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Review COMMONS

Upregulation of APP endocytosis by neuronal aging drives amyloid-dependent synapse loss

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Reviewer 1

Evidence, reproducibility and clarity

The authors used in vitro “aged” primary neurons (DIV28) and report increased levels of reproducibility lipofuscin staining and intracellular mouse Abeta as well as a decline in synaptic density. Interestingly, they also showed increased APP endocytosis, whereas other CME cargoes, such as TfR, were not affected. Notably, inhibition of Abeta production by a BACE inhibitor attenuated synaptic decline. One possible mechanism highlighted by the authors, causing the increased intracellular Abeta might be elevated Abeta endocytosis while in vitro aging.

1) The authors show increased intracellular Abeta immunoreactivity, as indicated by anti-Aß42 C-terminal specific antibody (12F4) (Figure 1). Specificity of the staining should be validated in APP KO or knock down primary neurons. Furthermore, the authors should provide co-stainings with APP to clarify if the anti-Aß42 antibody might also detect full length APP or CTFs. This could also be shown by expression of different murine APP mutants.
This control for specific intracellular Abeta detection is essential, as the authors also observed increased APP levels in "aged" primary neurons (Figure 2 and 3).

2) The authors observed increased levels of EEA1 staining in "aged" PN (Figure 3) and postulate increased levels of APP in EEA1 positive endosomes. Firstly, co-localization should be validated in a quantitative manner. Secondly, the authors should provide a control with another membrane protein, to ensure that APP is indeed increasing in early endosomes.

3) Biotinylation assays suggest strongly increased APP endocytosis in "aged" PN. The authors tried to validate these results with an APP antibody uptake assay. For this purpose they used 22C11. To our experience, the 22C11 works very well in WB analysis, but not in IC. Therefore, I strongly recommend to control either 22C11 immunoreactivity on APP KO cells or to use another antibody for the uptake assay.

4) For control, the authors showed very interestingly that Transferrin-receptor endocytosis was not increased in "aged" PN. For better direct comparison, the experiment with TfR should be

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carried out also with the bulk surface protein internalization assay, used for APP. Moreover, the authors should provide experimental evidence for this different behavior, as e.g. testing for different recycling rates of APP and TfR in "aged" PN.

5)The authors show in addition that inhibition of APP processing attenuates synaptic decline in "aged" PN. This is a pure correlative observation and should be experimentally validated by e.g. usage of APP KO PN or an APP knockdown approach combined with BACE inhibitor treatment.

Significance

The increased APP endocytosis in "aged" PN is a very interesting finding and has important impact on understanding of Abeta mediated neuronal cytotoxicity.

Researchers interested in how Abeta might cause synaptic loss and neuronal loss are potentially interested in this study. Furthermore, the study highlights the APP endocytosis mechanism as a potential target for future therapeutic approaches, which might be interesting for those, working on endocytosis mechanisms.

My field of expertise is on Amypoid precursor protein function and trafficking.

Reviewer 2

Evidence, reproducibility and clarity

This is an interesting article from the group that has been active in the field of membrane trafficking with a focus on Alzheimer disease. The paper by Burrinha et al. identifies the upregulation of APP endocytosis as a driving force of aging-dependent synapse loss and as a novel target to prevent late-onset AD. Using their own established model of aged mouse cultured neurons and material of aged mouse brains, the authors provide evidence that accelerated APP endocytosis potentiates APP processing and Abeta production. They also provide a mechanism for the upregulation of APP endocytosis, identifying clathrin, dynamin, and F-actin as key players. Techniques used in the study include ICC/IHC, WB, endocytosis, and trafficking assays.

Key conclusions are:

(i)Intracellular Abeta42 is increased with aging
(ii)APP processing and early endosomes increase with aging
(iii)APP endosomal localization and its endocytosis increase aging
(iv)Upregulation of APP endocytosis with aging requires clathrin and actin
(v)Age-dependent synapse is in part due to Abeta production

I found the concept interesting and the paper well presented. On the other hand, the conclusions driven from the results might be a bit preliminary and might require several additional controls.

Major comments:
My main concern is about the aging in-vitro model (see below). Additionally, I find conclusions ii), iii) and iv) (see my list above) are not convincing and preliminary.

1.Conclusion i):

I am not certain if embryonically isolated neurons are a good model for the study authors aimed to do. These neurons are usually expressing the doublecortin throughout their life in culture, which would say that they are stuck in a "new-born state". Additional control for doublecortin levels, as well as some additional markers of cellular senescence, should be used to verify the aging model.

2.Conclusion ii):

a.I am very confused with the data in Fig. 2A. Why the described CTFs in the text are not evident in the blot or am I missing something? The last marker is 15KD, while beta-CTFs are 12-14kD. It would

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be good to know what is quantified in Fig. 2B? Also, I suggest quantifying the APP processing into ß-CTFs (12-14kD) and ?-CTF separately, both for cultured neurons and brains. b.I agree that the data indicate a solid increase in EEA1 protein levels. But can one really draw a conclusion about the early endosome increase only from the EEA1 data? Some additional experiments might be required: i) the mRNA analysis of EEA1, ii) additional early endosomal markers (i.e. RAB5 levels), and iii) the authors should consider EM or super-resolution techniques (the EEA1 puncta in brain sections are in fact too big to be early endosomes). Alternatively, the claim for increased early endosomes should be re-evaluated (i.e. can be changed to EEA1-positive early endosomes instead).

c.The separation between cell body and dendrites, which authors make in the article is very confusing to follow since not all data are in fact always consistent, e.g. early endosomes increase more in dendrites with aging, but there is no difference in endocytosed APP and etc. I am not even sure why the authors need the cell body for their story. I would stay with neurites and synapses.

3. Conclusion iii):

a. APP surface levels are increased in aged neurons. Could this be that increased APP endocytosis is due to altered recycling of APP back plasma membrane (more APP is endocytosed because the APP is faster recycled)? A recycling assay should address this issue.

b.I am puzzled with a low amount of RAB5-positive endosomes containing the endocytosed APP (Fig. S2K,N). Can the rest be in recycling endosomes? ICC with endocytosed APP/RAB11 should be performed here.

4.Conclusion iv):

I found this conclusion is less convincing from all since all the data are generated using clathrin and dynamin inhibitors only. Pitstop2 and dynasore have multiple unspecific side effects (i.e. please see the Park et al., 2013, J. Cell Sci). To draw the conclusion that APP endocytosis requires clathrin and dynamin based on that is far too preliminary. I suggest either to down-tone throughout the paper (e.g. APP endocytosis is Pitsop-2 and Dynasore- sensitive) or perform the functional analysis using shRNAs or CRISPR/Cas.

5.Conclusion v):

Fig. 7 is loosely attached to the mainline of the manuscript. I suggest to either include this as a part of Fig.1 to illustrate an additional control for an in-vitro model or perform additional experiments showing the effect of APP endocytosis on synapse rescue in aged neurons (should be very straight-forward since authors can use the drugs mentioned above).

Generally, the data and the methods presented in a reproducible way, and all experiments adequately replicated and statistical analysis is adequate.

Minor:

1. Page 7, line 134- LAMP1 is not a pure lysosomal maker and will label MVBs and late endosomes as well.

2. FM1-43 is not an endosomal marker, but a lipophilic dye, which will insert into all membranes.

3. Please indicate whether the heavy or light clathrin chain was analyzed. 4.Discussion is too long and a bit diluted. I suggest shortening it by 1-2 pages.

5.This is correct that CALM can function as an adaptor for endocytosis of APP. Additionally, CALM can also regulate APP autophagic degradation (Tian et al. 2013). It would be good if the authors can re-consider their data according to this finding.

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Significance

The role of membrane trafficking in the pathophysiology of AD is currently under intense investigation. From that point of view, the study by Burrinha et al. is timely and of high significance for readers interested in cellular and molecular mechanisms of neurodegeneration.

Field of expertise: membrane trafficking, autophagy, endocytosis, neurodegeneration.

Reviewer 3

Evidence, reproducibility and clarity

SUMMARY

Burrinha et al. is a worthwhile study with potentially interesting findings on APP endocytosisin vitro and in vivo. The authors have developed systems to compare the aging of neurons in culture, derived from the cortex of E16 mice, as well as brains from young and old adult mice. They used two systems to compare APP endocytosis and processing in the neuronal samples over the time period and measured the level of AD as a link with Alzheimer disease. This study presents a significant body work with number of well-organized figures and extensive quantification and controls. However, many of the hypothesis/conclusions by the authors are not fully supported by the data. Also, and most importantly, a number of key controls are missing to support authors' conclusions. The balance of the discussion and conclusions need to be improved. Overall, if the author can improve the robustness of the data the manuscript could be a very useful contribution

MAJOR COMMENTS

Some of the key experiments need better controls to support the conclusion proposed by the authors (see below).
In Fig1, Abeta level after DAPT treatment needs to be included in the IF to demonstrate the specificity of staining by the Ab to Abeta40 or to Abeta42. The level of AD secreted needs to be quantified as well to determine the relationship between intracellular and secreted pools of Abeta.

In Fig. 2A, the quality of the western blot is poor and cannot be interpreted. There is no internal standard for normalization of loads between samples. The identity of the bands seems very speculative given the sizes of the bands do not correspond with their expected Mr and these need to be confirmed by different treatments. To increase the resolution for the lower bands, a different gel percentage can be used. As C99 bands are not identified specifically, conclusions on the AD pathway cannot be made from this blot. Moreover, APP signal is saturated in Fig. 2B and makes the quantification inaccurate. In Fig.2E-F, the level of EEA1 in 14DIV is missing and it would be relevant for a complete picture to show the data on the basal level of EEA1 (is the level stabilised between 14DIV and 21DIV or a continual increase?).

In Fig.3 it would be ideal to include APP staining in APP KO neurons as a control of APP specificity as some antibodies to APP can give high background. The specificity of staining needs to be demonstrated as the contribution of background staining may vary across the aged samples. This issue also applies to the labelling of endogenous APP at the cell surface (Fig. 4I-L), the specificity of this labelling needs to be established, particularly as only low levels of APP are considered to be at the PM.

Fig. 4A-E is missing a key control: a surface label and cleavage without a chase eg on ice, in order to determine the background for each DIV sample. This is a critical control to demonstrate that the labelling in each case is restricted to the cell surface. This control is also essential to be able to compare samples between 21DIV and 28DIV.

To complete Fig.6, effects of the drugs on transferrin endocytosis in aged neurons as well (here only 21DIV and not 28DIV) and for the styryl dye FM1-43 need to be check.

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In Fig7. the authors did not show if the BACE1 inhibitor and DAPT have actually blocked APP processing in their experiments. They have relied on the reported action of these drugs but it is essential that the level of inhibition in these experiments is documented. In the absence of information about APP processing conclusions cannot be drawn on the experiments in Fig 7.

Importantly, there is contradictory information in the different figures and quantification. In Fig.1, quantification of Lipofuscin is quite similar in the neurites at 21DIV and 28DIV but looks very different in the images. In Fig. 3A-B-C, the images show more APP in the cell body at 21DIV compared to 28DIV whereas the quantification is showing a similar level in the cell body. Moreover, Fig. 2 shows no difference in total APP levels between 21DIV and 28DIV yet Fig. 3C indicates that the neurite APP is higher at 28DIV.

One of the most important concerns in the study is the fact that the neuronal medium is not changed during the 28 days of neuron culture (line 576 - "Neurons maintained up to 28 days without changing the media"). This is a concern for neuron viability, and dendrites/synapses development. It may lead to an accumulation of APP and AD in the supernatant that will affect the results of the entire study. This is a really critical point and will need to be address by the authors. Notably, in Fig. 5 the reduction of the endocytosis of TfR in 28DIV is very substantial and might suggests that cells at day 28 are not healthy.

The authors did not mention potential contribution of anterograde trafficking APP and AD production along biosynthetic pathway and their statements (line 185) ignore locations other the endosomes. APP processing occurs in endosomes but also in the secretory pathway and AD has been shown to be produced in the Golgi apparatus as well. This issue needs to be discussed and integrated with their findings and their hypothesis (around line 416). Also, the paper argues for enhanced APP processing, however a reduction in secretion of Abeta would also be consistent with much of the data.

•Should the authors qualify some of their claims as preliminary or speculative, or remove them altogether?

•Would additional experiments be essential to support the claims of the paper? Request additional experiments only where necessary for the paper as it is, and do not ask authors to open new lines of experimentation.

The authors are able to observe APP CTFs fragments in 21DIV and 28DIV neurons by western blot (Fig. 2 A-B). However, CTFs in the 14DIV sample are not detected which is surprising. We would suggest treating neurons with different drugs (ie: DAPT/C3...) to inhibit APP processing and accumulate APP CTFs to be able to observe APP CTFs even at an early neuronal stage. In addition, the treatment with drugs will assist in the identification of each of the potential CTF bands. A different gel percentage should be used.

The authors have assessed the number of endosomes containing APP and observed an increased in early endosomes size. We would suggest staining the early endosomes with another marker (ie Rab5) for confirmation. It would be very informative to determine the distribution of APP across the different organelles within a cell as well. Lysosomes (numbers, shape and the localisation of APP in the lysosomes) should be included as APP is transported down the endolysosomal pathway (Toh et al. 2016). The authors have already performed some LAMP1 some LAMP1 staining in Fig.1 and an expansion to include APP levels in LAMP1 compartment would give a better picture of any changes in organelles in their aging models.

The authors have analysed APP endocytosis and AD production. However, AD can also be release in the supernatant. As the neuronal media is not changed during the 28 days of neuronal culture, it is possible that the accumulated AD in the culture medium will affect the aging of neurons and intracellular AD level.

To compliment the APP plasma membrane staining (with 22c11 Ab) at different stages (14DIV, 21DIV and 28DIV) (Fig. 4) it would be informative to quantify the level of APP at the plasma membrane and assess whether there are change in APP at the PM during aging.

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In the last part of the study, the authors investigated the synapse plasticity/loss using two markers PSD-95 and vGlut1 (Fig.7). EM will give direct evidence in the change in size/numbers of the synapses comparing mature and aged neurons. EM images will give a much higher impact to the study. However, this suggestion may be challenging if the authors do not have ready access to an EM facility.

•Are the data and the methods presented in such a way that they can be reproduced?
•Are the experiments adequately replicated and statistical analysis adequate?

In Fig.2 A-B, the quantification is not convincing as the APP bands are saturated. We would suggest decreasing the loading and using DAPT treatment to reveal the total APP CTFs/C99 produced in neurons. Using this technique, C99 and others CTFs are observable in young neurons (7DIV, cf Tan, Fourriere et al. 2019).
Quantification of APP in Fig.2C/D depends on the purity of the neuronal prep which is not stated in the paper.
APP staining in cell body is saturated in Fig. S2C/D which renders quantitation meaningless. In Fig. 4C, endocytosed APP should be represented in comparison to APP level at the plasma membrane at the beginning of the experiment as this level is increased in aged neurons (49% more).

It is not clear if the quantitation of IF was performed on optical sections or 3D reconstructions. If optical sections, how where they selected?

There is inconsistency in the age of mice used in the study between figures (either 6- and 18-months or 12- and 18-months).

Minor comments:

Line 165, the authors discuss the increase of APP processing with aging. It can be due to an increase of APP, of BACE1 or due to a change in APP and BACE1 intracellular trafficking with aging. Authors argued that BACE1 level is the same (Fig 2SAB). The authors should comment on this result as the literature reports stability in BACE1 mRNA (Bioal et al 2000, Irizavarry et al. 2001; Apelt et al. 2004) and BACE1 protein levels (RoDner et al. 2001; Fukumoto et al. 2004; Apelt et al. 2004). However, BACE1 activity is increased with aging in mouse and human brain (Fukumoto et al 2004). The authors need to comment on BACE1 activity in their system.

Line 275, "in young neurons, APP undergoes clathrin mediated endocytosis (CME)", a reference is needed.

The authors are focused on the endocytosis pathway of APP in aging neurons and brain. However, there is no mention of APP anterograde trafficking, and as APP is directly trafficked from the Golgi to the early endosomes, transport of APP from the anterograde transport pathway might play a role in the increase of APP in early endosomes as well (Xu et al. PNAS 1997; Siman R, Velji J. 2003; Choy et al. 2012; Toh et al. 2016...). The authors need to expand their conclusions to include alternative hypotheses from their study.

The text is generally well written although it has the feel of an early draft. Some parts of the text need to be rephrased (ie: line 58 "born embryonically"; line 62 "neurons are not only targets of AD" ...). The conclusion and discussion need to be clearer and more succinct.
Improvements here will provide a manuscript which is more accessible to readers in the cell and neurobiology fields.

A more definitive title could be used (if the control experiments are consistent) (ie: Up- regulation of APP endocytosis in aging neurons linked to synapses loss and Alzheimer development).
Abbreviations are not usually used in the summary (ie: D-amyloid instead of AD). Abstract can be rephrased to highlight better the in vivo and in vitro aspect of this study. The information is present, but it could be stated more clearly to show the strength of the study. D-amyloid and Alzheimer disease can be added to the key words.
See below our comments relative to specific sentence/statement/conclusion: Line 54 - "Cognitive decline develops with aging and often precedes AD"

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This statement could be improved as it is similar to the 'chicken or the egg' dilemma ... We don't really know which comes first - AD accumulation in the brain might appears first during early development of AD and will lead later to cognitive defects.

Line 77 "in familial AD, the progressive accumulation of Ab initiates the cascade..." true? Needs references.

Line 134 "the number of auto-fluorescent granules within lysosomes identified by the lysosome-associated membrane protein 1 (LAMP1) was significantly...."
Needs to be rephrase as it is confusing, LAMP1 is used as a lysosome marker to identify the quantity of granules/lipofuscin inside the lysosome.

Some parts of Fig. 2 are not commented in the text, we can observe an increase of EEA1 in aged neurons and aged brains and it need to be state/ commented in the text to be able to keep the actual conclusion "increased in APP processing in the aged neurons and aged brain".

Line 172 "Moreover, we found the levels of BACE1 and nicastrin, a ?-secretase subunit, unaltered in aged neurons when compared with mature neurons (Fig.S2 A, B)"
We would suggest being more cautious by saying "not significatively altered in our model..." instead of "unaltered".

Line 205 "their size and intensity increased in aged neurons especially in neurites"
The authors are making conclusions about early endosomes based on staining with the EEA1 marker. This conclusion is an overstatement as the size and intensity looks similar in the cell body compared with the neurites. Moreover, an increase of EEA1 staining doesn't mean an increase in endosomes as stated on line 179 in the manuscript.

Line 209 "Overall, these data indicate that with aging APP localizes more to enlarged early endosomes."
From this paragraph, the authors could have two interesting conclusions with the observation of enlarged endosomes in aged neurons and the increase of APP localisation in these enlarged endosomes. They have not discussed the potential reasons for enlarged endosomes

Line 217 "Ab production requires APP endocytosis"
This statement is not true. Abeta production can occur along the anterograde trafficking pathway, for example APP processing in the Golgi apparatus. Also APP can be transported from the Golgi directly to the endosomes without transport to the cell surface. The authors need to consider this option.

Line 301 "Overall, this data indicates that APP endocytosis is clathrin-, dynamin-, and actin-dependent in both mature and aged neurons ...". This conclusion is premature. In the supp data, Pitstop 2 doesn't affect APP endocytosis in 21DIV neurons. The difference in the effect of Pitstop 2 between 21DIV and 28DIV neurons need to be discussed in the main results section. Do APP endocytosis mechanisms change over time?

Line 321 and below "There are two endocytic adaptors genetically linked to Alzheimer's disease, CD2AP, and CALM..."
We would suggest removing the data about CD2AP and CALM as they are not really informative.

Line 354 "Importantly, the number of synapses increased significantly by 42% and 52% when we treated with DAPT or BACE inhibitor IV, respectively (Fig.7C, F, G, J, M, N)." The experiment may suggest a protection from loss rather than an increase. For example, the authors could indicate a stabilisation of the number of synapses between 21DIV and 28DIV or an inhibition of the decrease of the numbers of synapses instead of an increase of synapses...

Line 367 "fAD" needs to be defined.

In regard to the cell images in the figures, drawing the borders of the neurons would help to clarify the figures as some staining seems to be outside of the neurons/dendrites (ie on Fig.1 G-H, Fig.3 D/E/F/G). The means on the graphs are not always clearly visible and needs to be put in front (ie

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on Fig. 3 X/Y/Z/AA). It would help the reader if the colour code and order of the drugs in the figures is consistent between figures (Fig 6. and Fig.S3.); at the moment the order and colouring differ. Fig.2H-I panels are not commented in the text. They should be removed or discussed. In the model presented, endocytosis is enhanced due to increased levels of clathrin, by so why is TfR endocytosis decreased? This appears to be a contradiction.

Significance

In this study, Burrinha et al. report an increase of APP endocytosis with aging in aged neurons and aged brains. The strength of their experimental design is the inclusion of in vitro and in vivo experiments with a range of different cellular and molecular techniques together with very good quantification. However, their conclusions will need to be confirmed by inclusion of additional experiments and a number of key controls which are currently lacking. Reproducibility is also a concern given the methods used to culture neurons over extended periods. The relationship between APP processing, Abeta production, aging of neurons, and Alzheimer's disease is an important field that requires more research in order to understand the initiation and progression of Alzheimer's disease so that new treatments can be developed for neurodegenerative diseases.

Reviewer field of expertise: cell biology, neurobiology, intracellular trafficking of BACE1 and APP, confocal microscopy, neuron cell culture

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Author response to reviewers' comments

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

The authors used in vitro "aged" primary neurons (DIV28) and report increased levels of lipofuscin staining and intracellular mouse Abeta as well as a decline in synaptic density. Interestingly, they also showed increased APP endocytosis, whereas other CME cargoes, such as TfR, were not affected. Notably, inhibition of Abeta production by a BACE inhibitor attenuated synaptic decline. One possible mechanism highlighted by the authors, causing the increased intracellular Abeta might be elevated Abeta endocytosis while in vitro aging.

1) The authors show increased intracellular Abeta immunoreactivity, as indicated by anti-Aβ42 C-terminal specific antibody (12F4) (Figure 1). Specificity of the staining should be validated in APP KO or knock down primary neurons.

We thank the reviewer for raising an important point. We have controlled for 12F4 specificity in our previous work published in EMBO Reports in 2017 (Ubelmann et al., 2017). We did some critical controls, such as quantifying the decrease in Aβ42 immunofluorescence upon DAPT and BACE inhibitor treatment (Fig.EV1). Moreover, we used a second antibody against Aβ42 (H31L21) with similar results to 12F4. At Embo reports, one of the reviewers asked for the antibody specificity validation in APP KO neurons. We use the immunofluorescence protocol for Aβ42 developed in Gunnar Gouras lab (Almeida et al., 2006; Tampellini et al., 2007; Takahashi et al., 2004). Gouras has unpublished data showing the lack of staining in APP KO neurons with the 12F4 antibody that we included in the review process, but that is available in the peer review PDF (https://www.embopress.org/action/downloadSupplement?doi=10.15252/embr.201642738&file=embr201642738.reviewer_comments.pdf). Since we do not have the APP KO mice, these would be difficult, time-consuming, and costly experiments to repeat.

Furthermore, the authors should provide co-stainings with APP to clarify if the anti-Aβ42 antibody might also detect full length APP or CTFs. This could also be shown by expression of different murine APP mutants.This control for specific intracellular Abeta detection is essential, as the authors also observed increased APP levels in "aged" primary neurons (Figure 2 and 3).
We understand the reviewer's concern. We have imaged APP and Aβ42 extensively, and their staining is quite different regarding their cellular distribution. However, we think that the increase in APP in

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neurites of aged neurons, given its overall unchanged levels, indicates that APP trafficking is altered in aged neurons. In this paper, we started to unveil these aging-dependent alterations in APP trafficking, but there is still a lot to be done. We will prepare an image with co-staining with APP and Aβ42 to illustrate our observations.

2) The authors observed increased levels of EEA1 staining in "aged" PN (Figure 3) and postulate increased levels of APP in EEA1 positive endosomes. Firstly, colocalization should be validated in a quantitative manner. Secondly, the authors should provide a control with another membrane protein, to ensure that APP is indeed increasing in early endosomes.

We are unsure we understood the reviewer's concern about the validation of APP colocalization with early endosomes. To clarify we postulated that APP localization increased at early endosomes with aging based on three quantitative experiments:
1. We measured the colocalization of cellular APP quantitatively with EEA1 in "aged" neurons in vitro (Early endosomal APP, fig.3D-H). Because cellular staining of APP comprises APP at the plasma membrane, ER, Golgi, TGN, secretory vesicles, early and late endosomes, lysosomes, etc., the APP fraction signal that colocalizes with EEA1 is relatively small. But notably, it increased, especially in neurites of "aged" neurons by 64%.
2. We measured the colocalization of cellular APP quantitatively with EEA1 in "aged" neurons in vivo (Early endosomal APP, fig.3R-W, and Z). The colocalization of APP in early endosomes increased 88% in the aged brain.
3. We measured quantitatively APP endocytosis after 10 min, time at which APP is in early endocytic compartments as also observed in previous work (Ubelmann et al., 2017).
Regarding another control for endocytosis. We agree with the reviewer that this is an important point. Indeed, we aimed to control for another membrane protein by following the endocytosis of the transferrin receptor. Unfortunately, transferrin endocytosis changed with aging in opposite ways to APP. In turn, we present data using FM1-43 pulsed for 10 min, time at which it has been described to label early endosomes (Fomina et al., 2003). FM1-43 puncta increased with aging like APP and in contrast with transferrin. The increase in endocytosis with aging may be the more general phenotype. Furthermore, the literature (Blanpied et al., 2003; Alsaqati et al., 2018) supports our interpretation. We will try to identify another transmembrane marker.

3) Biotinylation assays suggest strongly increased APP endocytosis in "aged" PN. The authors tried to validate these results with an APP antibody uptake assay. For this purpose they used 22C11. To our experience, the 22C11 works very well in WB analysis, but not in IC. Therefore, I strongly recommend to control either 22C11 immunoreactivity on APP KO cells or to use another antibody for the uptake assay.

We agree with the reviewer that 22C11 may not be the best antibody in classical IC with fixed neurons. In contrast, we use 22C11 with live neurons, and the result is relatively consistent. The lack of fixation may expose the APP epitope in its native configuration and enable binding to 22C11.
We and others have used this antibody to follow APP endocytosis (Jung et al., 1996; Sun et al., 2019; Ubelmann et al., 2017). Initially, we also used P2-1, another anti-APP extracellular N-terminus with similar results in HeLa cells. But because this antibody does not recognize mouse APP, we decided to use 22C11. We have shown that N-terminal antibodies bind to the Abeta domain interfere with APP processing (Tampellini et al., 2007), precluding their use in this study.
When setting up this method, we used 9 DIV neurons, for which we could not detect any 22C11 positive endosomes. We had to overexpress APP to detect enough 22C11 in endosomes, indicating the need for high expression of APP for 22C11 binding. That is what we show in figure 3A-C and suggest in the paper (line 238-242). Only in mature neurons, there is enough APP at the plasma membrane to follow its endocytosis without overexpression. We agree that APP KO neurons would be a robust control, but unfortunately, we do not have easy and fast access to APP KO mice. If necessary, we can try to KD APP instead.

4) For control, the authors showed very interestingly that Transferrin-receptor endocytosis was not increased in "aged" PN. For better direct comparison, the experiment with TfR should be carried out also with the bulk surface protein internalization assay, used for APP.

We agree with the reviewer, and we have tried to do this experiment. By unclear technical reasons,

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we failed to detect biotinylated transferrin receptor endocytosis. Notably, the result using fluorescently labeled transferrin endocytosis was so dramatic that it left us quite convinced of the phenotype.

Moreover, the authors should provide experimental evidence for this different behavior, as e.g. testing for different recycling rates of APP and TfR in "aged" PN.

We thank the reviewer for this suggestion. The recycling of TfR in "aged" PN may be under the detection limit given that aged neurons endocytose very little Tf.
We will measure APP recycling in aged primary neurons.

5) The authors show in addition that inhibition of APP processing attenuates synaptic decline in "aged" PN. This is a pure correlative observation and should be experimentally validated by e.g. usage of APP KO PN or an APP knockdown approach combined with BACE inhibitor treatment.

We agree with the reviewer that our interpretation is based on a correlation between the inhibition of BACE1 and gamma-secretase activity (both block Aβ42 accumulation) and synaptic decline, at the expense of different precursors. We can only suggest that the decrease of synapses is attenuated by inhibition of Aβ42 production. Unfortunately, because APP full length has direct effects on synapses (Fogel et al., 2014; Martinsson et al., 2019; Priller et al., 2006; Lee et al., 2010; Weyer et al., 2011; Wang et al., 2017, 2009; Spires et al., 2005; Hoe et al., 2009; Müller et al., 1994) knockdown experiments would be difficult to interpret and to provide more direct proof of Aβ42 role at synapses in aged neurons.
Reviewer #1 (Significance (Required)):

The increased APP endocytosis in "aged" PN is a very interesting finding and has important impact on understanding of Abeta mediated neuronal cytotoxicity.

Researchers interested in how Abeta might cause synaptic loss and neuronal loss are potentially interested in this study. Furthermore, the study highlights the APP endocytosis mechanism as a potential target for future therapeutic approaches, which might be interesting for those, working on endocytosis mechanisms.
My field of expertise is on Amypoid precursor protein function and trafficking.

We thank the reviewer for the positive feedback and encouragement!

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

This is an interesting article from the group that has been active in the field of membrane trafficking with a focus on Alzheimer disease. The paper by Burrinha et al. identifies the upregulation of APP endocytosis as a driving force of aging-dependent synapse loss and as a novel target to prevent late-onset AD. Using their own established model of aged mouse cultured neurons and material of aged mouse brains, the authors provide evidence that accelerated APP endocytosis potentiates APP processing and Abeta production. They also provide a mechanism for the upregulation of APP endocytosis, identifying clathrin, dynamin, and F-actin as key players. Techniques used in the study include ICC/IHC, WB, endocytosis, and trafficking assays.

Key conclusions are:

1. Intracellular Abeta42 is increased with aging
2. APP processing and early endosomes increase with aging (iii)APP endosomal localization and its endocytosis increase aging
(iv)Upregulation of APP endocytosis with aging requires clathrin and actin (v)Age-dependent synapse is in part due to Abeta production

I found the concept interesting and the paper well presented. On the other hand, the conclusions driven from the results might be a bit preliminary and might require several additional controls.

We thank the reviewer for the positive feedback. We will perform additional experiments to solidify

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our conclusions.

Importantly, we would like to refer that we started this project in 2014, and took us six years to gather the data presented in this manuscript, mostly because aging takes time, but also because we use stringent criteria in all experiments to ensure that the neurons analyzed were aged and healthy.

Major comments:

My main concern is about the aging in-vitro model (see below). Additionally, I find conclusions ii), iii) and iv) (see my list above) are not convincing and preliminary.

1. Conclusion i):

I am not certain if embryonically isolated neurons are a good model for the study authors aimed to do. These neurons are usually expressing the doublecortin throughout their life in culture, which would say that they are stuck in a "new-born state". Additional control for doublecortin levels, as well as some additional markers of cellular senescence, should be used to verify the aging model.

We agree with the reviewer that this model of neuronal aging has limitations.
We did not develop the model we use. In the lab, we set-up a model of neuronal aging established and used by several groups, including Carlos Dotti, that established the primary cultures of neurons with Gary Banker and Bart de Strooper, a leader in the field of Alzheimer's disease research.
Some references in the literature:
(Kuroda et al., 1995; Lesuisse and Martin, 2002; Aksenova et al., 1999; Bertrand et al., 2011; Porter et al., 1997; Dong et al., 2011; Sapoznik et al., 2006; Kim, 1983; Brewer, 1997; Blanpied et al., 2003; Bigagli et al., 2016; Guix et al., 2012; Martín-Segura et al., 2019; Martin et al., 2010; Palomer et al., 2016; Martin et al., 2014, 2008, 2011; Simons et al., 1998; Li et al., 2004).
Several markers have been used to characterize these as "aged neurons," including Lipofuscin accumulation, protein carbonyl formation (protein oxidation), lipid alterations, and increased reactive oxygen species.
Because the "aged" neurons recapitulate essential aspects of the aging brain like the accumulation of lipofuscin in lysosomes, synapse decline, and beta-amyloid accumulation, we used these "aged" neurons to investigate changes in the mechanisms of neuronal cell biology, and we also confirmed, when possible, such changes in the aged mouse brain.
Nevertheless, we will strengthen the model by measuring doublecortin and senescence-associated ß-galactosidase in aged primary neurons.

2. Conclusion ii):

a.I am very confused with the data in Fig. 2A. Why the described CTFs in the text are not evident in the blot or am I missing something? The last marker is 15KD, while beta-CTFs are 12-14kD. It would be good to know what is quantified in Fig. 2B? Also, I suggest quantifying the APP processing into ß-CTFs (12-14kD) and η-CTF separately, both for cultured neurons and brains.

We thank the reviewer for this suggestion. To clarify, we quantified the ratio of all CTFs (α-, ß- and η- CTF) over APP because we can only identify the CTFs based on their approximate size, which is not very exact given that the electrophoretic mobility is not a linear fit when plotted against molecular weight (kDa). Another critical issue is that there are no commercially available antibodies to identify the mouse APP's different fragments.
Moreover, because BACE1 cleaves between the 1st and the 11th aminoacid of the amyloid sequence, originates C99 and C89, respectively. The C89 is virtually impossible to distinguish from C83 (α-CTF). We will try to resolve the CTFs better and to quantify them separately. Also, following on reviewer three suggestions, we will attempt to better identify each CTF band by treating primary neurons with DAPT alone, to accumulate all CTFs increasing their detection, and with DAPT plus BACE1 inhibitor to detect only α-CTF/C83 changes with aging.

b., I agree that the data indicate a solid increase in EEA1 protein levels. But can one really draw a conclusion about the early endosome increase only from the EEA1 data? Some additional experiments might be required: i) the mRNA analysis of EEA1, ii) additional early endosomal markers (i.e. RAB5

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levels), and iii) the authors should consider EM or super-resolution techniques (the EEA1 puncta in brain sections are in fact too big to be early endosomes). Alternatively, the claim for increased early endosomes should be re-evaluated (i.e. can be changed to EEA1-positive early endosomes instead).

i) the mRNA analysis of EEA1
We found an increase in EEA1 protein levels by WB in aged primary neurons and aged brains. Moreover, the analysis of each single EEA1-positive vesicle size and intensity increased in aged primary neurons and aged brains.
We agree that this finding needs further investigation. Not only investigating changes in EEA1 mRNA but also in EAA1 protein synthesis and degradation. As far as we could find, there is a fundamental lack of knowledge on EEA1 proteostasis or gene expression regulation. We consider this to be super interesting, and we will investigate this in-depth in a new line of investigation to be followed up subsequently.
ii) additional early endosomal markers (i.e. RAB5 levels),
We agree that the study would benefit from additional markers of early endosomes. We want to point out that clathrin, CALM, and CD2AP, which are markers of the early endocytosis machinery and are also up-regulated with aging. Nevertheless, we will investigate how other(s) markers of early endosomes change with aging.
iii) the authors should consider EM or super-resolution techniques (the EEA1 puncta in brain sections are in fact too big to be early endosomes).
Regarding the early endosomes size, we agree that there are EEA1 endosomes, or endosomes positive for EEA1, in the brain more prominent than the average size distribution of early endosomes. We obtained these measurements by analyzing the IHC images obtained by deconvolution of epifluorescence microscopy. We will now analyze more recent images acquired with the new Zeiss Airyscan 2.0 that reaches a 100 nm resolution to measure endosomes size more accurately.

c. The separation between cell body and dendrites, which authors make in the article is very confusing to follow since not all data are in fact always consistent, e.g. early endosomes increase more in dendrites with aging, but there is no difference in endocytosed APP and etc. I am not even sure why the authors need the cell body for their story. I would stay with neurites and synapses.

Thanks for the suggestion. Our finding that Aß42 accumulates more in neurites than in cell bodies prompt us to separate the cell body from the neurites. We agree that our results are more relevant in neurites and synapses. We will thus simplify the figures and the text accordingly.

3. Conclusion iii):

a. APP surface levels are increased in aged neurons. Could this be that increased APP endocytosis is due to altered recycling of APP back plasma membrane (more APP is endocytosed because the APP is faster recycled)? A recycling assay should address this issue.

We thank the reviewer for the alternative explanation for the increase in APP endocytosis. We will now test this hypothesis by measuring APP recycling.

b.I am puzzled with a low amount of RAB5-positive endosomes containing the endocytosed APP (Fig. S2K,N). Can the rest be in recycling endosomes? ICC with endocytosed APP/RAB11 should be performed here.

We were also puzzled with the low number of Rab5-positive APP endosomes. Our experience is that Rab5 antibodies are not very good. We tested more than one antibody against Rab5 in ICC and chose the one that gave the best results. We could only segment the Rab5 signal at 28DIV to quantify colocalization with endocytosed APP, probably because there is more rab5 per endosomes at 28DIV than at 21 DIV, thus increasing the antibody binding and improving the ICC. We are now going to quantify this to answer your point b.
We use automated segmentation and colocalization analysis with ICY bioimage analysis software to not introduce personal bias. However, when the signal is not clean, it underestimates the colocalization. We will try to measure the amount of Rab5 per APP endosomes to improve the analysis. We will perform ICC of endocytosed APP/RAB11 as suggested to clarify if part of the endocytosed APP is in recycling endosomes.

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4. Conclusion iv):

I found this conclusion is less convincing from all since all the data are generated using clathrin and dynamin inhibitors only. Pitstop2 and dynasore have multiple unspecific side effects (i.e. please see the Park et al., 2013, J. Cell Sci). To draw the conclusion that APP endocytosis requires clathrin and dynamin based on that is far too preliminary. I suggest either to down- tone throughout the paper (e.g. APP endocytosis is Pitsop-2 and Dynasore-sensitive) or perform the functional analysis using shRNAs or CRISPR/Cas.

We do provide additional data supporting that APP endocytosis increases with aging due to increased clathrin and actin-mediated endocytosis (Fig, 6 H-Z), namely:

i. Clathrin levels are up-regulated in aged neurons and aged brains;
ii. The density of clathrin puncta (sites of endocytic pits formation) increases in aged neurons;
iii. APP is more present at clathrin assemblies in aged neurons.
iv. F-actin is most often present in APP endosomes in aged neurons.
v. CALM, part of the machinery involved in the clathrin-mediated endocytosis of APP, and, CD2AP, an actin and endocytosis regulator, are most often present in APP endosomes in aged neurons.

Nevertheless, we agree with the reviewer that functional analysis with shRNA or Crispr/Cas9 should complement the endocytosis inhibitors, whenever technically possible. However, primary neurons are typically challenging to manipulate. To our knowledge, knockdown has not been done in long-term cultures of primary neurons. We have tried to do Crispr/Cas in our lab, and we were thus far successful in n2a, HEK, fibroblasts cells but not in primary neurons. Therefore, we propose to down-tone our interpretations throughout the paper.

5. Conclusion v):

Fig. 7 is loosely attached to the mainline of the manuscript. I suggest to either include this as a part of Fig.1 to illustrate an additional control for an in-vitro model or perform additional experiments showing the effect of APP endocytosis on synapse rescue in aged neurons (should be very straight-forward since authors can use the drugs mentioned above).

We agree with the reviewer. We will either provide new data on synapse rescue or include it as part of Fig.1.

Generally, the data and the methods presented in a reproducible way, and all experiments adequately replicated and statistical analysis is adequate.

Minor:

1. Page 7, line 134- LAMP1 is not a pure lysosomal maker and will label MVBs and late endosomes as well.

Since MVBs can be considered as late-endosomes, we have now defined LAMP1 as late-endosomes/lysosomes marker.

2. FM1-43 is not an endosomal marker, but a lipophilic dye, which will insert into all membranes.

We agree that FM1-43 is not an endosomal marker by excellence. But it has been used as a reporter for endocytosis, as well as for recycling depending on the time of the pulse, chase, and washing. It is cell impermeable, binding mostly to the plasma membrane. It will be enriched in endosomes by all kinds of endocytic routes in a time-dependent manner, so it is considered a non-specific marker of bulk endocytosis.
We have now a better description in the methods (line 689) and included a reference of a study of FM1-43 endocytosis using fluorescence imaging followed by electron microscopy, that identifies early endosomes as an endocytic compartment labeled by FM1-43 (Fomina, A. F., Deerinck, T. J., Ellisman,

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M. H., & Cahalan, M. D. (2003). Regulation of membrane trafficking and subcellular organization of endocytic compartments revealed with FM1-43 in resting and activated human T cells. *Experimental cell research*, *291*(1), 150–166. https://doi.org/10.1016/s0014-4827(03)00372-0).

3. Please indicate whether the heavy or light clathrin chain was analyzed.

We analyzed the heavy clathrin chain. This is now indicated in line 594.

4. Discussion is too long and a bit diluted. I suggest shortening it by 1-2 pages.

We will shorten the discussion by 1-2 pages.

5. This is correct that CALM can function as an adaptor for endocytosis of APP. Additionally, CALM can also regulate APP autophagic degradation (Tian et al. 2013). It would be good if the authors can re-consider their data according to this finding.

We thank the reviewer for triggering us to look at this possibility. We read carefully the Tian et.al 2013, and the data demonstrates that starvation-induced autophagy (n2a cells) increases CALM interaction with LC3 and LC3 with AP2. Although the authors present data showing that AP2 knockdown leads to the accumulation of APP CTFs, we could not find data showing CALM requirement. We found another paper supporting CALM's role in starvation-induced autophagy (HeLa cells) (Moreau et al. 2014). In this paper, CALM knockdown increases autophagy. The mechanism proposed is indirect via the endocytosis of VAMP2, apparently crucial for the fusion of autophagosomes with lysosomes. No link was established with APP degradation. Although possible, it remains an open question if CALM has a direct role in APP autophagic degradation.
Given that most CALM data point to a function in APP endocytosis, we found that APP degradation was not significantly altered in "aged" neurons (Fig.4G-H), we interpreted the data accordingly. Nevertheless, we agree that we need to investigate further the role of CALM in APP trafficking with aging. We will pursue this second line of research in the future and discuss the putative CALM role in APP autophagy in the discussion.

Reviewer #2 (Significance (Required)):

The role of membrane trafficking in the pathophysiology of AD is currently under intense investigation. From that point of view, the study by Burrinha et al. is timely and of high significance for readers interested in cellular and molecular mechanisms of neurodegeneration.

Field of expertise: membrane trafficking, autophagy, endocytosis, neurodegeneration.

We thank the reviewer for the positive and constructive feedback and for recognizing the importance of our work!

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

SUMMARY

Burrinha et al. is a worthwhile study with potentially interesting findings on APP endocytosis in vitro and in vivo. The authors have developed systems to compare the aging of neurons in culture, derived from the cortex of E16 mice, as well as brains from young and old adult mice. They used two systems to compare APP endocytosis and processing in the neuronal samples over the time period and measured the level of A as a link with Alzheimer disease. This study presents a significant body work with number of well-organized figures and extensive quantification and controls. However, many of the hypothesis/conclusions by the authors are not fully supported by the data. Also, and most importantly, a number of key controls are missing to support authors' conclusions. The balance of the discussion and conclusions need to be improved. Overall, if the author can improve the robustness of the data the manuscript could be a very useful contribution.

MAJOR COMMENTS

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Some of the key experiments need better controls to support the conclusion proposed by the authors (see below).

In Fig1, Abeta level after DAPT treatment needs to be included in the IF to demonstrate the specificity of staining by the Ab to Abeta40 or to Abeta42. The level of A secreted needs to be quantified as well to determine the relationship between intracellular and secreted pools of Abeta.

We thank the reviewer for raising an important point. Regarding the Aß specificity, we have controlled for 12F4 specificity in our previous work published in EMBO Reports in 2017 (Ubelmann et al., 2017), where we included essential controls, such as quantifying the decrease in Aß42 immunofluorescence upon DAPT and BACE inhibitor treatment (Fig.EV1). Moreover, we used a second antibody against Aß42 (H31L21) with similar results to 12F4.
Regarding Abeta40, we do have data on Abeta40. We observe that it does not colocalize significantly with Abeta42, and does not seem to change as much with aging, but we will quantify this data and include it in the paper.
Regarding secreted Aß, its aging changes have been previously reported to be also increased in aged neurons in similar experimental conditions (Guix et al., 2012). We can repeat these measurements in our experimental conditions.

In Fig. 2A, the quality of the western blot is poor and cannot be interpreted. There is no internal standard for normalization of loads between samples. The identity of the bands seems very speculative given the sizes of the bands do not correspond with their expected Mr and these need to be confirmed by different treatments. To increase the resolution for the lower bands, a different gel percentage can be used. As C99 bands are not identified specifically, conclusions on the A pathway cannot be made from this blot. Moreover, APP signal is saturated in Fig. 2B and makes the quantification inaccurate.

We will improve the quality of the blots and re-do the quantification.

In Fig.2E-F, the level of EEA1 in 14DIV is missing and it would be relevant for a complete picture to show the data on the basal level of EEA1 (is the level stabilised between 14DIV and 21DIV or a continual increase?).

This point was also raised by reviewer 2. We will analyze the levels of EEA1 at 14DIV in parallel with 21 and 28 DIV.

In Fig.3 it would be ideal to include APP staining in APP KO neurons as a control of APP specificity as some antibodies to APP can give high background. The specificity of staining needs to be demonstrated as the contribution of background staining may vary across the aged samples.

We agree that APP KO neurons would be a robust control, but unfortunately, we do not have easy and fast access to APP KO mice. If necessary, we can try to KD APP instead, although we are afraid that this may take time since we have not succeeded in doing knockdowns at 28 DIV.

This issue also applies to the labelling of endogenous APP at the cell surface (Fig. 4I-L), the specificity of this labelling needs to be established, particularly as only low levels of APP are considered to be at the PM.

Reviewer 1 also raised this point.
We agree with the reviewer that 22C11 may not be the best antibody in classical IC with fixed neurons. In contrast, we use 22C11 with live neurons, and the result is quite consistent. The neurons are not fixed and may expose the APP epitope in its native configuration and enable binding to 22C11.
We and others have used this antibody to follow APP endocytosis (Jung et al., 1996; Sun et al., 2019; Ubelmann et al., 2017). Initially, we also used P2-1, another anti-APP extracellular N-terminus with similar results in HeLa cells. But because this antibody does not recognize mouse APP, we decided to use 22C11. We have shown that N-terminal antibodies that bind to the Abeta domain interfere with APP processing (Tampellini et al., 2007), precluding their use in this study.
When setting up this method, we used 9 DIV neurons, for which we could not detect any 22C11 positive endosomes. We had to overexpress APP in order to detect enough 22C11 in endosomes. That

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is what we show in figure 3A-C and indicate in the paper (line 238-242). Only in mature neurons, there is enough APP at the plasma membrane to follow its endocytosis without overexpression. We will try to provide extra controls to address the reviewer's point.

Fig. 4A-E is missing a key control: a surface label and cleavage without a chase eg on ice, in order to determine the background for each DIV sample. This is a critical control to demonstrate that the labelling in each case is restricted to the cell surface. This control is also essential to be able to compare samples between 21DIV and 28DIV.
We agree with the reviewer that doing the labeling on ice is the best to determine surface labeling. We have done this successfully before with younger primary neurons (Ubelmann, 2017; Almeida et al. JNeurosci 2006) or other cell types (Almeida et al. NCB 2011). But by some unclear reason, the level of biotinylated proteins of mature neurons or aged neurons on ice was always inferior to the level observed upon incubation at 37C. We hypothesized that a reduction in biotin accessibility to plasma membrane proteins in mature neurons might be reduced. Instead, we observed that after a 10 min pulse at 37C, surface APP was consistently well biotinylated, and the amount of endocytosed APP (after stripping) represented a small fraction of the total APP at the surface (about 4 % at 21 DIV and 16 % at 28 DIV). Thus, we considered that we could still conclude APP endocytosis and APP surface levels using these experimental conditions. We have done ICC experiments with surface labeling of APP using 22C11. We propose to quantify the surface APP changes with aging.

To complete Fig.6, effects of the drugs on transferrin endocytosis in aged neurons as well (here only 21DIV and not 28DIV) and for the styryl dye FM1-43 need to be check.
We agree with the reviewer that the effect of the drugs on FM1-43 endocytosis is important to complete Fig.6. We disagree that analyzing the impact of the drugs on transferrin endocytosis at 28DIV would be informative. Because transferrin endocytosis is greatly reduced by aging (See fig.5 J-N), and as such, the effect of drugs in reducing transferrin endocytosis in aged neurons will probably be below detection. We will assess if FM1-43 endocytosis is inhibited with dynasore, pitstop2, CPZ, and actin drugs further to validate the effect of the drugs on bulk endocytosis.

In Fig7. the authors did not show if the BACE1 inhibitor and DAPT have actually blocked APP processing in their experiments. They have relied on the reported action of these drugs but it is essential that the level of inhibition in these experiments is documented. In the absence of information about APP processing conclusions cannot be drawn on the experiments in Fig 7.
Indeed, these are established inhibitors of APP processing. In our hands, we have observed that DAPT and BACE1 inhibitor block APP processing and reduce intracellular Abeta (Ubelmann 2017, Takahashi, 2004). Nevertheless, we can perform these experiments again and include the results in the paper.

Importantly, there is contradictory information in the different figures and quantification. In Fig.1, quantification of lipofuscin is quite similar in the neurites at 21DIV and 28DIV but looks very different in the images.
If we understand correctly, the reviewer refers to the difference in the number of auto-fluorescent granules present in aged neurites. We quantified the number of these in cell bodies and neurites, but the results are in supplementary figure 1. We defined lipofuscin as the granules present inside lysosomes. Thus we quantified the number of granules positive for lamp1 in the figure. The auto-fluorescent granules outside lysosomes could also be damaged mitochondria and other auto-fluorescent material. To clarify, we postulated that lipofuscin increased in the cell body and not in the neurites of aged neurons based on the detection of autofluorescent granules in lysosomes (LAMP1 positive organelles) in cell bodies of aged neurons.

In Fig. 3A-B-C, the images show more APP in the cell body at 21DIV compared to 28DIV whereas the quantification is showing a similar level in the cell body. Moreover, Fig. 2 shows no difference in total APP levels between 21DIV and 28DIV yet Fig. 3C indicates that the neurite APP is higher at 28DIV.
We agree with the reviewer that the images show more APP in the cell body at 21DIV than 28DIV. We will improve the figure to reflect the quantification better. By WB, we don't detect changes in APP total levels, but when we analyze APP's subcellular distribution, we found that it changes, increasing in the aged neurites. We interpret these subcellular changes as a result of trafficking alterations with aging. We believe that the impact of aging in other trafficking pathways of APP needs to be investigated for us to grasp why APP is increasing in neurites altogether. This result opens a new line of investigation for future studies.

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One of the most important concerns in the study is the fact that the neuronal medium is not changed during the 28 days of neuron culture (line 576 - "Neurons maintained up to 28 days without changing the media"). This is a concern for neuron viability, and dendrites/synapses development. It may lead to an accumulation of APP and A in the supernatant that will affect the results of the entire study. This is a really critical point and will need to be address by the authors. Notably, in Fig. 5 the reduction of the endocytosis of TfR in 28DIV is very substantial and might suggests that cells at day 28 are not healthy.

We agree that this model of neuronal aging has limitations. We set-up in the lab a model of neuronal aging established and used by several groups. Some references in the literature:
(Kuroda et al., 1995; Lesuisse and Martin, 2002; Aksenova et al., 1999; Bertrand et al., 2011; Porter et al., 1997; Dong et al., 2011; Sapoznik et al., 2006; Kim, 1983; Brewer, 1997; Blanpied et al., 2003; Bigagli et al., 2016; Guix et al., 2012; Martín-Segura et al., 2019; Martin et al., 2010; Palomer et al., 2016; Martin et al., 2014, 2008, 2011; Simons et al., 1998; Li et al., 2004).

Concerning neuron viability, in fig.1, we show that MAP2 and Tubulin were not compromised in "aged neurons" since the neurons' morphology is of viable neurons without the typical bead-like pattern of degenerating cells. Moreover, neither map2 neither synaptic proteins total were reduced in aged neurons, which would happen if the neurons would be dying. However, we do see the reduction of tfR in 28DIV, but we think that tfR reduction does not suggest that cells are not healthy. Instead, we found that this may be a specific alteration of TfR with aging, as previously reported (Lu et al., 2017).

The authors did not mention the potential contribution of anterograde trafficking APP and A production along biosynthetic pathway and their statements (line 185) ignore locations other the endosomes. APP processing occurs in endosomes but also in the secretory pathway and A has been shown to be produced in the Golgi apparatus as well. This issue needs to be discussed and integrated with their findings and their hypothesis (around line 416). Also, the paper argues for enhanced APP processing, however a reduction in secretion of Abeta would also be consistent with much of the data.

We agree with the reviewer, and we will mention the potential contribution of anterograde trafficking APP and Aß production along the biosynthetic pathway and will discuss and integrate the possible changes in Aß production in other cellular sites. Regarding secreted Aß, its aging changes have been previously reported to be increased in aged neurons using similar experimental conditions (Guix et al., 2012). However, we can repeat these measurements in our experimental conditions.

•Should the authors qualify some of their claims as preliminary or speculative, or remove them altogether?

•Would additional experiments be essential to support the claims of the paper? Request additional experiments only where necessary for the paper as it is, and do not ask authors to open new lines of experimentation.

The authors are able to observe APP CTFs fragments in 21DIV and 28DIV neurons by western blot (Fig. 2 A-B). However, CTFs in the 14DIV sample are not detected which is surprising. We would suggest treating neurons with different drugs (ie: DAPT/C3...) to inhibit APP processing and accumulate APP CTFs to be able to observe APP CTFs even at an early neuronal stage. In addition, the treatment with drugs will assist in the identification of each of the potential CTF bands. A different gel percentage should be used.

We thank the reviewer for the comment and suggestion. We do detect APP CTFs at 14DIV, but the higher CTFs are difficult to perceive because of their relatively low amount compared to 28DIV. As suggested, we will try to resolve the CTFs better and to quantify them separately. We will also attempt to better identify each CTF band, by treating primary neurons with DAPT alone, to accumulate all CTFs increasing their detection, and with DAPT plus BACE1 inhibitor to detect only α-CTF/C83 changes with aging.

The authors have assessed the number of endosomes containing APP and observed an increased in early endosomes size. We would suggest staining the early endosomes with another marker (ie Rab5) for confirmation.

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Reviewer 2 also raised this point. We agree with both reviewers that the study would benefit from an additional marker to measure early endosome size. Our experience is that the Rab5 antibodies are not very good, and we tested more than one antibody against Rab5 in ICC. We will try to improve the ICC of RAB5 and evaluate changes in size with aging.

It would be very informative to determine the distribution of APP across the different organelles within a cell as well. Lysosomes (numbers, shape and the localisation of APP in the lysosomes) should be included as APP is transported down the endolysosomal pathway (Toh et al. 2016). The authors have already performed some LAMP1 staining in Fig.1 and an expansion to include APP levels in LAMP1 compartment would give a better picture of any changes in organelles in their aging models.
We agree that we need to investigate further the distribution of APP across the different organelles within the cell. Since our "aged" primary neurons did not show alterations in APP degradation, we did not follow further this line of research. We do have data of Lamp1 and APP that we can analyze to determine APP localization in lysosomes. Regarding changes in the lysosome with aging, we agree that it is a fascinating point that we will pursue in the lab in the future.

The authors have analysed APP endocytosis and A production. However, A can also be release in the supernatant. As the neuronal media is not changed during the 28 days of neuronal culture, it is possible that the accumulated A in the culture medium will affect the aging of neurons and intracellular A level.

We agree with the reviewer. We will analyze the secreted Aß in neuronal media.

To compliment the APP plasma membrane staining (with 22c11 Ab) at different stages (14DIV, 21DIV and 28DIV) (Fig. 4) it would be informative to quantify the level of APP at the plasma membrane and assess whether there are change in APP at the PM during aging.

We agree with the reviewer that the level of APP at the plasma membrane would be relevant. We have the data need to analyze it further and assess changes at different stages.

In the last part of the study, the authors investigated the synapse plasticity/loss using two markers PSD-95 and vGlut1 (Fig.7). EM will give direct evidence in the change in size/numbers of the synapses comparing mature and aged neurons. EM images will give a much higher impact to the study. However, this suggestion may be challenging if the authors do not have ready access to an EM facility.

We agree that EM would give direct evidence for changes in synapses. However, we do not have ready access to an EM facility.

•Are the data and the methods presented in such a way that they can be reproduced?
•Are the experiments adequately replicated and statistical analysis adequate?

In Fig.2 A-B, the quantification is not convincing as the APP bands are saturated. We would suggest decreasing the loading and using DAPT treatment to reveal the total APP CTFs/C99 produced in neurons. Using this technique, C99 and others CTFs are observable in young neurons (7DIV, cf Tan, Fourriere et al. 2019).
We agree with the reviewer about APP bands' saturation. We will improve the quality of the blots. We will also perform western blot analysis of APP CTFs/C99 according to reviewer suggestions.

Quantification of APP in Fig.2C/D depends on the purity of the neuronal prep which is not stated in the paper.

We will state the purity of neuronal preparations in the paper. We only use neuronal preps that present a minimal presence of glia in our experiments.

APP staining in cell body is saturated in Fig. S2C/D which renders quantitation meaningless.

We agree that the contrast adjustment for displaying the APP staining in the cell body leads to signal saturation in the figure. But it does not affect the quantifications since these are done in images

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without any contrast adjustments and acquired without saturated pixels.

In Fig. 4C, endocytosed APP should be represented in comparison to APP level at the plasma membrane at the beginning of the experiment as this level is increased in aged neurons (49% more).

We chose to compare endocytosis separately because since the endocytosis increases by about 700%, we considered that this increase in endocytosis could not be masked by the 49% increase in the surface APP.

It is not clear if the quantitation of IF was performed on optical sections or 3D reconstructions. If optical sections, how where they selected?

The IF was performed on optical sections selected based on being best focused and on the quality of the IF signal.

There is inconsistency in the age of mice used in the study between figures (either 6- and 18- months or 12- and 18-months).
We agree with the reviewer. We would like to clarify that, for IHC, we only had 12-month-old brain available to perform the experiments. However, both ages, 6m and 12m, refer to adult mice.

Minor comments:
Line 165, the authors discuss the increase of APP processing with aging. It can be due to an increase of APP, of BACE1 or due to a change in APP and BACE1 intracellular trafficking with aging. Authors argued that BACE1 level is the same (Fig 2SAB). The authors should comment on this result as the literature reports stability in BACE1 mRNA (Bioal et al 2000, Irizavarry et al. 2001; Apelt et al. 2004) and BACE1 protein levels (Ro ner et al. 2001; Fukumoto et al. 2004; Apelt et al. 2004). However, BACE1 activity is increased with aging in mouse and human brain (Fukumoto et al 2004). The authors need to comment on BACE1 activity in their system.

In line 178-179 we commented on secretase activity according to the reviewer's suggestion.

Line 275, "in young neurons, APP undergoes clathrin mediated endocytosis (CME)", a reference is needed.

References have been added.

The authors are focused on the endocytosis pathway of APP in aging neurons and brain. However, there is no mention of APP anterograde trafficking, and as APP is directly trafficked from the Golgi to the early endosomes, transport of APP from the anterograde transport pathway might play a role in the increase of APP in early endosomes as well (Xu et al. PNAS 1997; Siman R, Velji J. 2003; Choy et al. 2012; Toh et al. 2016...). The authors need to expand their conclusions to include alternative hypotheses from their study.

We agree with the reviewer and will include these alternative hypotheses.

The text is generally well written although it has the feel of an early draft. Some parts of the text need to be rephrased (ie: line 58 "born embryonically"; line 62 "neurons are not only targets of AD"...). The conclusion and discussion need to be clearer and more succinct. Improvements here will provide a manuscript which is more accessible to readers in the cell and neurobiology fields.

We rephrased the sentences, as suggested by the reviewer. We will shorten and make a more straightforward discussion and conclusion.

A more definitive title could be used (if the control experiments are consistent) (ie: Up- regulation of APP endocytosis in aging neurons linked to synapses loss and Alzheimer development). Abbreviations are not usually used in the summary (ie: -amyloid instead of A). Abstract can be rephrased to highlight better the in vivo and in vitro aspect of this study. The information is present, but it could be stated more clearly to show the strength of the study. - amyloid and Alzheimer disease can be added to the key words.

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About the title, we and reviewer 2 consider that the synapse data could be stronger. Thus we would prefer to keep that data out of the title. We agree that abbreviations are not the best in summary, but the abstract gets so long and difficult to read that we would prefer to keep APP and Ab, but we removed LOAD. We enforced the in vivo and in vitro aspects of the work in the abstract.

See below our comments relative to specific sentence/statement/conclusion:

Line 54 - "Cognitive decline develops with aging and often precedes AD" This statement could be improved as it is similar to the 'chicken or the egg' dilemma ... We don't really know which comes first - A accumulation in the brain might appears first during early development of AD and will lead later to cognitive defects.

In this sentence, we refer to the higher probability of a person with cognitive decline to develop AD. We agree that Abeta starts accumulating during aging and precedes AD. We tried to allude to it in the following sentence: "The multifactorial mechanisms underlying this aging-associated cognitive decline are likely silent pathological mechanisms that will eventually trigger the onset of AD."

Line 77 "in familial AD, the progressive accumulation of Ab initiates the cascade..." true? Needs references.

We refer to experimental models like the triple transgenic and longitudinal studies of fAD patients where amyloid accumulation is the first change observed. References are now included.

Line 134 "the number of auto-fluorescent granules within lysosomes identified by the lysosome-associated membrane protein 1 (LAMP1) was significantly...." Needs to be rephrase as it is confusing, LAMP1 is used as a lysosome marker to identify the quantity of granules/lipofuscin inside the lysosome.

We have rephrased the sentence to clarify that we defined lipofuscin as the auto-fluorescent granules that localize within Lamp1-positive lysosomes.

Some parts of Fig. 2 are not commented in the text, we can observe an increase of EEA1 in aged neurons and aged brains and it need to be state/ commented in the text to be able to keep the actual conclusion "increased in APP processing in the aged neurons and aged brain".

We are unsure of which parts of the figure the reviewer would like us to state/comment on. Since in line 175-177, we wrote, "We started to investigate trafficking by analyzing EEA1 levels, an early endosomes marker, by western blot, and we found EEA1 increased in aged neurons (Fig.2E, F) and aged brain (Fig.2G, J). ".

Line 172 "Moreover, we found the levels of BACE1 and nicastrin, a γ-secretase subunit, unaltered in aged neurons when compared with mature neurons (Fig.S2 A, B)" We would suggest being more cautious by saying "not significatively altered in our model..." instead of "unaltered".

We have changed the text according to the reviewer's suggestion.

Line 205 "their size and intensity increased in aged neurons especially in neurites" The authors are making conclusions about early endosomes based on staining with the EEA1 marker. This conclusion is an overstatement as the size and intensity looks similar in the cell body compared with the neurites.

We agree that the absolute difference between the cell body and neurites is minimal, only the difference between 21 and 28 DIV is more significant. We have now removed the "especially in neurites".

Moreover, an increase of EEA1 staining doesn't mean an increase in endosomes as stated on line 179 in the manuscript.

We agree and have modified the text for "up-regulated endocytosis".

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Line 209 "Overall, these data indicate that with aging APP localizes more to enlarged early endosomes."
From this paragraph, the authors could have two interesting conclusions with the observation of enlarged endosomes in aged neurons and the increase of APP localisation in these enlarged endosomes. They have not discussed the potential reasons for enlarged endosomes

The reviewer may have missed in the discussion that we speculate on the potential reasons for enlarged endosomes.

"Early endosomes form by fusion of endocytic vesicles upon endocytosis. Thus, the up- regulation of early endosomes could result from a general increase in endocytosis and a specific increase in APP endocytosis. However, we cannot exclude the contribution of a deficit in the maturation into late endosomes and in lysosomal degradation with neuronal aging, despite APP degradation not being altered.
From a different perspective, the augmented recruitment of EEA1 to early endosomes could be due to the increased activation of Rab5 with aging (Ginsberg et al., 2011; Neefjes and van der Kant, 2014). Alternatively, an impairment of EEA1 degradation with aging may underlie its overall increase in aged neurons and the normal aging brain. Since alterations in proteostasis occur with aging (Hipp et al., 2019), research may reveal EEA1 proteostasis defects.
Overall, our findings indicate that early endosome enlargement results from increased endocytic uptake. "

Line 217 "Ab production requires APP endocytosis"
This statement is not true. Abeta production can occur along the anterograde trafficking pathway, for example APP processing in the Golgi apparatus. Also APP can be transported from the Golgi directly to the endosomes without transport to the cell surface. The authors need to consider this option.

Over the years, there has been a divergence in the sites of Abeta production. After reviewing the literature carefully and based on our findings, we believe that the divergence arises from the different experimental conditions used, e.g. APP overexpression, non-neuronal cell lines, non-differentiated neuronal cell lines, or immature primary neurons. There is nevertheless a trend for a consensus for the Abeta being produced in endosomes upon BACE1 and APP endocytosis that we reviewed recently (Guimas Almeida 2018). We will clarify this by mentioning the alternative pathways for the production of Abeta.

Line 301 "Overall, this data indicates that APP endocytosis is clathrin-, dynamin-, and actin-dependent in both mature and aged neurons ...". This conclusion is premature. In the supp data, Pitstop 2 doesn't affect APP endocytosis in 21DIV neurons. The difference in the effect of Pitstop 2 between 21DIV and 28DIV neurons need to be discussed in the main results section. Do APP endocytosis mechanisms change over time?

We understand the reviewers' doubts. To clarify, we used two inhibitors of clathrin-mediated endocytosis, the CPZ (chlorpromazine) and Pitstop2. CPZ was very efficient in blocking APP endocytosis at 21 and 28 DIV. Because Pitstop2 inhibited APP endocytosis less than CPZ even at 28 div, we assumed that PITSTOP2 was less efficient. Moreover, APP is endocytosed via clathrin-mediated endocytosis (Cirrito et al., 2008; Marquez-Sterling et al., 1997; Cossec et al., 2010). These data support our results and interpretation that APP is endocytosed via clathrin.
Furthermore, our main question was if APP endocytosis was dependent on clathrin. Since at 28DIV, both pitstop2 and CPZ inhibited APP endocytosis, we concluded that APP endocytosis in aged neurons was still mediated by clathrin. Our data suggest that the mechanisms don't change over time. Instead, there seems to exist an increase in the quantity of endocytosed APP. We will include this discussion in the main results section.

Line 321 and below "There are two endocytic adaptors genetically linked to Alzheimer's disease, CD2AP, and CALM..."
We would suggest removing the data about CD2AP and CALM as they are not really informative.

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We agree with the reviewer that this data does not prove the function of CALM and CD2AP in APP endocytosis with aging, but we found that the enrichment of CALM and CD2AP at APP endosomes so striking that we would like to share it with the community. It can potentiate research on analyzing the function of these two late-onset risk factors during aging. We would prefer to keep the data in the manuscript.

Line 354 "Importantly, the number of synapses increased significantly by 42% and 52% when we treated with DAPT or BACE inhibitor IV, respectively (Fig.7C, F, G, J, M, N)." The experiment may suggest a protection from loss rather than an increase. For example, the authors could indicate a stabilisation of the number of synapses between 21DIV and 28DIV or an inhibition of the decrease of the numbers of synapses instead of an increase of synapses...

We have altered the text to explain better the point raised by the reviewer.

Line 367 "fAD" needs to be defined.

Thanks for the careful review, we have now defined fAD.

In regard to the cell images in the figures, drawing the borders of the neurons would help to clarify the figures as some staining seems to be outside of the neurons/dendrites (ie on Fig.1 G-H, Fig.3 D/E/F/G). The means on the graphs are not always clearly visible and needs to be put in front (ie on Fig. 3 X/Y/Z/AA). It would help the reader if the colour code and order of the drugs in the figures is consistent between figures (Fig 6. and Fig.S3.); at the moment the order and colouring differ.

We will change all figures and graphs according to the reviewer's suggestions.

Fig.2H-I panels are not commented in the text. They should be removed or discussed.

The reviewer may have missed the text's reference because these panels are commented on with the in vitro data.

In the model presented, endocytosis is enhanced due to increased levels of clathrin, by so why is TfR endocytosis decreased? This appears to be a contradiction.

We agree that the reduction of TfR endocytosis is unexpected, given that it is the canonical marker of clathrin-mediated endocytosis. We found in the literature that iron homeostasis changes with aging, more specifically, that TfR is reduced in the aging brain and that increased oxidative stress prevents iron uptake by reducing the transferrin receptor at the surface (Malorni et al., 1998; Lu et al., 2017). These data would favor the scenario that with aging, there is the iron accumulation in the brain, and thus the iron uptake via transferrin endocytosis is reduced, leading to reduced expression of TfR. We did not pursue the characterization of iron transport in aged neurons because it opens a new research line. We decided to include this finding in the paper because it is the obvious control for the APP endocytosis. If necessary, we can expand the discussion of these results.

Reviewer #3 (Significance (Required)):

In this study, Burrinha et al. report an increase of APP endocytosis with aging in aged neurons and aged brains. The strength of their experimental design is the inclusion of in vitro and in vivo experiments with a range of different cellular and molecular techniques together with very good quantification. However, their conclusions will need to be confirmed by inclusion of additional experiments and a number of key controls which are currently lacking. Reproducibility is also a concern given the methods used to culture neurons over extended periods. The relationship between APP processing, Abeta production, aging of neurons, and Alzheimer's disease is an important field that requires more research in order to understand the initiation and progression of Alzheimer's disease so that new treatments can be developed for neurodegenerative diseases.

Reviewer field of expertise: cell biology, neurobiology, intracellular trafficking of BACE1 and APP, confocal microscopy, neuron cell culture

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We thank the reviewer for recognizing our data's strength and we are convinced that we will provide all the necessary requested controls. We understand the reviewer's concern with data reproducibility, but we believe we have had several students reproducing some of the findings, and thus, we believe that this system is reliable.

First decision letter

MS ID#: JOCES/2020/255752

MS TITLE: Up-regulation of APP endocytosis by neuronal aging

AUTHORS: Tatiana Burrinha, Ricardo Gomes, Ana Paula Terrasso, and Claudia G Guimas Almeida
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript based on the Referee reports in Review Commons and your suggested revision plan. I would be delighted to consider your manuscript for publication following this plan of work.

Please ensure that you clearly highlight all changes made in the revised manuscript, including figures. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers and if this revision does not follow the agreed plan of work. Please explain clearly why this is so.

First revision

Author response to reviewers' comments

Response to reviewers' comments
We would like to thank the reviewers for their comments and suggestions that we followed and improved the manuscript.
All substantive changes have been highlighted in yellow in the manuscript.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):
The authors used in vitro "aged" primary neurons (DIV28) and report increased levels of lipofuscin staining and intracellular mouse Abeta as well as a decline in synaptic density. Interestingly, they also showed increased APP endocytosis, whereas other CME cargoes, such as TfR, were not affected. Notably, inhibition of Abeta production by a BACE inhibitor attenuated synaptic decline. One possible mechanism highlighted by the authors, causing the increased intracellular Abeta might be elevated Abeta endocytosis while in vitro aging.

1) The authors show increased intracellular Abeta immunoreactivity, as indicated by anti-Aβ42 C-terminal specific antibody (12F4) (Figure 1). Specificity of the staining should be validated in APP KO or knock down primary neurons.

We thank the reviewer for raising an important point. We used the immunofluorescence protocol for Aβ42 developed in Gunnar Gouras lab (Almeida et al., 2006; Takahashi et al., 2004; Tampellini et al., 2007), and we have controlled for 12F4 specificity in our previous work published in EMBO Reports in 2017 (Ubelmann et al., 2017). We did some critical controls, such as quantifying the decrease in Aβ42 immunofluorescence upon DAPT and BACE inhibitor treatment in n2a cells (Fig.EV1; Ubelmann et al., 2017). Moreover, we used a second antibody against Aβ42 (H31L21) with

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similar results to 12F4.

We contacted Gunnar Gouras, and we have now included immunofluorescence images showing the lack of Aß42 staining in APP KO neurons with the 12F4 antibody (Fig. S2).

Furthermore, the authors should provide co-stainings with APP to clarify if the anti-Aß42 antibody might also detect full length APP or CTFs. This could also be shown by expression of different murine APP mutants.This control for specific intracellular Abeta detection is essential, as the authors also observed increased APP levels in "aged" primary neurons (Figure 2 and 3).

We understand the reviewer's concern. We have imaged APP and Aß42 extensively, and their staining is quite different. Moreover, in the EMBO reports paper figure EV1 (Ubelmann et al., 2017), we show that when we overexpress the APP CTF (C99) and immunolabel it with APPY188 and anti-Aß42, the signals do not overlap.

We prepared an image with co-staining with APP and Aß42 to illustrate our observations (Fig. S2).

2) The authors observed increased levels of EEA1 staining in "aged" PN (Figure 3) and postulate increased levels of APP in EEA1 positive endosomes. Firstly, colocalization should be validated in a quantitative manner. Secondly, the authors should provide a control with another membrane protein, to ensure that APP is indeed increasing in early endosomes.

We are unsure we understood the reviewer's concern about the validation of APP colocalization with early endosomes. To clarify, we postulated that APP localization increased at early endosomes with aging based on three quantitative experiments:

1. We measured the colocalization of cellular APP quantitatively with EEA1 in "aged" neurons **in vitro** (Early endosomal APP, Fig.3 I, J). Because cellular staining of APP comprises APP at the plasma membrane, ER, Golgi, TGN, secretory vesicles, early and late endosomes, lysosomes, etc., the APP fraction signal that colocalizes with EEA1 is relatively small. However, notably, it increased in neurites of "aged" neurons by 64%.
2. We measured the colocalization of cellular APP quantitatively with EEA1 in "aged" neurons **in vivo** (Early endosomal APP, fig.3 M, O). The colocalization of APP in early endosomes increased 88% in the aged brain.
3. We measured APP endocytosis quantitatively after 10 min, the time at which APP is in early endocytic compartments as also observed in previous work (Fig. 4) (Ubelmann et al., 2017a).

Now we included additional data or re-analysis as follows:

New quantitative analysis of APP colocalization with EEA1 using a novel ICY plugin (Lagache, T., Grassart, A., Dallongeville, S., Faklaris, O., Sauvonnet, N., Dufour, A., Danglot, L. and Olivo-Marin, J.-C. (2018). Mapping molecular assemblies with fluorescence microscopy and object- based spatial statistics. Nat. Commun. 9, 698.), demonstrate that APP colocalization with EEA1 increases with aging by 87% and 63% in aged neurons and aged brain, respectively (Fig.3).

New quantitative analysis of APP colocalization with LAMP1, a marker of late-endosomes, shows that it does not increase with aging (Fig. 3 K, L).

New quantitative analysis of endocytosed APP colocalization with Rab11a, in addition to Rab5a (Fig. S3), shows that endocytosed APP colocalizes more with Rab5a than with Rab11a in aged neurons.

We provide new data showing an increase in Rab5a per APP endosome (Fig.S3 E, F).

We obtained new data showing that an increase in Rab5+ endosomes (Fig.3 E-H).

3) Biotinylation assays suggest strongly increased APP endocytosis in "aged" PN. The authors tried to validate these results with an APP antibody uptake assay. For this purpose they used 22C11. To our experience, the 22C11 works very well in WB analysis, but not in IC. Therefore, I strongly recommend to control either 22C11 immunoreactivity on APP KO cells or to use another antibody for the uptake assay.

We thank the reviewer for the suggestion of a critical control experiment.
We and others have used this antibody to follow APP endocytosis in live neurons, that may expose the epitope differently from IC (Acevedo et al., 2011; Chaufty et al., 2012; Jung et al., 1996; Sullivan

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et al., 2014; Sun et al., 2019; Ubelmann et al., 2017a; Ubelmann et al., 2017b). Initially, we also used P2- 1, another anti-APP extracellular N-terminus with similar results in HeLa cells. But, because this antibody does not recognize mouse APP, we decided to use 22C11.
Initially, we used 9 DIV neurons, for which we could not detect any 22C11 positive endosomes. We had to overexpress APP to detect enough 22C11 endocytosis, indicating the need for a high APP expression for 22C11 binding. Only in mature neurons, there is enough APP at the plasma membrane to follow its endocytosis with 22C11 without overexpression (Fig. 4) that is mentioned in the results section.
Thanks to Gunnar Gouras, we have included in the paper immunofluorescence of 22C11 in APP KO neurons compared to WT, with the APP KO neurons showing a 50% reduction in labeling (Fig. S3).

4) For control, the authors showed very interestingly that Transferrin-receptor endocytosis was not increased in "aged" PN. For better direct comparison, the experiment with TfR should be carried out also with the bulk surface protein internalization assay, used for APP.

We agree with the reviewer, and we have tried to do this experiment. For unclear technical reasons, we failed to detect biotinylated transferrin receptor endocytosis. Notably, the result using fluorescently labeled transferrin endocytosis was so dramatic that it left us quite convinced of the phenotype. Alternatively, we now examined the endocytosis of LDL and found it increased in intensity and size in aged neurons (Fig. 5K-N). Thus, transferrin decreased endocytosis seems to be the exception and not the rule. In summary, the increase in APP endocytosis is more pronounced than FM1-43 and LDL with aging.

Moreover, the authors should provide experimental evidence for this different behavior, as e.g. testing for different recycling rates of APP and TfR in "aged" PN.

We thank the reviewer for this suggestion. According to the reviewer's suggestion, we measured APP recycling in aged primary neurons (Fig. S3 J, K). The recycling of TfR in "aged" PN may be under the detection limit, given that aged neurons endocytose very little Tf.

5) The authors show in addition that inhibition of APP processing attenuates synaptic decline in "aged" PN. This is a pure correlative observation and should be experimentally validated by e.g. usage of APP KO PN or an APP knockdown approach combined with BACE inhibitor treatment.

We agree with the reviewer that our interpretation is based on a correlation between the inhibition of BACE1 and gamma-secretase activity (both block Aß42 accumulation) and synaptic decline at the expense of different precursors. We can only suggest that the decrease of synapses is attenuated by inhibition of Aß42 production. Unfortunately, because APP full length has direct effects on synapses (Fogel et al., 2014; Hoe et al., 2009; Lee et al., 2010; Martinsson et al., 2019; Müller et al., 1994; Priller et al., 2006; Spires et al., 2005; Wang et al., 2009; Wang et al., 2017; Weyer et al., 2011) knockdown experiments would be difficult to interpret and to provide more direct proof of Aß42 role at synapses in aged neurons.
We experimentally validated this result by overexpressing Rab5a, which increases endocytosis and Aß production (Grbovic et al., 2003), which we verified in mature neurons to recapitulate the decrease in synapses observed in aged neurons (Fig.7).

Reviewer #1 (Significance (Required)):

The increased APP endocytosis in "aged" PN is a very interesting finding and has important impact on understanding of Abeta mediated neuronal cytotoxicity.

Researchers interested in how Abeta might cause synaptic loss and neuronal loss are potentially interested in this study. Furthermore, the study highlights the APP endocytosis mechanism as a potential target for future therapeutic approaches, which might be interesting for those, working on endocytosis mechanisms.
My field of expertise is on Amypoid precursor protein function and trafficking.

We thank the reviewer for the positive feedback and encouragement!

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Reviewer #2 (Evidence, reproducibility and clarity (Required)):

This is an interesting article from the group that has been active in the field of membrane trafficking with a focus on Alzheimer disease. The paper by Burrinha et al. identifies the upregulation of APP endocytosis as a driving force of aging-dependent synapse loss and as a novel target to prevent late-onset AD. Using their own established model of aged mouse cultured neurons and material of aged mouse brains, the authors provide evidence that accelerated APP endocytosis potentiates APP processing and Abeta production. They also provide a mechanism for the upregulation of APP endocytosis, identifying clathrin, dynamin, and F-actin as key players. Techniques used in the study include ICC/IHC, WB, endocytosis, and trafficking assays.

Key conclusions are:

(i) Intracellular Abeta42 is increased with aging
(ii) APP processing and early endosomes increase with aging
(iii) APP endosomal localization and its endocytosis increase aging
(iv) Upregulation of APP endocytosis with aging requires clathrin and actin
(v) Age-dependent synapse is in part due to Abeta production

I found the concept interesting and the paper well presented. On the other hand, the conclusions driven from the results might be a bit preliminary and might require several additional controls.

We thank the reviewer for the positive feedback. We will perform additional experiments to solidify our conclusions.

Importantly, we would like to refer that we started this project in 2014, and it took us six years to gather the data presented in this manuscript, mostly because aging takes time, but also because we use stringent criteria in all experiments to ensure that the neurons analyzed were aged and healthy.

Major comments:

My main concern is about the aging in-vitro model (see below). Additionally, I find conclusions ii), iii) and iv) (see my list above) are not convincing and preliminary.

1. Conclusion i):

I am not certain if embryonically isolated neurons are a good model for the study authors aimed to do. These neurons are usually expressing the doublecortin throughout their life in culture, which would say that they are stuck in a "new-born state". Additional control for doublecortin levels, as well as some additional markers of cellular senescence, should be used to verify the aging model.

We agree with the reviewer that this model of neuronal aging has limitations.
We set-up a model of neuronal aging established and used by several groups, including Carlos Dotti, that established the primary cultures of neurons with Gary Banker and Bart de Strooper, a leader in the field of Alzheimer's disease research.
Some references in the literature:
(Aksenova et al., 1999; Bertrand et al., 2011; Bigagli et al., 2016; Blanpied et al., 2003; Brewer, 1997; Dong et al., 2011; Guix et al., 2012; Kim, 1983; Kuroda et al., 1995; Lesuisse and Martin, 2002; Li et al., 2004; Martin et al., 2008; Martin et al., 2010; Martin et al., 2011; Martin et al., 2014; Martín-Segura et al., 2019; Palomer et al., 2016; Porter et al., 1997; Sapoznik et al., 2006; Simons et al., 1998).
Several markers have been used to characterize these as "aged neurons," including Lipofuscin accumulation, protein carbonyl formation (protein oxidation), lipid alterations, and increased reactive oxygen species.
Because the "aged" neurons recapitulate essential aspects of the aging brain like the accumulation of lipofuscin in lysosomes, synapse decline, and Aß accumulation, we used these "aged" neurons to investigate changes in the mechanisms of neuronal cell biology, and we also confirmed, when possible, that such changes occur in the aged mouse brain.
Nevertheless, we strengthened the model by measuring the reduction of doublecortin (Fig. S1) and

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the accumulation of senescence-associated ß-galactosidase (Fig. 1) in aged primary neurons.

2. *Conclusion ii):*

a.I am very confused with the data in Fig. 2A. Why the described CTFs in the text are not evident in the blot or am I missing something? The last marker is 15KD, while beta-CTFs are 12-14kD. It would be good to know what is quantified in Fig. 2B? Also, I suggest quantifying the APP processing into ß-CTFs (12-14kD) and η-CTF separately, both for cultured neurons and brains.

We apologize for not presenting the data in a more straightforward way. We thank the reviewer for the suggestion. To clarify, we quantified the ratio of all CTFs (α-, ß- and η-CTF) over APP because we can only identify the CTFs based on their approximate size, which is not very exact given that the electrophoretic mobility is not a linear fit when plotted against molecular weight (kDa). Another critical issue is that there are no commercially available antibodies to identify the mouse APP's different fragments.
BACE1 cleaves between the 1st and the 11th aminoacid of the amyloid sequence originating C99 and C89. The C89 is virtually impossible to distinguish from C83 (α-CTF).

According to the reviewer suggestion we resolved the CTFs better by running 12% Bis-Tris gels and quantified them separately (Fig. 2).

b., I agree that the data indicate a solid increase in EEA1 protein levels. But can one really draw a conclusion about the early endosome increase only from the EEA1 data? Some additional experiments might be required: i) the mRNA analysis of EEA1, ii) additional early endosomal markers (i.e. RAB5 levels), and iii) the authors should consider EM or super-resolution techniques (the EEA1 puncta in brain sections are in fact too big to be early endosomes). Alternatively, the claim for increased early endosomes should be re-evaluated (i.e. can be changed to EEA1-positive early endosomes instead).

i. the mRNA analysis of EEA1
We found an increase in EEA1 protein levels by WB in aged primary neurons and aged brains. Moreover, the analysis of each single EEA1-positive vesicle size and intensity increased in aged primary neurons and aged brains.
We agree that these findings need further investigation. There is a fundamental lack of knowledge on EEA1 proteostasis or gene expression regulation as far as we could find. We consider this to be super interesting, and we will investigate this in-depth in a new line of investigation to be followed up subsequently.

ii. additional early endosomal markers (i.e. RAB5 levels),
We agree that the study would benefit from additional markers of early endosomes. We have now included data on Rab5a levels that confirm the increase in early endosomes (Fig. 3), despite the total levels of Rab5a not increasing in aged neurons or aged brain lysates (Fig. 2). This is in accordance with previous observations (Alsaqati et al., 2018). The lack of change in total Rab5 could be due to the dynamic association of Rab5 with endosomes (GTP form) and the cytosol (GDP form) or to the low sensitivity of the anti-rab5 antibody. We want to point out that clathrin, CALM, and CD2AP markers of the early endocytosis machinery, were also up-regulated with aging (Fig. 6).

iii. the authors should consider EM or super-resolution techniques (the EEA1 puncta in brain sections are in fact too big to be early endosomes).
We agree that there are EEA1+ endosomes in the brain bigger than early endosomes' average size. We have re-analyzed the endosome size and found some inconsistencies that have now been corrected (Fig. 3). We thank the reviewer for such a careful review. In the time available, we were unable to do EM or super-resolution. Thus we focused on the relative change in endosome size with aging. We also analyzed Rab5+endosomes *in vitro* and *in vivo* that corroborated the initial findings.

c. The separation between cell body and dendrites, which authors make in the article is very confusing to follow since not all data are in fact always consistent, e.g. early endosomes increase more in dendrites with aging, but there is no difference in endocytosed APP and etc. I am not even sure why the authors need the cell body for their story. I would stay with neurites and synapses.

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Thanks for the suggestion. Our finding that Aß42 accumulates more in neurites than in cell bodies prompts us to separate the cell body from the neurites. We agree that our results are more relevant in neurites and synapses. We agree with the reviewer, and after figure 1 we focused on neurites, simplifying the figures and the text.

3. Conclusion iii):

a. APP surface levels are increased in aged neurons. Could this be that increased APP endocytosis is due to altered recycling of APP back plasma membrane (more APP is endocytosed because the APP is faster recycled)? A recycling assay should address this issue.

We thank the reviewer for the alternative explanation for the increase in APP endocytosis. We measured APP recycling (Fig. S3 J, K) and found that it was increased by 25%. The magnitude of the increase does not seem enough to account for the increase in endocytosis (700%) (Fig. 4). We do agree that the increase in surface APP, resulting from recycled and the secretory pathway may increase the availability of APP to be endocytosed. We mentioned this now in the manuscript and will tackle this novel question in the future.

*b.I am puzzled with a low amount of RAB5-positive endosomes containing the endocytosed APP (Fig. S2K,N). Can the rest be in recycling endosomes? ICC with **endocytosed APP/RAB11 should be performed here**.*

We were also puzzled by the low number of Rab5a-positive APP endosomes detected. Our experience is that Rab5 antibodies are not very good. We tested more than one antibody against Rab5 in ICC and chose the one that gave the best results. Nevertheless, we could only segment the Rab5a signal at 28DIV for colocalization, probably because there is more rab5a per endosome at 28DIV than at 21 DIV, thus increasing the antibody binding and improving the signal to noise in ICC. To address the reviewer's concern, we segmented endocytosed APP and measured the amount of Rab5a. We found a significant 71% increase in Rab5a per APP endosome in aged neurons (Fig. S3 F). Moreover, we performed ICC of endocytosed APP/Rab11a as suggested and found that only 12% of endocytosed APP localizes to recycling endosomes, and this is not due to a reduction in Rab11a levels, which we found unchanged in aged neurons (Fig. S3 E-I).

4. Conclusion iv):

*I found this conclusion is less convincing from all since all the data are generated using clathrin and dynamin inhibitors only. Pitstop2 and dynasore have multiple unspecific side effects (i.e. please see the Park et al., 2013, J. Cell Sci). To draw the conclusion that APP endocytosis requires clathrin and dynamin based on that is far too preliminary. I suggest either to **down- tone throughout the paper (e.g. APP endocytosis is Pitsop-2 and Dynasore-sensitive) or perform the functional analysis using shRNAs or CRISPR/Cas**.*

We agree with the reviewer that functional analysis with shRNA or Crispr/Cas9 should complement the endocytosis inhibitors whenever technically possible. However, primary neurons are typically challenging to manipulate. To our knowledge, knockdown has not been done in aged cultures of primary neurons. We have tried to do Crispr/Cas9 in our lab, and we were thus far successful in n2a, HEK, fibroblasts cells but not in primary neurons. Therefore, we have down-toned our interpretations throughout the paper.
In addition to the use of inhibitors, we do provide data that support that APP endocytosis increases with aging due to increased clathrin and actin-mediated endocytosis (Fig. 6 C-Q), namely:
6. Clathrin levels are up-regulated in aged neurons and aged brains;
7. The density of clathrin puncta (sites of endocytic pits formation) increases in aged neurons;
8. APP is more present at clathrin assemblies in aged neurons than in mature neurons.
9. F-actin is most often present in APP endosomes in aged neurons.
10. CALM, part of the machinery involved in the clathrin-mediated endocytosis of APP, and CD2AP, an actin and endocytosis regulator, are most often present in APP endosomes in aged neurons.
Nevertheless, we have down-toned our interpretations throughout the paper.

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5. Conclusion v):

Fig. 7 is loosely attached to the mainline of the manuscript. I suggest to either include this as a part of Fig.1 to illustrate an additional control for an in-vitro model or perform additional experiments showing the effect of APP endocytosis on synapse rescue in aged neurons (should be very straightforward since authors can use the drugs mentioned above).

We thank the reviewer for motivating us to go the extra mile. We have induced endocytosis up-regulation by overexpressing Rab5 in mature (not aged neurons) and recapitulated the increase in intracellular Aß and decreased synapses observed in aged neurons. We have included this new and exciting finding in Fig. 7.

Generally, the data and the methods presented in a reproducible way, and all experiments adequately replicated and statistical analysis is adequate.

Minor:

1. Page 7, line 134- LAMP1 is not a pure lysosomal maker and will label MVBs and late endosomes as well.

Since MVBs can be considered as late-endosomes, we have now defined LAMP1 as late- endosomes/lysosomes marker.

2. FM1-43 is not an endosomal marker, but a lipophilic dye, which will insert into all membranes.

We agree that FM1-43 is not an endosomal marker by excellence. However, it has been used as a reporter for endocytosis and recycling depending on the time of the pulse, chase, and washing. It is cell impermeable, binding mostly to the plasma membrane. It will be enriched in endosomes by all kinds of endocytic routes in a time-dependent manner, so it is considered a non-specific marker of bulk endocytosis.
We have now included a better description in the results and methods and a reference of a study of FM1-43 endocytosis using fluorescence imaging followed by electron microscopy that identifies early endosomes as an endocytic compartment labeled by FM1-43 (Fomina, A. F., Deerinck, T. J., Ellisman,
M. H., & Cahalan, M. D. (2003). Regulation of membrane trafficking and subcellular organization of endocytic compartments revealed with FM1-43 in resting and activated human T cells. Experimental cell research, 291(1), 150–166. https://doi.org/10.1016/s0014-4827(03)00372-0).

3. Please indicate whether the heavy or light clathrin chain was analyzed.

We analyzed the heavy clathrin chain. This is now indicated in the methods section.

4. Discussion is too long and a bit diluted. I suggest shortening it by 1-2 pages.

We have shortened the discussion by almost 2 pages.

5. This is correct that CALM can function as an adaptor for endocytosis of APP. Additionally, CALM can also regulate APP autophagic degradation (Tian et al. 2013). It would be good if the authors can re-consider their data according to this finding.

We thank the reviewer for triggering us to look at this possibility. We carefully read the Tian et al. 2013, and the data demonstrate that starvation-induced autophagy (n2a cells) increases CALM interaction with LC3 and LC3 with AP2. Although the authors present data showing that AP2 knockdown leads to APP CTFs accumulation, we could not find data showing CALM requirement. We found another paper supporting CALM's role in starvation-induced autophagy (HeLa cells) (Moreau et al. 2014). In this paper, CALM knockdown increases autophagy. The mechanism proposed is indirect via the endocytosis of VAMP2, apparently crucial for the fusion of autophagosomes with lysosomes.

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No link was established with APP degradation. Although possible, it remains an open question if CALM has a direct role in APP autophagic degradation.
Given that most CALM data point to a function in APP endocytosis (Chae et al., 2020; Sahlender et al., 2013; Tebar et al., 1999; Xiao et al., 2012), and we found that APP degradation was not significantly altered in "aged" neurons (Fig.4 K-M), we interpreted the data accordingly. Nevertheless, we agree that we need to investigate further the role of CALM in APP trafficking with aging. We will pursue this line of research in the future.

Reviewer #2 (Significance (Required)):

The role of membrane trafficking in the pathophysiology of AD is currently under intense investigation. From that point of view, the study by Burrinha et al. is timely and of high significance for readers interested in cellular and molecular mechanisms of neurodegeneration.

Field of expertise: membrane trafficking, autophagy, endocytosis, neurodegeneration.

We thank the reviewer for the positive and constructive feedback and for recognizing the importance of our work!

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

SUMMARY

Burrinha et al. is a worthwhile study with potentially interesting findings on APP endocytosis in vitro and in vivo. The authors have developed systems to compare the aging of neurons in culture, derived from the cortex of E16 mice, as well as brains from young and old adult mice. They used two systems to compare APP endocytosis and processing in the neuronal samples over the time period and measured the level of A as a link with Alzheimer disease. This study presents a significant body work with number of well-organized figures and extensive quantification and controls. However, many of the hypothesis/conclusions by the authors are not fully supported by the data. Also, and most importantly, a number of key controls are missing to support authors' conclusions. The balance of the discussion and conclusions need to be improved. Overall, if the author can improve the robustness of the data the manuscript could be a very useful contribution.

MAJOR COMMENTS

Some of the key experiments need better controls to support the conclusion proposed by the authors (see below).

In Fig1, Abeta level after DAPT treatment needs to be included in the IF to demonstrate the specificity of staining by the Ab to Abeta40 or to Abeta42. The level of A secreted needs to be quantified as well to determine the relationship between intracellular and secreted pools of Abeta.

We thank the reviewer for raising an important point. Regarding the Aß specificity, we have controlled for 12F4 specificity in our previous work published in EMBO Reports in 2017 (Ubelmann et al., 2017), where we included essential controls, such as quantifying the decrease in Aß42 immunofluorescence upon DAPT and BACE inhibitor treatment (Fig.EV1, Ubelmann et al. 2017). Moreover, we used a second antibody against Aß42 (H31L21) with similar results to 12F4. Nevertheless, we included the impact of DAPT and BACE inhibitor on Aß42 and APP levels by immunofluorescence and WB for APP in Fig. S6 G-M.
Regarding Aß40, we quantified it and found it also increased in aged neurons (Fig. S2 C, D).
Regarding secreted Aß, its aging changes have been previously reported to be also increased in aged neurons in similar experimental conditions (Guix et al., 2012). We repeated these measurements in our experimental conditions and could not detect differences in secreted Aß (Fig. S2 E-G). It could be due to our different experimental conditions, namely, we used the ELISA kit from Invitrogen, which could be less sensitive than the Wako kit used by Guix et al. that, unfortunately, we did not have access to.

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In Fig. 2A, the quality of the western blot is poor and cannot be interpreted. There is no internal standard for normalization of loads between samples. The identity of the bands seems very speculative given the sizes of the bands do not correspond with their expected Mr and these need to be confirmed by different treatments. To increase the resolution for the lower bands, a different gel percentage can be used. As C99 bands are not identified specifically, conclusions on the A pathway cannot be made from this blot. Moreover, APP signal is saturated in Fig. 2B and makes the quantification inaccurate.

According to the reviewer's suggestion, we ran 12 % Bis-Tris gels to better resolve APP CTFs. We significantly improved the blots' quality and re-did the quantification, analyzing C99 bands individually (Fig. 2). Additionally, we present the blot for APP alone in order to avoid saturation.

In Fig.2E-F, the level of EEA1 in 14DIV is missing and it would be relevant for a complete picture to show the data on the basal level of EEA1 (is the level stabilised between 14DIV and 21DIV or a continual increase?).

This point was also raised by reviewer 2. We have included the levels of EEA1 at 14DIV in Fig. 2. We did not find a significant difference in EEA1 between 14 and 21 DIV, indicating that the level of EEA1 is stabilized between 14 and 21 DIV and increases at 28 DIV.

In Fig.3 it would be ideal to include APP staining in APP KO neurons as a control of APP specificity as some antibodies to APP can give high background. The specificity of staining needs to be demonstrated as the contribution of background staining may vary across the aged samples.

We agree that control for APP y188 specificity is essential. A previous paper comparing different APP antibodies showed APP Y188 specificity using APP KO (Guo et al., 2012). Thanks to Gunnar Gouras, now an author, we verified the absence of the APPY188 labeling in APP KO neurites (Fig. S2). Nevertheless, the increase in the background would be an issue common to all antibodies used, and we did not observe or quantified an increase at 28 DIV for all, namely, for TfR and GluA2 that decrease (Fig. 5, 7).

This issue also applies to the labelling of endogenous APP at the cell surface (Fig. 4I-L), the specificity of this labelling needs to be established, particularly as only low levels of APP are considered to be at the PM.

We thank the reviewer for the suggestion of an important control experiment, that reviewer 1 also raised.
We and others have used this antibody to follow APP endocytosis in live neurons, that may expose the epitope differently from IC (Acevedo et al., 2011; Chaufty et al., 2012; Jung et al., 1996; Sullivan et al., 2014; Sun et al., 2019; Ubelmann et al., 2017a; Ubelmann et al., 2017b). Initially, we also used P2- 1, another anti-APP extracellular N-terminus with similar results in HeLa cells. However, because this antibody does not recognize mouse APP, we decided to use 22C11.
Initially, we used 9 DIV neurons, for which we could not detect any 22C11 positive endosomes. We had to overexpress APP to detect enough 22C11 endocytosis, indicating the need for a high APP expression for 22C11 binding. Only in mature neurons, there is enough APP at the plasma membrane to follow its endocytosis with 22C11 without overexpression (Fig. 4) mentioned in the results section. Thanks to Gunnar Gouras, we have included in the paper immunofluorescence of 22C11 in APP KO neurons compared to WT, with the APP KO neurons showing a 50% reduction in labeling (Fig. S3 C, D).

Fig. 4A-E is missing a key control: a surface label and cleavage without a chase eg on ice, in order to determine the background for each DIV sample. This is a critical control to demonstrate that the labelling in each case is restricted to the cell surface. This control is also essential to be able to compare samples between 21DIV and 28DIV.
We agree with the reviewer that doing the labeling on ice is the best to determine surface labeling. We have done this successfully before with younger primary neurons (Ubelmann, 2017; Almeida et al. JNeurosci 2006) or other cell types (Almeida et al. NCB 2011). But for some unclear reason, the

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level of biotinylated proteins of mature neurons or aged neurons on ice was always inferior to the level observed upon incubation at 37ºC. We hypothesized that the biotin accessibility to plasma membrane proteins in mature neurons might be reduced. We choose the 10 min time point, because we observed that after a 10 min pulse at 37ºC, surface APP was consistently well biotinylated, and the amount of endocytosed APP (after stripping) represented a small fraction of the total APP at the surface (about 4 % at 21 DIV and 16 % at 28 DIV). Thus, we considered that we could still interpret the data as APP surface levels using these experimental conditions. We have done ICC experiments with surface labeling of APP using 22C11 in non-permeabilized neurons, included in Fig. 4.

To complete Fig.6, effects of the drugs on transferrin endocytosis in aged neurons as well (here only 21DIV and not 28DIV) and for the styryl dye FM1-43 need to be check.
We agree with the reviewer that the effect of the drugs on FM1-43 endocytosis is essential. We have now performed the experiment and added it to Fig. S4. We disagree that analyzing the impact of the drugs on transferrin endocytosis at 28DIV would be informative. Because transferrin endocytosis is greatly reduced by aging (See Fig.5 E, F), and as such, the effect of drugs in reducing transferrin endocytosis in aged neurons will probably be below detection.

In Fig7. the authors did not show if the BACE1 inhibitor and DAPT have actually blocked APP processing in their experiments. They have relied on the reported action of these drugs but it is essential that the level of inhibition in these experiments is documented. In the absence of information about APP processing conclusions cannot be drawn on the experiments in Fig 7.
Indeed, these are established inhibitors of APP processing. In our hands, we have observed that DAPT and BACE1 inhibitor block APP processing and reduce intracellular Aß (Ubelmann 2017, Takahashi, 2004). Nevertheless, we performed these experiments and included the results in Fig. S6 G-M.

Importantly, there is contradictory information in the different figures and quantification. In Fig.1, quantification of lipofuscin is quite similar in the neurites at 21DIV and 28DIV but looks very different in the images.
If we understand correctly, the reviewer refers to the difference in the number of auto-fluorescent granules present in aged neurites. We quantified the number of these in cell bodies and neurites, and the results are in figure S1. To clarify, we concluded that lipofuscin increased in the cell body and not in the neurites of aged neurons based on the detection of autofluorescent granules in lysosomes (LAMP1 positive organelles) in cell bodies of aged neurons (Fig. 1 F, G). The auto-fluorescent granules outside lysosomes could also be damaged mitochondria and other auto-fluorescent material.

In Fig. 3A-B-C, the images show more APP in the cell body at 21DIV compared to 28DIV whereas the quantification is showing a similar level in the cell body. Moreover, Fig. 2 shows no difference in total APP levels between 21DIV and 28DIV yet Fig. 3C indicates that the neurite APP is higher at 28DIV.
We agree with the reviewer that the images show more APP in the cell body at 21DIV than 28DIV. We improved the figure to reflect the quantification better. By WB, we did not detect APP total levels changes, but when we analyzed APP's subcellular distribution, we found that it changes, increasing in the aged neurites. We interpret these subcellular changes because of trafficking alterations with aging. We believe that the impact of aging in other trafficking pathways of APP needs to be investigated to grasp why APP is increasing in neurites altogether. This result opens a new line of investigation for future studies.

One of the most important concerns in the study is the fact that the neuronal medium is not changed during the 28 days of neuron culture (line 576 - "Neurons maintained up to 28 days without changing the media"). This is a concern for neuron viability, and dendrites/synapses development. It may lead to an accumulation of APP and A in the supernatant that will affect the results of the entire study. This is a really critical point and will need to be address by the authors. Notably, in Fig. 5 the reduction of the endocytosis of TfR in 28DIV is very substantial and might suggests that cells at day 28 are not healthy.
We agree that this model of neuronal aging has limitations, but it does recapitulate important features of neuronal aging and it has been used by several groups. Some references in the literature: (Aksenova et al., 1999; Bertrand et al., 2011; Bigagli et al., 2016; Blanpied et al., 2003; Brewer, 1997; Dong et al., 2011; Guix et al., 2012; Kim, 1983; Kuroda et al., 1995; Lesuisse and Martin, 2002; Li et al., 2004; Martin et al., 2008; Martin et al., 2010; Martin et al., 2011; Martin et

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al., 2014; Martín-Segura et al., 2019; Palomer et al., 2016; Porter et al., 1997; Sapoznik et al., 2006; Simons et al., 1998).

Concerning neuron viability, in Fig. 1, we show that MAP2 and Tubulin were not compromised in "aged neurons" since the neurons' morphology is of viable neurons without the typical bead-like pattern of degenerating cells. Moreover, neither MAP2 neither synaptic proteins' total levels were reduced in aged neurons (Fig. S6), which would happen if the neurons would be dying. However, we do see the reduction of TfR in 28DIV, but we think that TfR reduction does not suggest that cells are not healthy. Instead, we found that this may be a specific alteration of TfR with aging, as previously reported (Lu et al., 2017). We have clarified this in the manuscript.

The authors did not mention the potential contribution of anterograde trafficking APP and A production along biosynthetic pathway and their statements (line 185) ignore locations other the endosomes. APP processing occurs in endosomes but also in the secretory pathway and A has been shown to be produced in the Golgi apparatus as well. This issue needs to be discussed and integrated with their findings and their hypothesis (around line 416). Also, the paper argues for enhanced APP processing, however a reduction in secretion of Abeta would also be consistent with much of the data.

We agree with the reviewer. We tried to clarify the potential contribution of APP trafficking and Aß biosynthetic production throughout the manuscript.
Regarding secreted Aß, its aging changes have also been previously reported to increase in aged neurons in similar experimental conditions (Guix et al., 2012). We repeated these measurements in our experimental conditions and could not detect differences in secreted Aß (Fig. S2 E-G). Due to our different experimental conditions, namely, we used the ELISA kit from Invitrogen, which could be less sensitive than the Wako kit used by Guix et al. that, unfortunately, we did not have access to.

•Should the authors qualify some of their claims as preliminary or speculative, or remove them altogether?*

•Would additional experiments be essential to support the claims of the paper? Request additional experiments only where necessary for the paper as it is, and do not ask authors to open new lines of experimentation.

The authors are able to observe APP CTFs fragments in 21DIV and 28DIV neurons by western blot (Fig. 2 A-B). However, CTFs in the 14DIV sample are not detected which is surprising. We would suggest treating neurons with different drugs (ie: DAPT/C3...) to inhibit APP processing and accumulate APP CTFs to be able to observe APP CTFs even at an early neuronal stage. In addition, the treatment with drugs will assist in the identification of each of the potential CTF bands. A different gel percentage should be used.
We thank the reviewer for the comment and suggestion. We detect APP CTFs at 14 DIV and even at 9 DIV (Ubelmann et. al., 2017), but maybe they did not show well in the previous figure. As suggested, we resolved the APP CTFs better using 12% Bis-Tris gels and succeed in visualizing and quantifying them individually (Fig. 2).

The authors have assessed the number of endosomes containing APP and observed an increased in early endosomes size. We would suggest staining the early endosomes with another marker (ie Rab5) for confirmation.

Reviewer 2 also raised this point. We agree with both reviewers that the study would benefit from an additional marker to measure early endosome size. We have now evaluated Rab5a changes, and although the antibody is not excellent, we found that Rab5a also increased *in vitro* and *in vivo aging (Fig3)*.

It would be very informative to determine the distribution of APP across the different organelles within a cell as well. Lysosomes (numbers, shape and the localisation of APP in the lysosomes) should be included as APP is transported down the endolysosomal pathway (Toh et al. 2016). The authors have already performed some LAMP1 staining in Fig.1 and an expansion to include APP

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levels in LAMP1 compartment would give a better picture of any changes in organelles in their aging models.

We agree that we need to investigate further the distribution of APP across the various organelles within the cell. Since our "aged" primary neurons did not show alterations in APP degradation, we did not research further the potential late-endosome changes with aging. We now have analyzed the colocalization of LAMP1 with APP, which does not change with aging (Fig. 3 K, L). Regarding changes in the lysosome with aging, we agree that it is a fascinating point that we are pursuing in the lab.

The authors have analysed APP endocytosis and A production. However, A can also be release in the supernatant. As the neuronal media is not changed during the 28 days of neuronal culture, it is possible that the accumulated A in the culture medium will affect the aging of neurons and intracellular A level.

We agree with the reviewer. We cannot exclude that Ab endocytosis from the media increases with aging. We have included this point in the discussion. Regarding secreted Aß, its aging changes have also been previously reported to increase in aged neurons in similar experimental conditions (Guix et al., 2012). We repeated these measurements in our experimental conditions and could not detect differences in secreted Aß (Fig. S2 E-G). It could be due to our different experimental conditions. Namely, we used the ELISA kit from Invitrogen, which could be less sensitive than the Wako kit used by Guix et al. that, unfortunately, we did not have access to.

To compliment the APP plasma membrane staining (with 22c11 Ab) at different stages (14DIV, 21DIV and 28DIV) (Fig. 4) it would be informative to quantify the level of APP at the plasma membrane and assess whether there are change in APP at the PM during aging.

We agree with the reviewer that quantifying the level of APP at the plasma membrane would be relevant. We have now included data on surface APP during aging (Fig. 4 N, O).

In the last part of the study, the authors investigated the synapse plasticity/loss using two markers PSD-95 and vGlut1 (Fig.7). EM will give direct evidence in the change in size/numbers of the synapses comparing mature and aged neurons. EM images will give a much higher impact to the study. However, this suggestion may be challenging if the authors do not have ready access to an EM facility.

We agree that EM would give direct evidence for changes in synapses. However, we did not have ready access to an EM facility.

•Are the data and the methods presented in such a way that they can be reproduced?
•Are the experiments adequately replicated and statistical analysis adequate?

In Fig.2 A-B, the quantification is not convincing as the APP bands are saturated. We would suggest decreasing the loading and using DAPT treatment to reveal the total APP CTFs/C99 produced in neurons. Using this technique, C99 and others CTFs are observable in young neurons (7DIV, cf Tan, Fourriere et al. 2019).

We agree with the reviewer about the APP bands' saturation. We performed western blot analysis using 12% Bis-Tris gels to better resolve the APP CTFs/C99 and quantified them individually according to the reviewer suggestions (Fig. 2).

Quantification of APP in Fig.2C/D depends on the purity of the neuronal prep which is not stated in the paper.

We have now analyzed by Western blot the GFAP level, marker of glial cells, in our neuronal preparations (Fig. S1 E, F) and found that, although a bit variable, that it did not significantly increase with time in culture. We now state that we only use neuronal cultures that present minimal glia in our experiments in the methods.

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APP staining in cell body is saturated in Fig. S2C/D which renders quantitation meaningless.

We agree that the contrast adjustment for displaying the APP staining in the cell body leads to signal saturation in the figure. However, it does not affect the quantifications since these are done in axons and dendrites, always using images without any contrast adjustments and acquired without saturated pixels.

In Fig. 4C, endocytosed APP should be represented in comparison to APP level at the plasma membrane at the beginning of the experiment as this level is increased in aged neurons (49% more).

We agree that representing endocytosis compared to APP level at the plasma membrane at the beginning of the experiment would be ideal. However, we found that comparing endocytosis between 21 and 28 DIV better showed the differences observed. Since endocytosis increased by about 700% and the surface APP by 49%, we considered that the increase in endocytosis could not be accounted for by the surface APP increase.

It is not clear if the quantitation of IF was performed on optical sections or 3D reconstructions. If optical sections, how where they selected?

The IF was performed on optical sections selected based on being best focused and on the IF signal's quality.

There is inconsistency in the age of mice used in the study between figures (either 6- and 18-months or 12- and 18-months).

We agree with the reviewer. We would like to clarify that, for IHC, we only had 12-month-old brain available to perform the experiments. However, both ages, 6-month-old, and 12-month-old, refer to adult mice.

Minor comments:

Line 165, the authors discuss the increase of APP processing with aging. It can be due to an increase of APP, of BACE1 or due to a change in APP and BACE1 intracellular trafficking with aging. Authors argued that BACE1 level is the same (Fig 2SAB). The authors should comment on this result as the literature reports stability in BACE1 mRNA (Bioal et al 2000, Irizavarry et al. 2001; Apelt et al. 2004) and BACE1 protein levels (Ro ner et al. 2001; Fukumoto et al. 2004; Apelt et al. 2004). However, BACE1 activity is increased with aging in mouse and human brain (Fukumoto et al 2004). The authors need to comment on BACE1 activity in their system.

We commented on secretase activity according to the reviewer's suggestion.

Line 275, "in young neurons, APP undergoes clathrin mediated endocytosis (CME)", a reference is needed.

References have been added.

The authors are focused on the endocytosis pathway of APP in aging neurons and brain. However, there is no mention of APP anterograde trafficking, and as APP is directly trafficked from the Golgi to the early endosomes, transport of APP from the anterograde transport pathway might play a role in the increase of APP in early endosomes as well (Xu et al. PNAS 1997; Siman R, Velji J. 2003; Choy et al. 2012; Toh et al. 2016...). The authors need to expand their conclusions to include alternative hypotheses from their study.

We agree with the reviewer and included these alternative hypotheses in the discussion.

The text is generally well written although it has the feel of an early draft. Some parts of the text need to be rephrased (ie: line 58 "born embryonically"; line 62 "neurons are not only targets of

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AD"...). The conclusion and discussion need to be clearer and more succinct. Improvements here will provide a manuscript which is more accessible to readers in the cell and neurobiology fields.

We rephrased the sentences, as suggested by the reviewer. We shortened the discussion and hope it is straightforward.

A more definitive title could be used (if the control experiments are consistent) (ie: Up- regulation of APP endocytosis in aging neurons linked to synapses loss and Alzheimer development). Abbreviations are not usually used in the summary (ie: -amyloid instead of A). abstract can be rephrased to highlight better the in vivo and in vitro aspect of this study. The information is present, but it could be stated more clearly to show the strength of the study. - amyloid and Alzheimer disease can be added to the key words.

We have added important new data on synapse loss driven by up-regulation of endocytosis linked to intraneuronal Aß and critical controls as suggested by the reviewers. We have thus changed the title according to the reviewer's suggestion.
We agree that abbreviations are not the best in summary, but the abstract gets too long and difficult to read without the abbreviations that we would prefer to keep APP and Aß, but we removed LOAD. We enforced the *in vivo* and *in vitro* aspects of the work in the abstract.

See below our comments relative to specific sentence/statement/conclusion:

Line 54 - "Cognitive decline develops with aging and often precedes AD" This statement could be improved as it is similar to the 'chicken or the egg' dilemma ... We don't really know which comes first - A accumulation in the brain might appears first during early development of AD and will lead later to cognitive defects.

In this sentence, we refer to the higher probability of a person with cognitive decline developing AD. We agree that Aß starts accumulating during aging and precedes AD. We tried to allude to it in the following sentence: "The multifactorial mechanisms underlying this aging-associated cognitive decline are likely silent pathological mechanisms that will eventually trigger the onset of AD."

Line 77 "in familial AD, the progressive accumulation of Ab initiates the cascade..." true? Needs references.

We refer to experimental models like the triple transgenic and longitudinal studies of fAD patients where amyloid accumulation is the first change observed. References are now included.

Line 134 "the number of auto-fluorescent granules within lysosomes identified by the lysosome-associated membrane protein 1 (LAMP1) was significantly...." Needs to be rephrase as it is confusing, LAMP1 is used as a lysosome marker to identify the quantity of granules/lipofuscin inside the lysosome.

We have rephrased the sentence to clarify that we defined lipofuscin as the auto-fluorescent granules that localize within LAMP1-positive lysosomes.

Some parts of Fig. 2 are not commented in the text, we can observe an increase of EEA1 in aged neurons and aged brains and it need to be state/ commented in the text to be able to keep the actual conclusion "increased in APP processing in the aged neurons and aged brain".

We are unsure of which parts of the figure the reviewer would like us to state/comment on. In the results section, we wrote, "We started to investigate trafficking by analyzing EEA1 levels, an early endosome marker, by western blot, and we found EEA1 increased in aged neurons (Fig. 2 A, F) and aged brain (Fig.2 H, M) ".

Line 172 "Moreover, we found the levels of BACE1 and nicastrin, a γ-secretase subunit, unaltered

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in aged neurons when compared with mature neurons (Fig.S2 A, B)" We would suggest being more cautious by saying "not significatively altered in our model..." instead of "unaltered".

We have changed the text according to the reviewer's suggestion.

Line 205 "their size and intensity increased in aged neurons especially in neurites" The authors are making conclusions about early endosomes based on staining with the EEA1 marker. This conclusion is an overstatement as the size and intensity looks similar in the cell body compared with the neurites.

We agree that the absolute difference between the cell body and neurites is minimal. Only the difference between 21 and 28 DIV is more significant. We have now removed the "especially in neurites."

Moreover, an increase of EEA1 staining doesn't mean an increase in endosomes as stated on line 179 in the manuscript.

We agree and have modified the text for "up-regulated endocytosis".

Line 209 "Overall, these data indicate that with aging APP localizes more to enlarged early endosomes."
From this paragraph, the authors could have two interesting conclusions with the observation of enlarged endosomes in aged neurons and the increase of APP localisation in these enlarged endosomes. They have not discussed the potential reasons for enlarged endosomes

The reviewer may have missed that we speculate on the potential reasons for enlarged endosomes in the discussion section “Early endosome up-regulation with neuronal aging”.

Line 217 "Ab production requires APP endocytosis"
This statement is not true. Abeta production can occur along the anterograde trafficking pathway, for example APP processing in the Golgi apparatus. Also APP can be transported from the Golgi directly to the endosomes without transport to the cell surface. The authors need to consider this option.

Over the years, there has been a divergence in the sites of Aß production. After reviewing the literature carefully and based on our findings, we believe that the divergence arises from the different experimental conditions used, e.g., APP overexpression, non-neuronal cell lines, non-differentiated neuronal cell lines, or immature primary neurons. There is nevertheless a trend for a consensus for the Aß being produced in endosomes upon BACE1 and APP endocytosis that we reviewed recently (Guimas Almeida 2018). We now mention and reference the alternative pathways to produce Aß.

Line 301 "Overall, this data indicates that APP endocytosis is clathrin-, dynamin-, and actin-dependent in both mature and aged neurons ...". This conclusion is premature. In the supp data, Pitstop 2 doesn't affect APP endocytosis in 21DIV neurons. The difference in the effect of Pitstop 2 between 21DIV and 28DIV neurons need to be discussed in the main results section. Do APP endocytosis mechanisms change over time?

We understand the reviewers' doubts. To clarify, we used two inhibitors of clathrin-mediated endocytosis, the CPZ (chlorpromazine) and Pitstop2. CPZ was very efficient in blocking APP endocytosis at 21 and 28 DIV. Because Pitstop2 inhibited APP endocytosis less than CPZ even at 28 DIV, we assumed that Pitstop2 was less efficient. Moreover, APP was known to internalize via clathrin-mediated endocytosis (Cirrito et al., 2008; Cossec et al., 2010; Marquez-Sterling et al., 1997), that together with our data support our interpretation that APP is endocytosed via clathrin. Furthermore, our main question was if APP endocytosis in aged neurons was dependent on clathrin. Since at 28DIV, both Pitstop2 and CPZ inhibited APP endocytosis, we concluded that APP endocytosis in aged neurons was mediated by clathrin. Our data suggest that the mechanisms do not change over time. Instead, there seems to exist an increase in the quantity of clathrin-mediated endocytosed APP. We have clarified this in the main results section.

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Line 321 and below "There are two endocytic adaptors genetically linked to Alzheimer's disease, CD2AP, and CALM..."
We would suggest removing the data about CD2AP and CALM as they are not really informative.

We agree with the reviewer that this data does not prove the function of CALM and CD2AP in APP endocytosis with aging, but we found that the enrichment of CALM and CD2AP at APP endosomes so striking that we would like to share it with the community. It can potentiate research on analyzing the function of these two late-onset risk factors during aging. We would prefer to keep the data in the manuscript.

Line 354 "Importantly, the number of synapses increased significantly by 42% and 52% when we treated with DAPT or BACE inhibitor IV, respectively (Fig.7C, F, G, J, M, N)." The experiment may suggest a protection from loss rather than an increase. For example, the authors could indicate a stabilisation of the number of synapses between 21DIV and 28DIV or an inhibition of the decrease of the numbers of synapses instead of an increase of synapses...

We have altered the text to explain better the point raised by the reviewer.

Line 367 "fAD" needs to be defined.

Thanks for the careful review. We have now defined fAD.

In regard to the cell images in the figures, drawing the borders of the neurons would help to clarify the figures as some staining seems to be outside of the neurons/dendrites (ie on Fig.1 G-H, Fig.3 D/E/F/G). The means on the graphs are not always clearly visible and needs to be put in front (ie on Fig. 3 X/Y/Z/AA). It would help the reader if the colour code and order of the drugs in the figures is consistent between figures (Fig 6. and Fig.S3.); at the moment the order and colouring differ.

We have changed all figures and graphs according to the reviewer's suggestions.

Fig.2H-I panels are not commented in the text. They should be removed or discussed.

The reviewer may have missed the text's reference because these panels (now Fig. 2I-L) are commented together with the in vitro data (lines 196-212).

In the model presented, endocytosis is enhanced due to increased levels of clathrin, by so why is TfR endocytosis decreased? This appears to be a contradiction.

We agree that the reduction of TfR endocytosis is unexpected, given that it is the canonical marker of clathrin-mediated endocytosis. In the literature, we found that iron homeostasis changes with aging, more specifically, that TfR is reduced in the aging brain and that increased oxidative stress prevents iron uptake by reducing the transferrin receptor at the surface(Lu et al., 2017; Malorni et al., 1998). These data are compatible with an iron accumulation in the brain, and a compensatory reduction in iron uptake via transferrin, leading to reduced expression of TfR. We did not pursue iron transport characterization in aged neurons because it opens a new research line. We decided to include this finding in the paper because it is the obvious control for APP endocytosis. We expanded the discussion of these results.
Furthermore, we now examined the endocytosis of LDL, another cargo of clathrin-mediated endocytosis, and found it increased in intensity and size in aged neurons (Fig. 5K-N). Thus, transferrin decreased endocytosis seems to be the exception and not the rule. In summary, the increase in APP endocytosis is more pronounced than FM1-43 and LDL; thus, there might be a higher sensitivity of APP endocytosis to aging.

Reviewer #3 (Significance (Required)):

In this study, Burrinha et al. report an increase of APP endocytosis with aging in aged neurons and aged brains. The strength of their experimental design is the inclusion of in vitro and in vivo

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experiments with a range of different cellular and molecular techniques together with very good quantification. However, their conclusions will need to be confirmed by inclusion of additional experiments and a number of key controls which are currently lacking. Reproducibility is also a concern given the methods used to culture neurons over extended periods. The relationship between APP processing, Abeta production, aging of neurons, and Alzheimer's disease is an important field that requires more research in order to understand the initiation and progression of Alzheimer's disease so that new treatments can be developed for neurodegenerative diseases.

Reviewer field of expertise: cell biology, neurobiology, intracellular trafficking of BACE1 and APP, confocal microscopy, neuron cell culture

We thank the reviewer for recognizing our data's strength, and we are convinced that we provided the essential controls. We understand the reviewer's concern with data reproducibility, but we have had several students reproducing some of the key findings, and thus, we believe that this system is reliable.

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Second decision letter

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MS ID#: JOCES/2020/255752

MS TITLE: Up-regulation of APP endocytosis by neuronal aging drives amyloid dependent-synapse loss

AUTHORS: Tatiana Burrinha, Isak Martinsson, Ricardo Gomes, Ana Paula Terrasso, Gunnar K Gouras, and Claudia Guimas Almeida
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This paper improved significantly since the last time I've reviewed it. I only have minor suggestions, as listed below.

Comments for the author

1. Page 8: The fact that the amount of secreted Abeta40 and A42 is unaltered in the media of aged neurons could also indicate a problem with Abeta secretion. Please discuss this possibility.
2. The title on page 8 contains RAB5. I am not sure I can find the evidence in this paragraph for that. Qualifications in Fig. 2 indicate unchanged and even decreased levels of RAB5.
3. The authors should discuss how recently failed trials with Verubecestat reconcile with their data in Fig. 7.
4. Finally, I could not find the information if the quantification for the mean intensity levels e.g. Fig. 1I, K is taking into account the area? Please provide this information. I assume the data are normalized to 1µm2 since otherwise, an increase in Abeta and APP in aged neurons could simply reflect their increased neuronal complexity and size with age (obvious in Fig. 1J).

Reviewer 2

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Advance summary and potential significance to field

In this manuscript from the laboratory of C. Almeida, who is an expert in Alzheimer's research and APP trafficking, the authors provide evidence that APP endocytosis increases by aging, which in turn accelerates Abeta production and loss of synapses. The manuscript is mainly based on studies of primary neurons differentiated for 20 or 28 days in vitro, modeling mature and aged neurons, respectively. Some key findings, including accumulation of APP in early endosomes and altered processing, were also validated in aged (18 month old) mice.

Comments for the author

Major points

1) In this study, the authors were able to show that a typical marker for ageing neurons, lipofuscin, is increased in DIV 28 but not in DIV21 neurons. They were also able to show that synaptic density decreases from DIV21 to DIV28. Accordingly, they considered DIV21 neurons as mature non-aged neurons and compared them with DIV28 neurons. For better comparability with other studies, the authors should provide information on the number of astrocytes in the culture. In addition, it would be helpful if the culture was also checked with markers for apoptosis (tunnel staining, caspase level).

2) Other studies reported enlarged early endosomes in aged AD models and AD patients. Here, a contribution of APPL1 was reported. It would be interesting to see if APPL1 and Rab5 levels are also increased in DIV28 neurons.

3) The authors show that DIV28 neurons have an increased level of Abeta in neurites, as indicated by 12F4 antibody stainings. To validate specificity of the observed immunoreactivity, control stainings of DIV28 APP KO primary neurons and aged mouse brains should be included in the study.

4) The authors show an enrichment of higher molecular weight CTFs, possibly representing beta or eta CTFs. For clearer assignment, loading controls with corresponding heterologously expressed CTF constructs should be shown. In case, increased expression of eta CTFs can be verified, the authors should also discuss its possible involvement in aging and loss of synapses. Here, experimental evidence clarifying the impact of Abeta or Aeta would be helpful.

5) The authors show in biotinylation experiments with DIV21 and DIV28 neurons that APP gets endocytosed increasingly in DIV28 neurons. At the same time, an increased amount of surface APP was detected in DIV28 neurons. This seems contradictory, as elevated endocytosis should cause a reduction and no increase of APP levels at the surface. One explanation might be that anterograde transport of APP to the cell surface is increased or that APP shedding is decreased in DIV28 neurons. The authors should provide experimental evidence to clarify this point.

6) The authors show an accumulation of APP in endosomes. The 22C11 antibody was used for this purpose. Based on our experience, the 22C11 antibody only recognizes denatured APP in e.g. Western Blot analyses. Therefore, APP KO controls should be used to demonstrate specificity of 22C11 staining for immunohistochemistry and immunocytochemistry.

7) Very interestingly is the observation that specifically APP endocytosis, but not canonical endocytosis, nor transferrin endocytosis is increased in DIV28 neurons. The authors should give a putative explanation for those findings.

8) Another highly interesting finding is that DIV28 neurons exhibit an increased influence of clathrin-independent endocytosis on surface APP and a stronger involvement of F-actin. Unfortunately, the authors did not provide any experimental data to address the underlying mechanism. Studies with siRNA against PICALM or other discussed candidates could help to clarify this point.

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9) The data shown in Figure 7 suggest that inhibition of BACE and gamma secretases can at least partially reverse the decline of glutamatergic synapses in DIV28 neurons. These results show significant but very minor changes. As these findings were not further validated in aged mice, the results should be interpreted more cautiously.

Minor points

- Abstract: As DIV28 neurons are a limited aging model, the statement in the abstract: “We identify the upregulation of the amyloid precursor protein actin- and clathrin-dependent endocytosis as a mechanism used by normal neuronal aging to increase the intracellular production of Aβ.” should be toned down.
- Abstract: The sentence: “We modeled normal neuronal aging using aged mouse primary neurons, that accumulate lysosomal lipofuscin and show synapse loss, and normal aged brain.” appears incomplete and should be corrected.
- Introduction: The flow of the text in the first part of the introduction is disturbed by disjointed sentences. This should be revised.
- Introduction: page 4 line 75: …, considered as the cellular aging hallmark (Mattson and Magnus, 2006).” should be changed to “…, considered as a cellular aging hallmark (Mattson and Magnus, 2006).”.
- The authors observed that the number of lipofuscin granules was significantly higher in the cell body of DIV28 neurons, whereas LAMP1 revealed unchanged. The authors should provide an explanation for this finding.
- Figure 3: The neuron shown in panel B appears non-vital. This image should be replaced.
- The authors used much higher n-values to demonstrate significant differences in neurites as in cell bodies. I assume that this is due to the fact that detection in neurites is more challenging. However direct comparisons between differences in neurites and cell bodies should be taken with more caution.

Reviewer 3

Advance summary and potential significance to field

In this study, Burrinha et al. report an increase of APP endocytosis with aging in aged neuronal cultures and aged brains. The strength of their experimental design is the inclusion of in vitro and in vivo experiments with a range of different cellular and molecular techniques together with very good quantification. The integration of high resolution confocal imaging, biochemical assays of APP processing, cell biological trafficking assays and neuronal function provides an in depth analyses of events using a model system of neuronal aging. The findings represent a significant contribution to the field.

Comments for the author

I previously reviewed this manuscript through Reviewer Commons and suggested an extensive set of additional experiments and key controls to improve the quality of the data and to strengthen the conclusions. To their credit, the authors have now performed and included all the essential experiments suggested in this current manuscript and the data are now very robust and conclusions are well supported and convincing. I have no additional concerns regarding the quality of data, reproducibility or conclusions.

One minor comment in relation to the details of the quantitative analyses of images. Previously, we indicated that it was not clear if the quantitation of IF was performed on optical sections or 3D reconstructions. If optical sections, how where they selected?

The authors have indicated in their reviewer comments that "The IF was performed on optical sections selected based on being best focused and on the IF signal's quality." However, this information appears to be absent in the manuscript text. A statement to this effect should be included in the methods section.

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Second revision

Author response to reviewers' comments

We thank the reviewers for their careful review of the revised manuscript.

Reviewer 1 Advance Summary and Potential Significance to Field:
This paper improved significantly since the last time I've reviewed it. I only have minor suggestions, as listed below.

Reviewer 1 Comments for the Author:
1) Page 8: The fact that the amount of secreted Abeta40 and A42 is unaltered in the media of aged neurons could also indicate a problem with Abeta secretion. Please discuss this possibility.

TEXT CHANGES We included on page 8 that Abeta secretion may be reduced.

2) The title on page 8 contains RAB5. I am not sure I can find the evidence in this paragraph for that. Qualifications in Fig. 2 indicate unchanged and even decreased levels of RAB5.

TEXT CHANGES We changed the title on page 8 for "APP processing and early endosome EEA1 increase with aging".

3) The authors should discuss how recently failed trials with Verubecestat reconcile with their data in Fig. 7.
The failure of BACE inhibitors may be multifactorial, including problems in patient's selection, the level of BACE1 inhibition, and the stage of AD at which to treat for optimal efficacy, but maybe more important are the secondary targets of BACE1 inhibition (Coimbra et al., 2018; Moussa-Pacha et al., 2020; Vassar, 2014). The experiment we performed aims at advancing our knowledge on the mechanisms of neuronal aging. We do not claim that this will be a therapeutic strategy, at least with the current inhibitors.
Given the journal space restrictions and the multiple reasons why the clinical trials failed, we find the discussion of this matter, although essential, out of this paper's scope.

4) Finally, I could not find the information if the quantification for the mean intensity levels e.g. Fig. 1I, K is taking into account the area? Please provide this information. I assume the data are normalized to 1µm2 since otherwise, an increase in Abeta and APP in aged neurons could simply reflect their increased neuronal complexity and size with age (obvious in Fig. 1J).

We provided information on how we analyzed the data normalizing the mean intensity per area indicated in the methods (page 28).

Reviewer 2 Advance Summary and Potential Significance to Field:
In this manuscript from the laboratory of C. Almeida, who is an expert in Alzheimer's research and APP trafficking, the authors provide evidence that APP endocytosis increases by aging, which in turn accelerates Abeta production and loss of synapses. The manuscript is mainly based on studies of primary neurons differentiated for 20 or 28 days in vitro, modeling mature and aged neurons, respectively. Some key findings, including accumulation of APP in early endosomes and altered processing, were also validated in aged (18 month old) mice.

Reviewer 2 Comments for the Author:

Major points

1) In this study, the authors were able to show that a typical marker for ageing neurons, lipofuscin, is increased in DIV 28 but not in DIV21 neurons. They were also able to show that synaptic density decreases from DIV21 to DIV28. Accordingly, they considered DIV21 neurons as mature non-aged neurons and compared them with DIV28 neurons. For better comparability with other studies, the authors should provide information on the number of astrocytes in the culture.

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In addition, it would be helpful if the culture was also checked with markers for apoptosis (tunnel staining, caspase level).

In the revision of the paper, we included western blot data for GFAP, which shows that some glia cells in our cultures do not increase consistently with aging, and thus, the astrocytes putative contribution to the western blots analysis was not considered as significant. In the immunofluorescence analysis, we only analyzed neurons, distinguished by morphology and by MAP2 labeling.
Regarding apoptosis, in previous work, we worked with primary fAD neurons that showed synapse loss at 19 DIV (Almeida et al., 2005), we measured apoptosis and cell death, and we did not detect changes.
Aging neurons show synapse loss without processes degeneration that precedes apoptosis and neuronal death (Yankner et al., 2008). Here, using wild-type neurons aged in culture whenever cultures showed processes degeneration were excluded. Primary cultures show a degree of variability, and we used a stringent selection of our cultures for the studies presented in this manuscript.

2) Other studies reported enlarged early endosomes in aged AD models and AD patients. Here, a contribution of APPL1 was reported. It would be interesting to see if APPL1 and Rab5 levels are also increased in DIV28 neurons.
Thanks for the suggestion. Here, we are modeling normal aging, and we found rab5 positive endosomes increased (Fig. 3). We are also intrigued by the mechanisms underlying the early endosomes' increase and find the link with APPL1 attractive. We believe that this paper will provide food for thought and fuel novel research avenues, including investigating APPL1 positive pre-early endosomes or signaling endosomes in aging neurons in future work.

3) The authors show that DIV28 neurons have an increased level of Abeta in neurites, as indicated by 12F4 antibody stainings. To validate specificity of the observed immunoreactivity, control stainings of DIV28 APP KO primary neurons and aged mouse brains should be included in the study.
Because several groups have shown intracellular Abeta42 to increase in the normal aged brain (Baker- Nigh et al., 2015; Blair et al., 2014; Kikuchi et al., 2011; Lesné et al., 2013; Marks et al., 2017), we decided to use the difficult to obtain aged brains to analyze APP processing and APP localization in EEA1 positive early endosomes and used the last sections of old brains for analyzing Rab5 as reviewers requested in the first revision. We are now aging mice but do not foresee the next 12 months to have enough tissue available. Upon reviewer's request we included APP KO neurons at 19DIV, showing virtually no labeling by 12F4. We used this antibody in a previous work where we showed that DAPT and BACE inhibitor reduces 12F4 labeling. We consider that these controls are sufficient to support our interpretation that Abeta42 increases in neurites with aging in culture as it does in vivo in mice and humans, as we wrote in the rebuttal letter of the first revision.

4) The authors show an enrichment of higher molecular weight CTFs, possibly representing beta or eta CTFs. For clearer assignment, loading controls with corresponding heterologously expressed CTF constructs should be shown. In case, increased expression of eta CTFs can be verified, the authors should also discuss its possible involvement in aging and loss of synapses. Here, experimental evidence clarifying the impact of Abeta or Aeta would be helpful.
Although we cannot exclude the contribution of eta-CTFs toxicity (Willem et al., 2015), given the overwhelming amount of data proving Abeta synaptotoxicity, we interpreted the data accordingly. We believe that better identifying the APP CTFs will not substantively change the central message of the paper.

5) The authors show in biotinylation experiments with DIV21 and DIV28 neurons that APP gets endocytosed increasingly in DIV28 neurons. At the same time, an increased amount of surface APP was detected in DIV28 neurons. This seems contradictory, as elevated endocytosis should cause a reduction and no increase of APP levels at the surface. One explanation might be that anterograde transport of APP to the cell surface is increased or that APP shedding is decreased in DIV28 neurons. The authors should provide experimental evidence to clarify this point.
The other reviewers brought up this issue in the first revision. We observed, as previously reported, that only a small fraction of surface APP undergoes endocytosis, does even with the increase in endocytosis observed, it may not be sufficient to reduce surface APP. We observed an

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increase in APP recycling that may contribute to this surface increase. Nevertheless, we agree that there is probably an increase in anterograde or a decrease in retrograde APP trafficking that we will address experimentally. We discussed this possibility in the discussion (page 19).

6) The authors show an accumulation of APP in endosomes. The 22C11 antibody was used for this purpose. Based on our experience, the 22C11 antibody only recognizes denatured APP in e.g. Western Blot analyses. Therefore, APP KO controls should be used to demonstrate specificity of 22C11 staining for immunohistochemistry and immunocytochemistry.
This point was also raised in the first revision. Please see the rebuttal letter and the supplementary figure S3, where we show that APP KO has reduced 22C11 staining.
We used 22C11 in immunocytochemistry for detecting surface APP, which we also measured by biotinylation. We mostly used 22C11 to follow APP endocytosis in live neurons, which may enhance specificity due to the lack of fixative.

7) Very interestingly is the observation that specifically APP endocytosis, but not canonical endocytosis, nor transferrin endocytosis is increased in DIV28 neurons. The authors should give a putative explanation for those findings.
We thank the reviewer for considering our findings very interesting. We want to point the reviewer to our discussion where we provide a putative explanation for the increase in APP endocytosis.

8) Another highly interesting finding is that DIV28 neurons exhibit an increased influence of clathrin- independent endocytosis on surface APP and a stronger involvement of F-actin. Unfortunately, the authors did not provide any experimental data to address the underlying mechanism. Studies with siRNA against PICALM or other discussed candidates could help to clarify this point.
We thank the reviewer again for considering our results as highly interesting. We want to clarify that 28DIV shows an increased influence of clathrin-DEPENDENT endocytosis, which we also discovered to be DEPENDENT on F-ACTIN. We would love to pursue the dissection of the mechanisms involved, but we have first to solve a technical challenge related to siRNA use in aged primary cultures. We will work on this, hoping in the future to discover the mechanism whereby aging increases APP endocytosis.

9) The data shown in Figure 7 suggest that inhibition of BACE and gamma secretases can at least partially reverse the decline of glutamatergic synapses in DIV28 neurons. These results show significant but very minor changes. As these findings were not further validated in aged mice, the results should be interpreted more cautiously.

TEXT CHANGES We found the reduction in synapse number in aging neurons to be small. We agree that these should be confirmed in vivo. We interpreted the results more cautiously.

Minor points

Abstract: As DIV28 neurons are a limited aging model, the statement in the abstract: "We identify the upregulation of the amyloid precursor protein actin- and clathrin-dependent endocytosis as a mechanism used by normal neuronal aging to increase the intracellular production of Aß." should be toned down.

TEXT CHANGES We have changed this sentence, not including the mechanism part for which we have primarily in vitro data.

Abstract: The sentence: "We modeled normal neuronal aging using aged mouse primary neurons, that accumulate lysosomal lipofuscin and show synapse loss, and normal aged brain." appears incomplete and should be corrected.

TEXT CHANGES We have changed the sentence according to the reviewer's suggestion.

Introduction: The flow of the text in the first part of the introduction is disturbed by disjointed sentences. This should be revised.
TEXT CHANGES We revised the first part of the introduction.

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Introduction: page 4 line 75: …, considered as the cellular aging hallmark (Mattson and Magnus, 2006)." should be changed to "…, considered as a cellular aging hallmark (Mattson and Magnus, 2006).".

TEXT CHANGES We changed the text accordingly.

The authors observed that the number of lipofuscin granules was significantly higher in the cell body of DIV28 neurons, whereas LAMP1 revealed unchanged. The authors should provide an explanation for this finding.

We observe that not all LAMP1 positive endolysosomes contain lipofuscin. One reason could be that LAMP1 also identifies late-endosomes, which are unknown to accumulate lipofuscin.

Figure 3: The neuron shown in panel B appears non-vital. This image should be replaced.

We are unsure what the reviewer refers to in panel B. Panel B in figure 3 is a graph. If the author refers to the second panel in figure 3A, the image shows EEA1 labeling after background subtraction that eliminates the background that would classically show the neuronal morphology. Although this presentation favors the evaluation of endosomes does not facilitate the evaluation of the cellular viability.

The authors used much higher n-values to demonstrate significant differences in neurites as in cell bodies. I assume that this is due to the fact that detection in neurites is more challenging. However direct comparisons between differences in neurites and cell bodies should be taken with more caution.

We understand the reviewer's concern and would like to clarify that, for each neuron, we analyze its one cell body and, on average, three neuritic segments, and that's why the number of neurites is at least 3 times higher than the number of cell bodies. Nevertheless, we always compare the average differences per experiment in addition to the statistics of individual neurites. We chose these plots because it represents the actual measures, according to the most recent recommendations.

Reviewer 3 Advance Summary and Potential Significance to Field:
In this study, Burrinha et al. report an increase of APP endocytosis with aging in aged neuronal cultures and aged brains. The strength of their experimental design is the inclusion of in vitro and in vivo experiments with a range of different cellular and molecular techniques together with very good quantification. The integration of high resolution confocal imaging, biochemical assays of APP processing, cell biological trafficking assays and neuronal function provides an in depth analyses of events using a model system of neuronal aging. The findings represent a significant contribution to the field.

Reviewer 3 Comments for the Author:
I previously reviewed this manuscript through Reviewer Commons and suggested an extensive set of additional experiments and key controls to improve the quality of the data and to strengthen the conclusions. To their credit, the authors have now performed and included all the essential experiments suggested in this current manuscript and the data are now very robust and conclusions are well supported and convincing. I have no additional concerns regarding the quality of data, reproducibility or conclusions. One minor comment in relation to the details of the quantitative analyses of images. Previously, we indicated that it was not clear if the quantitation of IF was performed on optical sections or 3D reconstructions. If optical sections, how where they selected?

The authors have indicated in their reviewer comments that "The IF was performed on optical sections selected based on being best focused and on the IF signal's quality." However, this information appears to be absent in the manuscript text. A statement to this effect should be included in the methods section.

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TEXT CHANGES

We thank the reviewer for the positive assessment of our work. We included this info in the methods section.

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Third decision letter

MS ID#: JOCES/2020/255752

MS TITLE: Up-regulation of APP endocytosis by neuronal aging drives amyloid dependent-synapse loss

AUTHORS: Tatiana Burrinha, Isak Martinsson, Ricardo Gomes, Ana Paula Terrasso, Gunnar K Gouras, and Claudia Guimas Almeida
ARTICLE TYPE: Research Article

Thank you for sending your manuscript to Journal of Cell Science through Review Commons.

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

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