic vesicles.

We argued that these data could be further, and stronger, supported by an orthogonal approach, through expanding our electrophysiology experiments. While we documented in our initial submission only LTP, we now extended this to paired pulse and train stimulations in both the CA3 and CA1 regions, for comparison and to identify circuit specific effects (Fig.4 new panels h to j (paired pulse), panels k-l (train stimulations)). These new and independent data confirm and strengthen the observed morphological differences in synaptic vesicle pools observed by EM. This is now added to the Result section:

“APPxPSEN2KO mice showed increased synaptic facilitation in the CA1, while in the CA3, PSEN2KO rescued the lowered response seen in APP KI to wild-type levels. In contrast, APPxFADPSEN2 mice displayed clear synaptic depression in the CA3 circuit, with normal responses in CA1 (Fig. 4i-j). To investigate the effects on presynaptic dynamics during periods of more demanding synaptic activity, we performed train stimulations in both hippocampal regions. In line with the paired pulse data, we observed increased synaptic facilitation in the APPxPSEN2KO predominantly in CA1 and partially in CA3, while APPxFADPSEN2 mice showed clear synaptic depression, exclusively in the CA3 circuit (Fig. 4k-l). Taken together, these results indicate alterations to synaptic efficacy in both the APPxPSEN2KO and APPxFADPSEN2 mice, likely involving presynaptic changes in the organization of the synaptic vesicle pool (see below). In case of the APPxFADPSEN2 this is more specific to the CA3 region whereas for the APPxPSEN2KO this is observed in both CA3 and CA1 but without impacting LTP in the CA1.”

Although the results are generally convincing, the data analysis could be improved, as follows:

4) In Fig.6A, colocalization analysis should complement the representative figure, and the number of examples should be reduced to facilitate the perception of colocalization to 1 or 2 examples. We recommend using the Fiji plugin ComDET because it allows triple colocalization, which would enable quantitatively determining if the "Organelles positive for both antibodies identify APP β -CTF build up, whereas 82e1-only organelles point to A β accumulation" differ with the genotypes.

We thank the reviewer for the statement that the results are in general convincing, and appreciated the suggestion for improvement. We have used the ComDET plugin to quantify the overlap between LAMP1 and 82e1/APP (Fig. 7c). We have additionally used this plugin

to quantify the PSD95 overlap with PSEN2/LAMP1 (Fig/ 6c).

5) Although the WB in 6B shows an increase in APP CTFs, no statistics are indicated in the quantification presented in panel 4D. The increase seems small and may not significantly differ from APP KI; thus, the interpretation needs to be toned down. "Finally, western blot analysis also showed higher levels of APP-CTFs in APPxPSEN2KO and APPxFADPSEN2 hippocampal neurons (Fig. 6b,d)." Moreover, as mentioned before, the authors could complement this data by analyzing APP CTFs in brain lysates.

We have increased the number the number of replicates and updated the graph. However, due to the large variation in the data (inherent to western blots as it is only semi-quantitative), the data has not reached significance. But when looking at the average, both the APPxPSEN2KO and APPxFADPSEN2 show approximately 60% increase in the APPCTF:APPFL ratio. This combined with the imaging data indicate increased APPCTFs in these models. We have therefore, as suggested, toned down the interpretation. As for the APP-CTFs in the brain, this turned out to be also very variable for quantification. Also here, immunostaining for APP in brain sections (Fig. 2 and Fig. S2) is more clear, demonstrating a visible increase in both APPxPSEN2KO and APPxFADPSEN2.

6) Fig. 6C Colocalization should be presented in % of overlap over Lamp1 since LAMP1 labeling increases (fig. 6F).

APP-CTFs are broader localized and accumulation is not restricted to LAMP1+ organelles. Indeed, the LAMP1+ area increases in our PSEN2 genotypes, but even in that context we still see more APP-CTF accumulating in LAMP1+ organelles, indicating a selective accumulation in these organelles and the point we wanted to make. Furthermore, we have now changed the quantification from a Mander's coefficient to the %overlap calculated by the ComDET plugin. Herein, there is a clear increase in % of LAMP1 positive vesicles associated with 82e1/APP. Therefore, if the APP accumulation would stay the same between genotypes one would anticipate a decrease in the %overlap if LAMP1 area is increased. Which is not the case.

7) Fig6 E-H, the analysis of % of the area to conclude on endosome enlargement or upregulation is indirect and not ideal. Instead it should be measured the size of LAMP1, EEA1, and VPS35 and the density (#/um2) similarly to what the authors did in Fig4C, D for PSD-95,

In figure 4C-D we indeed measured size of PSD95+ puncta, because these are limited to dendrites and more easily and reliably discernable as individual spots. We agree that this is a better measure also for endosomal and lysosomal compartments. However, and despite having used Airyscan imaging, this turned out to be not easy given organelles cluster, particularly in somatic regions, or have a more tubular morphology like VPS35+ endosomal compartments.

Other points:

8) line 301: "In conclusion, both APPxPSEN2KO and APPxFADPSEN2 neurons presented distinct accumulation of APP fragments that may impair endolysosomal homeostasis, as reported in other models (see above)." It should clearly state which APP fragments accumulate to clarify the conclusion instead of being referred to (see above).

We have adjusted this and clarified that it is APP-CTFs and A β that accumulate.

The general concern with experiment design and data analysis relates to the indication that two or three independent experiments were performed using mouse neuronal cultures. There is an enormous concern with reproducibility in biomedical research, and performing only twice an experiment is a major concern regarding its reproducibility.

We have performed additional biological replicates and have now the data presented from at least 3 or more independent neuronal cultures.

9) We assume that performing twice was the exception, but each statistical analysis must indicate the exact number of independent experiments.

We thank the reviewer for this remark and have for all graphs involving neuronal experiments included the total number of cells (n) analyzed (circles) and the different independent neuronal cultures (N) (triangles).

10) Moreover, it is indicated that multiple comparisons were performed, but it is necessary to indicate what was compared for each comparison. The indication of the p values in the figures is essential. Still, it needs to be indicated the comparisons between the experimental models, APP KI Psen2 KO vs. APP Psen2 KI and vs. APP KI and vs. wt (ideally, this group should be included in all experiments).

We have now included all the multiple comparisons in the graphs with a line and corresponding p-value. In addition, we have added in the figure legends which comparisons were made.

11) Notably, a smaller p-value indicates that the reproducibility of that result is higher. However, it does not mean a bigger reduction or increase (PMID: 25161440). The magnitude of the decrease or increase must be significantly different.

We thank the reviewer for this comment and we have carefully looked into the Result section and avoided the terms 'bigger' or 'smaller' when statistically different.

Figure labeling and legends need improvement and review. For example:

12) The identification of the different groups is missing from Fig. S1, panels D, and f.

This is corrected.

13) Fig.2a Thioflavin labeling the brain slices needs to be indicated in the figure and indicate which Thioflavin was used.

This is corrected.

14) Fig.3d, G-j, statistical comparison between groups is missing; data should be plotted as in Fig. S3.

For the working memory, we have not statistically compared the different genotypes because that is not the purpose of this test. Here, the aim is for the animals to decrease swimming distance compared to their first swim, therefore, these are the statistical comparisons are performed. We have consulted with our collaborating behavioral expert to ensure that this is the correct way to analyze this experiment.

15) Fig.3F-J number of animals analyzed is missing

This has now been added to the figure.

16) Fig5 Control WT is missing.

We have added the wild type control for a select few experiments but do not believe that this comparison is within the scope and research question of this manuscript (as argued above).

17) Fig5A Requires quantification of PSD-95 density and PSEN2 colocalization with LAMP1 and PSD-95.

We have quantified this and added it to main Fig 6.c

18) Fig5b second panel with PSEN2 in APP NLGF KI misses a white square

This has been corrected

19) Fig5b magnified insets are saturated, precluding a clear assessment of colocalization.

These images were not over-saturated during the image acquisition. However, we slightly balanced the staining to more clearly indicate examples of colocalization within dendrites.

20) Fig 5i has a label floating on top of the figure.

This has been corrected

21) Fig6N should use the same lut colors as in other panels; red lut should not be used as color-blind people cannot assess colocalization.

We have adjusted the color scheme

22) Fig6B is missing the label of proteins detected

The proteins detected have been added to the figure.

23) Fig6O-P p values labels should not be on top of the data

We thank the reviewer for the suggested improvements: we have adjusted all of the above mentioned comments in our new version of the figures.

Other minor points

24) Line 221 figS4a should be Fig.4a

This is corrected.

25) The APPNGLF mice could have been introduced in line 27, "APP (KI) knock-in mouse model."

This is done.

26) In the Title, "synaptic signaling" should be substituted for synapses as signaling often refers to regulating synaptic activity mediated by kinases, phosphatases, or other signals.

We have changed 'synaptic signaling' to 'synapse function'.

27) The discussion and the introduction should be more concise.

Compared to the original submission and taken into consideration the newly added datasets, we still have reduced the introduction and discussion sections by approx. 20% each, providing a more concise text.

Generally, the methods provided enough detail for the work to be reproduced, except for the following:

28) The methodology used for image analysis is somewhat incomplete; for example, the % area of synaptic and endosomal proteins is not clearly explained.

This has now been explained in more detail.

29) In the analysis of % surface area, what is it relative to?

This is relative to the total surface area of the neuron.

We thank the reviewer for these textual suggestions and these have been adapted and added in the revised manuscript.

Reviewer #3 (Remarks to the Author):

This is an **outstanding and highly original manuscript** describing how the most prevalent presenilin 2 (PSEN2) mutation linked to familial Alzheimer's disease (AD), and also the knockout of PSEN2, impact the neuron cell biology, pathology and phenotypes (behavior & physiology) of AD mouse models. Such a comprehensive and high-level mechanistic cell to in vivo studies is rare in the field of experimental AD research.

We thank reviewer#3 for the overall very strong and enthusiastic appreciation of our manuscript.

Specific points

In the Introduction, line 83, they write: "Post-embryonic full knock out of γ -secretase, obtained by conditionally knocking out all three APH1 isoforms, results in clear c-terminal fragment (CTF) accumulation of various substrates, neurodegeneration and neuroinflammation (25), underscoring the centralized role γ -secretase plays in AD pathogenesis". It is not clear why this is a model of AD pathogenesis if there are no plaques while AD patients with PSEN 1 & PSEN2 variants invariably develop amyloid plaques. Or are there cases of PSEN variants having neurodegeneration but no amyloid plaques? Can they later on then really speak of AD pathogenesis when it comes to PSEN2 KO?

We agree with reviewer#3 on this. This paper underscores a central importance for γ -secretase in brain, but not necessarily restricted to AD pathology. We have corrected this sentence and included more appropriate references.

On line 183 it is not clear what is meant by “and alike APP-FADPSEN2”. Do they mean that the dystrophies were increased at 9 months in the KO mice similar to what is seen with the PSEN2 mutants? This could be rewritten for clarity.

That is indeed what we mean. We have adjusted the sentence for clarity.

Line 189, this heading would seem to read better as “Altering PSEN2 expression in APP NLG F mice impacts working memory”

We thank the reviewer for this suggestion and have adjusted this accordingly.

Line 217 “significantly” instead of “significant”. Also, at the end of this sentence, don’t they mean APPxPSEN2KO and APPxFADPSEN2 here?

The sentence has been adjusted for clarity.

On line 252 they mention: “..white arrowheads, in Fig.4h,…” I was not able to see these arrowheads in Figure 4h.

We apologize for having forgotten the arrowheads. They are now included in the revised version.

On line 353 they write: “The lack of PSEN2 expression drives all APP metabolism towards PSEN1/ γ -secretase, which resides at the cell surface and in early/recycling endosomes.” Is there a reference for this localization of PSEN1 specifically in the setting of PSEN2 KO?

This is based on our 2016 Cell paper (Sannerud et al, Cell 2016) where we have used PSEN2KO cells stably rescued with either GFP-PSEN1 or GFP-PSEN2, in fact mimicking single KO lines as well. In the absence of PSEN2, only PSEN1-containing complexes can be formed, and hence processing of APP is driven towards the remaining complexes. In the same paper, we have more elaborately explored the different subcellular locations of PSEN2 vs PSEN1, using antibodies to PSEN2 and combining this with aforementioned rescue lines. Moreover we have used in addition cell surface biotinylation and analysis of magnetically isolated LE/Lys which also confirmed the preferential enrichment of PSEN1 complexes at the cell surface and PSEN2 complexes in LE/Lys. We would like to refer the reviewer to Sannerud et al., 2016 for more details.

Related to the above point, on line 358 they write “These findings provide in vivo support for PSEN2/ γ -secretase being the main generator of intracellular A β , which is significantly increased in an FAD setting whilst PSEN1 preferentially contributes to the secreted pool (23).” Ref 23 might be the reference asked for in the point above?

Yes, this is correct.

They further suggest then that without PSEN2 increased plaques come from elevated Abeta secretion. Might it not be possible that the disrupted endolysosomal biology induced by PSEN2KO leads to an altered subcellular PSEN1 localization into the more abundant LE/Lys compartments and thereby aberrant/elevated iAbeta42 in LE/Lys by PSEN1? However, they then nicely show elevated Abeta secretion in primary APP mutant neurons with PSENKO and importantly also show that Abeta42 in cell lysates is reduced, which argues against the possibility raised in my prior sentence.

Yes, indeed. We would like to point out that although PSEN2 is restricted to LE/Lys, it reaches this through transport via the cell surface. So, small amounts are present outside LE/Lys. This is not a black/white issue. Likewise, most PSEN1 is on the cell surface and in early/recycling endosomes, but a small part we also find in LE/Lys (see eg Sannerud et al., 2016). The point is that the balance is changed and more of the available APP will be processed through PSEN1/ γ -secretase: given its major residence at the cell surface and

early/recycling endosomes, it follows that it will be generating an A β pool that is more readily secreted. The fact that in PSEN2KO, the secreted pool is indeed increased supports this.

However, when one looks at the MSD results in Figure 1 it is intriguing that there are not only decreases in both 40 and 42 in lysates but also in 42 secretion in the primary APPxPSEN2KO compared with just the APP(NLF) neurons, while 40 shows a trend for a decrease but certainly not an increase. This does not really argue for just elevated PSEN1 secretion then causing increased plaques in the setting of PSEN2 KO.

It is true that A β 42 is decreased and A β 40 has a tendency to decrease. It must be noted that the MSD results represent % of total; they do not say anything about absolute amounts.

Therefore, it can be concluded that overall APPxPSEN2KO neurons secrete more A β 38 compared to the other species. Even though these are shorter peptides that have a decreased tendency to aggregate, they still do which we believe also partially explains the distinct morphologies of the plaques, with more compact plaques in the FADPSEN2 context (caused by decreased processivity and increase aggregation prone abeta42) and more loose plaques in the PSEN2KO context (more processivity and relatively more shorter peptides). This is as such described in the Results.

However, to say that this is the sole reason for increased plaque deposition is premature and other mechanisms cannot be ruled out based on our data. Another possibility is that, for example, due to the endolysosomal defects we observed, lysosomes lose their integrity, leading to neuronal cell death: the remaining intracellular A β may next act as seeds for plaques.

They use the terms lysosome, endolysosomes and late endosomes/lysosomes somewhat interchangeably in the manuscript. There are somewhat different definitions for the term lysosome. Typically, lysosomes are considered to be predominantly perinuclear and in the cell bodies of neurons; it is e.g. considered one of the vulnerabilities of neurons that they need to traffic cargo back along their processes all the way to their cell bodies for degradation.

Lysosomes by most definitions have lower pH than LEs and it would seem unlikely that what they at times call lysosomes in terminals have the required low pH of lysosomes.

Alternatively, lysosomes were traditionally defined by their appearance on electron micrographs. This reviewer is fine with the term LE/Lys of endolysosomes but believes that using just 'lysosomes' when it comes to neuronal terminals as suboptimal. On line 437 for example they write: "At the post-synapse, lysosomal Ca²⁺ release and lysosomal exocytosis...". At the synapse these will more likely be LEs; in particular MVBs are common in or near to synaptic terminals from where they release their inner vesicles: exosomes.

However, since this specific point is just one of semantics, this reviewer is fine with flexibility around these terms and is not demanding any changes.

We agree with this point, and only using LAMP1 staining or LT will not entirely differentiate between the subpopulations. We tried to use as much as possible LE/Lys (or endolysosomes) and have carefully checked the text for consistency.

Is it possible that some of the changes in plaque morphology with altered PSEN2 might even relate in some degree to microglia? This reviewer is not clear about the expression of PSEN2 in activated microglia and thereby a possible contribution. - This point actually is then addressed in their Discussion where they even describe a manuscript in preparation about this.

We indeed observe a significant change in microglial activation and recruitment to plaques, with a clear difference between PSEN2KO and FAD-PSEN2 backgrounds and which could indeed contribute to the observed pathology. However, this aspect requires a more detailed analysis to explain the observed differences that would be difficult to combine with this, already elaborated, study. We are currently indeed preparing a manuscript on this aspect in particular and decided for this paper to focus on the neuronal aspects of altered PSEN2

expression.

On line 385 and in several other places they have no space between the # and “months”:
“6months”

This has been corrected throughout the manuscript.

Page 467: “In support, in sporadic AD forms, only splice variants are found in PSEN2 but PSEN1 genes that result in truncated/lowered transcripts...” Please correct/rewrite for clarity.

This sentence has been rewritten for clarity.

It is not clear why APP labelling is so apparent in the APP but not in the APPxPSEN2KO and APPxFADPSEN2 mice in Figure 2f.

We acknowledge this remark. This is likely due to a higher background staining in APPKI compared to the other genotypes.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #5 (Remarks to the Author):

As requested by the editor, this review relates only to the generation of the new genetically modified (GM) mice, specifically the PSEN2 KI line (i.e., N141I), given that the APP KI and the PSEN2 KO lines have already been characterized and published.

- As per figure S1, there are only two silent mutations (not three), and they are located on the binding site, not on the PAM. This needs to be corrected (line 512).

There were three silent mutations added to the donor. This has been corrected in the figure.

- Which algorithm (to prevent off target) has been used to design the selected sgRNA? Please add this information in the Methods section.

The Crispor webtool was used and this has been added to the method section.

- The provided genomic sequences on Figure S1 are not correct. Indeed, they contain the 3 expected mutations, however, when aligned to the WT (Mus Musculus) sequence, extra mismatches are present. This needs to be corrected.

We have re-checked the sequence and adapted it. Based on the Sanger sequencing we performed (Fig. S1c), there are no other mismatches in the genomic sequence besides the anticipated mutations. We have, in the same figure, aligned it to the mouse sequence, confirming no additional mismatching.

- The primers used to genotype the Founders are to be presented by pairs (M&M section). It is currently impossible to check for the expected band sizes.

We have added the primer information in the materials and methods.

- The identification of potential founders by PCR is highly risky and insufficient because additional (unwanted) mutations often occur when using CRISPR. The authors mentioned that the genomic sequence of the selected Founder has been sequenced (line 519). Which sequencing was used (Sanger, NGS..)? The raw data for the sequencing should be provided.

We used Sanger sequencing to confirm the absence of unwanted additional mutations. This information has now been added to the supplementary figure. We apologize to have it not included in the first version.

- The doses of CRISPR reagents used for microinjection are extremely high (e.g., 1µg/ul).

Such high concentrations are usually used for electroporation (not microinjection) of zygotes.
Can this be reviewed and justified?

This is indeed a mistake. We wrote down the stock solutions instead of the working solutions.
This has been adjusted in the revised manuscript.

NCOMMS-24-16020-T: point-to-point rebuttal

Reviewer #1 (Remarks to the Author):

The authors have adequately addressed the comments of the previous review. We thank Reviewer #1 for the very positive assessment of our revised manuscript.

Reviewer #2 (Remarks to the Author):

We acknowledge the improvement of the data now supporting better the results.

We appreciate Reviewer #2's recognition of the enhancements made in our revised manuscript

The significance of this paper is limited by the choice of studying the cumulative effect of two familial mutations of AD that, to my knowledge, have not been identified in AD mutations. Nevertheless, the field will benefit from the publication of these results. The mechanisms whereby mutations in PS2 alone are sufficient to drive AD pathology remain unclear.

We value Reviewer #2's positive feedback on the impact our study will have in the field. We have made additional changes in the message conveyed to more clearly state the limitations of the study (see in particular response to point 1 below).

The authors addressed many of the suggested revisions, but some critical points remain and should be revised:

1) We thank the authors for explaining the inconsistent use of WT mice as healthy controls. However, the manuscript must include the rationale provided in the rebuttal to clarify the experimental approach used for Nature Communication readers.

Respectfully, the problem the authors propose to solve in the abstract and introduction is understanding the mechanisms whereby a sole mutation of PSEN2 causes AD.

Example:

Abstract "Rare mutations in the gene encoding presenilin2 (PSEN2) cause familial Alzheimer's disease (FAD); however, the underlying molecular mechanisms remain elusive."

Introduction:

"Given its LE/Lys localization, we hypothesized that changes in PSEN2 function, through KO or FAD-linked mutations, may directly impact on LE/Lys homeostasis through regulating $\text{iA}\beta$ and APP-CTF levels."

The authors do not use an experimental design that allows them to (dis)prove this hypothesis.

Instead, the authors investigated how PSEN2 changes amyloidosis, endolysosomal abnormalities, and synaptic function caused by the knock-in of mutant APP. This choice must be better communicated (written) in the abstract and the introduction.

The authors can strengthen the manuscript by explaining why the APPKI background was essential for their conclusions. Ideally, the authors should mention that the PSEN2 mutation does not cause amyloidosis in mice, thus requiring a cross with the APP mice.

As it is, the results provide novel and exciting findings supporting that PSEN2 loss of function has a cumulative harmful effect on already diseased mice. However, they do not provide proof that PSEN2 loF-dependent mechanisms are sufficient to trigger AD in a mouse model, indicating that for PSEN2, other models may be more relevant. The authors need to discuss this limitation in the manuscript. An alternative model could be the Humanized Abeta KI mice (developed by model-AD), which is available and could be a promising alternative for future research.

We understand the point of the reviewer and have now modified the cited sections as well as added the discussed rationale in the manuscript.

Specifically, the abstract was adapted as follows to consider the reviewer's suggestion:

Lines 28-40

Rare mutations in the gene encoding presenilin2 (PSEN2) are known to cause familial Alzheimer's disease (FAD). Here, we explored how altered PSEN2 expression impacts on the amyloidosis,

endolysosomal abnormalities, and synaptic dysfunction observed in the APP knock-in mouse model. We demonstrate that PSEN2 knockout (KO) as well as the FAD-associated N141I KI mutant accelerate AD-related pathologies in female mice. Both models showed significant deficits in working memory that linked to elevated PSEN2 expression in the hippocampal CA3 region. The mossy fiber circuit of APPxPSEN2KO and APPxFADPSEN2 mice had smaller pre-synaptic compartments, distinct changes in synaptic vesicle populations and significantly impaired long term potentiation compared to APPKI mice. At the cellular level, altered PSEN2 expression resulted in endolysosomal defects and lowered surface expression of synaptic proteins. As PSEN2/ γ -secretase is restricted to late endosomes/lysosomes, we propose PSEN2 impacts endolysosomal homeostasis, affecting synaptic signaling in AD-relevant vulnerable brain circuits; which could explain how mutant PSEN2 accelerates AD pathogenesis."

The mentioned sentence in the introduction was modified as follows:

Lines 99-100:

"Given its LE/Lys localization, we hypothesized that changes in PSEN2 function, through KO or FAD-linked mutations, **may affect A β pathogenesis, potentially through altering iA β and APP-CTF levels.**"

In addition, mentions to the rationale for the choice of models have been added to both the Results and Discussion sections:

Lines 149-151 in the Results section:

"To note, wild-type mice were not included in these analyses as they do not develop amyloid pathology. Indeed, the murine A β sequence differs from the human one by three amino acids at N-terminal positions 5, 10, and 13 and therefore does not readily aggregate."

Lines 325-327 in the Discussion section:

"Importantly, the use of an APPKI mouse model was necessary to address this question since, as previously mentioned, wild-type mice do not develop A β pathology."

Lines 439-442 in the Discussion section:

"It would be interesting to extend this study to a humanized APP mouse model, as this would allow to investigate the direct potential of altered PSEN2 expression to induce amyloid pathology instead of the impact on existing amyloid pathology as investigated here."

With these changes, we hope the reviewer will agree that the message conveyed in our manuscript has been sufficiently adjusted to avoid any overstatement.

2) Regarding supplementary Fig. S1D (former 1C), although one can perceive the appearance of a higher molecular weight band potentially being APP-bCTF (C99), the prominent one band detected in wild-type, that corresponds to both alpha (C83) and beta-CTF (C89) decreases in PSEN 2 KO and FAD neurons. In the manuscript reads:

Line 125 Furthermore, in PSEN2KO and FADPSEN2 mice, the APP-CTF profile included an additional lower mobility band, likely representing APP- β CTF,
127 not detected in wild-type mice (Fog/S1d).

The authors must revise the text (or data) to include a description of the observations concerning the decrease in APP CTFs and to interpret these results.

We understand the point of the reviewer and we believe that deciphering the exact profile of APP- α CTF vs APP- β CTF in our models would require more investigation with specific targeted antibodies.

In order to avoid any over-interpretation, we chose to remove the APP profiles from our Fig.S1d.

The corresponding text now reads:

Lines 125-127

"These mice, which show no overt phenotypic differences, have higher endogenous mutant PSEN2 levels, suggesting more stable PSEN2/ γ -secretase levels (Fig. S1d) or a broader distribution (Fig. 4a). FADPSEN2 and PSEN2KO mice (18) were crossed with the APP NL-G-F KI (APPKI) AD model to investigate AD pathology (hereafter referred to APPxPSEN2KO and APPxFADPSEN2) (49)."

We believe this alleviates this point of discussion.

3) Regarding Figure 4 and its description in the results section:

Indication of p-values is missing from panels of figure 4e, 4f, 4i, 4k till 4O, as mentioned in the figure legend (All graphs are represented as mean $\pm$ SEM and include p-values.), is needed to support the description of the data:

Line 220-228: "We then explored short-term presynaptic plasticity using paired-pulse stimulations in the CA1 and CA3 regions. APPxPSEN2KO mice showed increased synaptic facilitation in the CA1, while in the CA3, PSEN2KO rescued the lowered response seen in APP KI to wild-type levels. In contrast, APPxFADPSEN2 mice displayed clear synaptic depression in the CA3 circuit, with normal responses in CA1 (Fig. 4h-k). To investigate the effects on presynaptic dynamics during periods of more demanding synaptic activity, we performed train stimulations in both hippocampal regions. In line with the paired pulse data, we observed increased synaptic facilitation in the APPxPSEN2KO predominantly in CA1 and partially in CA3, while APPxFADPSEN2 mice showed clear synaptic depression, exclusively in the CA3 circuit (Fig. 4l-o, Fig. S4h)."

Or the description of the data needs to be revised.

The statistical analyses have been added to the graphs and corresponding legends.

3) While in Figure 4, the number of animals and cells is indicated in the figure legends, it should be indicated for each experiment in the figure legends in the other figures. This is essential for transparency and reproducibility.

Although 'n' was always provided, the manuscript was adapted to better describe the nature of entity for 'n' in the legend(s) of figure(s) 1b-g; 3b, e, g-j; 4g.

4) The magnified insets in Figures 6B and 7N are still saturated, preventing a clear assessment of colocalization. The authors should address the saturation issue to ensure the data can be accurately interpreted.

We thank the reviewer for their observation regarding the magnified insets in Figures 6B and 7N. To ensure consistency and reliable comparisons between genotypes, all images were acquired using identical microscope settings across all conditions and were not individually processed or altered, in line with Nature guidelines. We recognize that this approach can result in slight saturation in certain genotypes/conditions, as seen here, to maintain comparability across samples. We hope this explains our imaging strategy and the steps taken to provide unbiased, reproducible data.

Reviewer #3 (Remarks to the Author):

This is a revised and now even further improved manuscript showing how PSEN2 deficiency compared to the familial Alzheimer's disease (AD)-associated PSEN2 N141I mutation affect AD pathogenesis when crossed into an AD APP knockin mouse model. I had viewed this work as outstanding already in my initial review due to the multiple excellent and diverse (cell biology, pathology, behaviour and physiology) methods used and the high level of scientific importance to this work. This reviewer believes that the authors extensively addressed the points of the original reviews. Reviewer 2 did a particularly thorough and excellent review, although I agree with the authors that the requests to extend all their work to wild-type and just PSEN2 KO mice (as the authors point out, they do look at these for many experiments) is requesting too much more work given that the central question is how PSEN2 deficiency differs from an AD mutation in PSEN2 in the setting of AD pathogenesis. Their work contains intriguing findings showing e.g. that either PSEN2 deficiency or AD mutation worsen AD pathogenesis. Although I don't fully agree with their interpretation of increased A β pathology with PSEN2 deficiency originating from elevated A β secretion, their data does point to this interpretation, and they certainly might be correct. One alternate possibility is that ageing might change the results they detected in their primary neurons, although I consider that requesting to explore such a complex possibility would be very unreasonable given not only

the difficulty of the task but also the extensive work that they have already presented in this excellent manuscript.

I have no further comments for this now even better manuscript.

We value Reviewer #3's very positive assessment of both our initial and revised manuscript.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We thank Reviewer #4 for their participation to the assessment of our work.

Reviewer #5 (Remarks to the Author):

All comments related to the generation of the mutant mice have now been satisfactorily addressed and I have no other comments.

One important typo might still need correction though: the M& M section (mice) mentions that Exon 4 has been targeted using CRISPR, when in fact it was directed to Exon 6.

We thank Reviewer #5 for acknowledging the improvements made in our revised manuscript. We have corrected the mistake that was remaining in the M&M section and thank the reviewer for pointing our attention to it.