EMBO
reports

Manuscript EMBO-2016-42738

Bin1 and CD2AP polarise the endocytic generation of beta-amyloid

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Review timeline:

Submission date:	18 May 2016
Editorial Decision:	20 June 2016
Revision received:	07 September 2016
Editorial Decision:	11 October 2016
Revision received:	14 October 2016
Accepted:	19 October 2016

Editor: Esther Schnapp

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 20 June 2016

Thank you for the submission of your research manuscript to EMBO reports. We have now received the referee reports that are pasted below.

As you will see, all referees agree that the findings are interesting and novel. However, they also have several suggestions for how the study could be improved, and given that all concerns and comments are rather straightforward, partially overlap, and include only little new experimentation, they should all be addressed.

We would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

REFEREE REPORTS

Referee #1:

In their manuscript, Ubelmann and colleagues have investigated how altering expression levels of Bin1 and CD2AP in cells and neurons affects Abeta generation. Bin1 and CD2AP emerged from GWAS studies as LOAD risk factors but how they impact on the disease remains largely uncharted territory. The authors explored the effects of KD and OE in two cellular models, (differentiated)

Neuro2A and differentiated primary hippocampal neurons. Particularly the latter has been very instrumental in identifying a role for both endocytic regulators in the polarized generation of Abeta. The most exciting part of their story is the fact that they discovered different sorting events that regulate the encountering of the substrate, APP, with the sheddase, BACE1. Bin1 seems to act mainly in axons where it regulates recycling of BACE1, while CD2AP may be a gatekeeper for endocytic sorting of APP in dendrites. KD of either proteins increases the residence time of APP and BACE1 in early/sorting endosomes, resulting in net accumulations of Abeta. Overall this is a very well documented manuscript with highly relevant data and findings; the authors use majorly imaging approaches and which are of high quality and well controlled. It provides significant progress in a still poorly explored area and a strong validation for the genetic studies. I include below some major and minor comments that need to be addressed to further improve the paper and make it suitable for publication.

Major comments:

The authors developed a semi-quantitative imaging approach for intracellular endogenous Abeta42 which provides an important read-out for studying the effects of altered expression of Bin1 and CD2AP. Although the 12F4 is often use to detect Abeta42, the endogenous levels in murine neurons remain extremely low for an easy detection by classical ICC and confocal microscopy. Given its weight for the paper, the authors should include more controls for their semi-quantitative immunofluorescence based Abeta42 assay, for instance using g-secretase and BACE1 inhibitors for an extended time: in both cases, specific Abeta42 immunofluorescencs should dramatically decrease underscoring the specificity of the assay. Secondly, the authors should rule out that some crossreactivity occurs with the C99 C-terminal fragment: an easy assay could be to transfect cells with C99-GFP in the absence or presence of a saturating doses of g-secretase inhibitor, or even better, in wt and PSEN dKO cells. The 12F4 should not give any signal in the latter cells or very low in the presence of inhibitors.

I wonder whether the immunoprecipitation data are really needed. Firstly, these are single experiments not independently confirmed by other methods and require more scrutiny. Secondly, without further identification of for instance interacting domains, these data remain suggestive. I don't see the importance of including them and at least it does not affect the paper by omitting them. The Discussion section is rather poor and requires improvement. The authors largely limit the discussion to re-iterating on their findings without much relation to the literature, next questions or future directions. For instance I miss some more in depth discussion on the extent that Bin1 and CD2AP are potential important risk factors. While there is no clear idea how expression levels are altered in LOAD, the current data suggest that it might be likely lower expression of Bin1 and CD2AP variants that contribute to disease. How is this studied for other candidate risk factors? Thirdly, the authors measure mostly intracellular Abeta when they evaluate the effects of altered expression of Bin1 and CD2AP. Interestingly, intracellular Abeta accumulation is an early feature in disease progression and in particular Abeta42 accumulates in MVBs where the more acidic environment promotes aggregation underscoring pathological relevance (reviewed in Peric and Annaert, 2015). On the other hand endo/lysosomal abnormalities are also among the earliest features observed in AD pathogenesis. Also Cell reported just now a prominent role for PSEN2 in contributing most to this intracellular Abeta pool, which occurs, due to its restricted localization in MVBs/lysosomes (Sannerud et al., 2016, Cell). Although speculative at the moment, it might be worth discussing whether LOAD risk factors may more pronouncedly affect the intracellular pool?

Minor (compulsory) comments:

Line 30: Excessive generation of Abeta is only a trigger in a subset of FAD. In many FAD mutations, like in PSENs, there is in fact less Abeta but the ratio shifts to longer Abeta.

Line 107 to 112: Reference to fig.2 is wrong and should be Fig. S2 (and appropriate panels), no? In this section, the authors suggest that the 40% loss after 60 min chase is in agreement with APP degradation in the lysosome. As supported by several papers, I do not dispute that APP is not degraded in this route, but I am less convinced that this occurs via classical lysosomal degradation. There are namely g-secretases in this route as well (see also Sannerud et al., 2016, but also earlier proteomic studies on isolated lysosomes (Pasternak et al)), and hence also this pool could follow the canonical degradation by dual processing. In fact, no real study exists that makes a balance sheet

between the pool degraded through beta/alpha-gamma-secretase or lysosomal enzymes and the latter pool might be rather small.

Line 163. Here an alternative explanation could be that CD2AP depletion results as well in halting cargo delivery from maturing endosomes in dendrites to mature lysosomes in cell bodies. This could explain the increase 22C11 in dendrites and decrease in cell bodies.

Line 225: '... recapitulating the early endosome enlargement that occurs early in AD.' This is too speculative here, and should be rather included in the discussion (see major comment).

Line 237: the authors state here that downregulation of Bin1 as well as CD2AP results in an increased colocalization of APP with BACE1. If so, why are opposite effects observed for APP-CTF (Fig. 1h-i)?

Line 254 and following: The authors measure here circularity and length of each carrer and conclude that they are increased in Bin1-depleted axons. However, I find this set of data a bit far-fetched given the low resolution of classic confocal microscopy. I would limit it here by stating that endosomes appear more extended and enlarged (so measure volume) as the actual evidence of a failure of tubule scission is far more convincing with the live imaging data (fig 6e-f).

Line 289 and following: the authors report here that upon inhibitor treatment or CD2AP combined with Rab5QL, APP is in the limiting membrane. This has been demonstrated already much earlier (then the by several other groups (Schneider, Rajendran et al 2006; Schneider, Rajendran et al 2008; Sannerud et al. 2011) and should be included. Secondly, it is an overstatement to say that APP localized to the lumen of enlarged Rab5 QL endosomes while in CD2AP it moved to the limiting membrane as the authors cannot distinguish between full-length APP and APP-CTF. Additional controls with BACE1 inhibitors (e.g. see Esselens et al 2012; Sannerud et al., 2011; Rajendran et al. 2006) might be considered.

Line 313: the authors didn't study KO of Bin1 and CD2AP but KD. So 'absence' is an overstatement.

Line 319: the link to cellular propagation of seeds is too speculative and lacks support.

Line 369: 'We uncover the mechanisms by which APP and BACE1 segregate at early endosomes..." Although the authors have revealed novel sorting regulation, they ignore other mechanisms that also regulate the encounter/segregation of APP and BACE1. For instance Sannerud et al (2011) demonstrated that APP and BACE1 follow distinct internalization routes in cells and neurons which should be included in the discussion. Moreover, worth mentioning is the fact that the contribution of axonally produced Abeta is very low compared to dendritic Abeta: this is relevance in a discussion on the relative contributions of Abeta pools to the disease or to propagation.

Specific remarks on the figures:

- For both Bin1 and CD2AP independent siRNA should be tested on a few basic readouts to be sure that the effects are not caused by off-targets.
- Fig. 1I shows a decrease in APP-CTF/APP while there is no effect on this ratio in FigS1d. Please explain.
- It gives a better overview if panels S4d-e are included in Figure 3 (connected to panel I).
- I find the presentation of the data in figures S2 and S3 confusing. A better way might be to cluster the data on APP in S2 and on BACE1 in S3.
- Fig S4b: immunostaining for Bin1/Ank is not convincing. It should demonstrate Bin1 in axons but most is in dendrites (inset).

Referee #2:

In this study, Ubelmann et al explore the role of Bin1 and CD2AP, two genes implicated as risk factors for late-onset Alzheimer's disease (AD). Both are regulators of endocytic trafficking, but their role in development of AD is yet unknown. The authors argue that both Bin1 and CD2AP depletion increase production of amyloid beta by increasing the interaction between APP and BACE1, though they act differently in the dendritic and axonal compartments. The authors report

that Bin1 depletion prevents BACE1 recycling back to the plasma membrane in the axon, whereas CD2AP depletion prevents APP lysosomal degradation in the dendrite. The route of APP through the endo-lysosomal pathway and its points of regulation are of significant interest to the field of AD, both to understand how AD pathology develops and to identify potential targets for AD therapeutics.

Overall, the paper is well laid out and the experiments are clear. The authors extensively studied the differential regulation of APP processing in axons versus dendrites and provided mechanistic clues as to the potential role of Bin1 and CD2AP in AD pathology. However, there are a few elements which need to be addressed before publication and are as follows:

<u>Major critiques:</u>

1. In light of a recent manuscript that have come out regarding Bin1 and its role in BACE1 trafficking (Miyagawa et. al., 2016), the authors should discuss how their data supports or refutes these new findings.

2. The authors interpret that the residual 22C11 signal seen in many of their siCD2AP experiments (for example, Figure 2d) is due to a lack of APP lysosomal degradation because APP is not sorted into intraluminal vesicles (ILVs). However, lysosomal degradation is not specifically tested by any kind of inhibitor (bafilomycin, NH4Cl) and in fact the retention of 22C11 signal could be due to an inhibition of cleavage of the N-terminal APP due to APP sequestration in ILVs. The authors should specifically test lysosomal degradation using both immunofluorescence and biochemical techniques or adjust their interpretation accordingly.

<u>Other critiques:</u>

3. In figure 1, it is important to show the specificity of AB42 staining, perhaps by conducting experiments in an APP knockdown background or with BACE1 or gamma-secretase inhibitors.

4. In figure 1, what happens to intracellular levels of AB40? Since there are changes in extracellular secreted AB40, intracellular levels should be examined as well. Additionally, the authors state that there is a tendency for higher secretion of AB42, which is not clear in figure (1g). How do they explain a decrease in extracellular AB40?

5. In figure 1 and S1, the method of normalization of APP-CTF levels is inconsistent, either to full-length APP (Fig. 1h) or tubulin (Fig. S1d). The authors should be consistent.

6. In figure 2, the authors should include label of 22C11 antibody staining, and include time of chase for each experiment

7. In figure 3d, should the y-axis label be % of time point 0?

8. In figures 6 & 7, the quantifications lack error bars. Even if they are % values from pooled experiments, the authors should express the variability from independent experiments. Data should be quantified such that the amount of variability between experiments can be expressed. Alternatively, they can use more appropriate statistical tests, such as chi-square.

Referee #3:

In this manuscript, authors examined the role of Bin1 and CD2AP in Abeta production and vesicular trafficking in neurons. They found that downregulation of Bin1 and CD2AP increased the production of Abeta by distinct mechanisms. Bin1 regulates BACE1 recycling from early endosome, and CD2AP controls the sorting of APP to the degradation pathway. Depletion of CD2AP and Bin1 increased the encounter of APP and BACE1 at early endosome, thereby increasing the Abeta production. The research design is adequate, the methods used for this study and data obtained are solid. I have several comments and suggestions for the authors to consider improving the quality of the paper:

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1) They have shown that knockdown of CD2AP and Bin1 altered the levels of APP-CTFs (Fig. 1h). As gamma-secretase is also involved in the regulation of APP-CTF metabolism, the authors should show the levels of the gamma-secretase (e.g., nicastrin) in the lysates of siRNA-treated cells to exclude the possibility that the gamma-secretase activity was controlled.

2) It is general concern that tagging to intracellular region of the cargos affects their vesicular trafficking. Authors should examine the subcellular localization of endogenous APP as well as BACE1 in Bin1 or CD2AP-depleted N2a cells (or primary neurons) by immunocytochemistry.

3) Authors indicated that knockdown of CD2AP inhibited the sorting of APP to the ILVs. However, the levels of APP holoprotein was now altered in siCD2AP treated cells. Why? Authors should discuss this issue.

4) Bin1 depletion inhibited the tubule scission for BACE1 in primary neurons. Is this phenomenon is specific to the BACE1?

5) Recently, similar result for Bin1 was reported (Miyagawa et al., Hum Mol Genet 2016 in press). Authors should refer this and discuss the functional aspect of Bin1 in BACE1 trafficking.

1st Revision - authors' response 07 September 2016

We would like to thank you for the opportunity to revise our EMBOR-2016-42738V1 manuscript entitled "Bin1 and CD2AP polarise the endocytic generation of beta-amyloid" based on the positive initial reviews. We believe that our work is a very important contribution to the understanding of the cell biological mechanisms of late-onset Alzheimer's disease (AD). We would also like to thank the three reviewers for their constructive comments, which helped us to significantly improve the manuscript. We have addressed all points raised by the reviewers.

Importantly, regarding reviewer 1 and 2 concerns with the sensitivity and specificity of the antibodies used in our novel method to detect endogenous Aβ42 we have added a whole new figure (EV1) that includes all control experiments suggested. Moreover, we included, for review only, data from Pr. Gunnar K. Gouras (Lund University) using APP knockout neurons.

Regarding reviewer's 1 suggestion of removing the co-IP data from the paper (point 2), we would like to ask your opinion. We agree with the reviewer that co-IP does not prove interaction. Thus we altered our interpretation of the data to indicative of association but would prefer to keep the data in the paper since it strengthens our findings.

Regarding reviewer's 1 and 3 suggestions we expand our discussion with two new subsections and discussed the recent findings by Miyagawa et. al 2016.

Regarding reviewer's 1 and 2 concerns about APP degradation in the lysosome we have performed the suggested experiments of APP degradation in the presence of lysosomal inhibitor (Fig. EV3C and EV3D). We address each point brought up by the three reviewers below and highlighted in the text all changes in yellow.

Referee #1

Major comments:

The authors developed a semi-quantitative imaging approach for intracellular endogenous Abeta42 which provides an important read-out for studying the effects of altered expression of Bin1 and CD2AP. Although the 12F4 is often use to detect Abeta42, the endogenous levels in murine neurons remain extremely low for an easy detection by classical ICC and confocal microscopy.

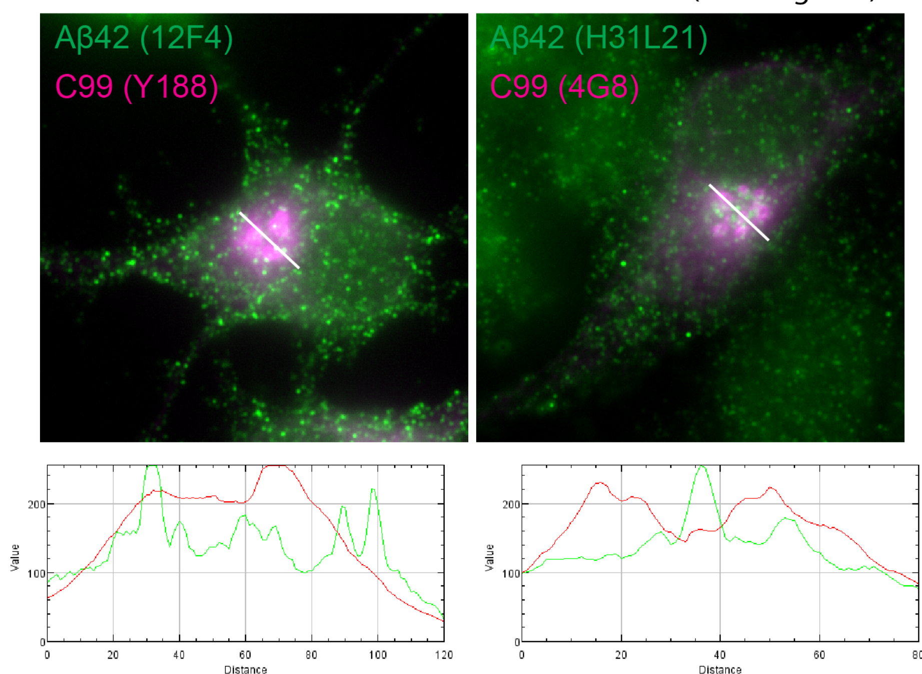
Given its weight for the paper, the authors should include more controls for their semi-quantitative immunofluorescence based Abeta42 assay, for instance using g-secretase and BACE1 inhibitors for an extended time: in both cases, specific Abeta42 immunofluorescencs should dramatically decrease underscoring the specificity of the assay.

Response: We understand reviewer 1's concern relative to the difficulty of detecting endogenous levels in murine neurons. Until recently the intracellular pool of Aβ42 has been understudied mainly because extracellular Aβ is more easily detected. We have been successful many years ago in detecting intracellular Aβ42 by immunofluorescence in neurons overexpressing APP with the AD familial Swedish mutation(Almeida et al., 2006; Takahashi et al., 2004). Now we have been able by optimizing our assay to consistently detect endogenous Aβ42, and significant positive and negative changes of its levels.

As suggested, we have now done additional experiments to control Aβ42 detection with 12F4 by immunofluorescence (Fig. EV1). Additionally, since we also use the anti-Aβ42 (H31L21) we included the requested controls also for this antibody. We found that treatment with the gamma-secretase inhibitor DAPT for an extended time (48h) more efficiently reduced the levels of Aβ42 detected with 12F4 or H31L21. BACE1 inhibition with compound IV also reduced Aβ42 levels. Although endogenous intracellular Aβ42 is not as easily detected as when APP is overexpressed our previous and suggested controls confirm the reliability and robustness of our semi-quantitative assay of endogenous intracellular Aβ42.

Secondly, the authors should rule out that some crossreactivity occurs with the C99 C-terminal fragment: an easy assay could be to transfect cells with C99-GFP in the absence or presence of a saturating doses of g-secretase inhibitor, or even better, in wt and PSEN dKO cells. The 12F4 should not give any signal in the latter cells or very low in the presence of inhibitors.

Response: According to reviewer 1's suggestion, we expressed a C99 C-terminal fragment construct (made by Virginia Lee) in N2a cells treated or not with DAPT (Fig.EV1C and EV1D). We observed no clear crossreactivity since the signal of Aβ42 (12F4 and H31L21) did not overlap with that of C99, see also below that their intensity profiles do not overlap. We also found that DAPT treatment reduced Aβ42 signal despite the overexpression and excess of APP CTFs. In addition, the residual signal did not overlap with APP CTFs. This rules out some crossreactivity with C99 and further confirms the specificity of the anti- Aβ42 used in this study. Moreover, we suggest reading the reply to reviewer 2 other critiques point 1, where additional Aβ42 immunofluorescence of APP knockout neurons with 12F4 and H31L21, show no specific signal.



I wonder whether the immunoprecipitation data are really needed. Firstly, these are single experiments not independently confirmed by other methods and require more scrutiny. Secondly, without further identification of for instance interacting domains, these data remain suggestive. I don't see the importance of including them and at least it does not affect the paper by omitting them.

Response: We understand the reviewer's point on coimmunoprecipitation experiments being only suggestive of interaction. We observed co-immunoprecipitation of BACE1 with Bin1 and APP with CD2AP which reinforces the colocalization observed by immunofluorescence. We would prefer to tone down the interpretation of this experiment than removing it completely. We believe that together these two pieces of evidence confirm that Bin1 is associated with BACE1 and CD2AP with APP. We also believe the inclusion of this data will steer the scientific community to further study the interacting domains and show direct interaction. Thus we removed "interaction" and reinforced that the co-immunoprecipitation indicates cellular proximity and is only suggestive of interaction (line 191; line 211). We also removed "interaction" from the discussion line 401.

The Discussion section is rather poor and requires improvement. The authors largely limit the discussion to re-iterating on their findings without much relation to the literature, next questions or future directions. For instance I miss some more in depth discussion on the extent that Bin1 and CD2AP are potential important risk factors. While there is no clear idea how expression levels are altered in LOAD, the current data suggest that it might be likely lower expression of Bin1 and CD2AP variants that contribute to disease. How is this studies for other candidate risk factors? Secondly, the authors measure mostly intracellular Abeta when they evaluate the effects of altered expression of Bin1 and CD2AP. Interestingly, intracellular Abeta accumulation is an early feature in disease progression and in particular Abeta42 accumulates in MVBs where the more acidic environment promotes aggregation underscoring pathological relevance (reviewed in Peric and Annaert, 2015). On the otherhand endo/lysosomal abnormalities are also among the earliest features observed in AD pathogenesis. Also Cell reported just now a prominent role for PSEN2 in contributing most to this intracellular Abeta pool, which occurs, due to its restricted localization in MVBs/lysosomes (Sannerud et al., 2016, Cell). Although speculative at the moment, it might be worth discussing whether LOAD risk factors may more pronouncedly affect the intracellular pool?

Response: We appreciate reviewer 1 support of intracellular Aβ and of the role of endocytic abnormalities in AD. This topic is central to our current and past research (Almeida et al., 2005, 2006; Snyder et al., 2005; Takahashi et al., 2004).

Regarding the potential of Bin1 and CD2AP as AD risk factors we took reviewer's suggestion and extended our discussion with a full separate subsection (See paragraph starting on line 418). Moreover, concerning the point on the expression levels of AD risk factors we have extended the introduction on the expression of Bin1 and CD2AP in Alzheimer's including a reference to a recent paper by the Thinakaran lab(De Rossi et al., 2016) and on other endocytic risk factors (line 44).

Regarding reviewer 1's last point on the role of the endocytic risk factors on the intracellular pool of Aβ. We agree that this pool of Aβ is often unconsidered in the progression of the disease. In previous work we showed that oligomerization of Aβ starts in MVBs and correlates with pathology(Takahashi et al., 2002, 2004); that Aβ targets synapses post-synaptically(Almeida et al., 2005; Snyder et al., 2005) and that Aβ promotes a decrease of glutamate receptors from synapses by increasing their endocytosis(Snyder et al., 2005). Importantly, it has been very recently reported that familial AD mutations in PS2 increase intracellular more than extracellular Aβ(Sannerud et al., 2016). In the current study we mostly concentrated on intracellular Aβ because we wanted to determine if there were subcellular differences in Aβ production in axons vs. dendrites. However, our data revealed that Bin1 and CD2AP loss of function increased mainly intracellular Aβ. These effects could be explained by our findings on Bin1 and CD2AP regulation of Aβ generation, which occurs intracellularly. Extracellularly, we did not observe an increase in Aβ42 and observed a decrease in Aβ40 secretion. Future experiments will be needed to determine how Bin1 and CD2AP affect the levels of extracellular Aβ. Please also read the answer to reviewer 2 other critiques point 3 regarding the mechanisms of Aβ secretion. In the future we also agree that it will be interesting to determine the relative contribution of other risk factors in regulating the intra- vs extracellular pools of Aβ. We have now addressed the raised point in a new subsection of the discussion about intracellular Aβ starting on line 344.

Minor (compulsory) comments:

Line 30: Excessive generation of Abeta is only a trigger in a subset of FAD. In many FAD mutations, like in PSENs, there is in fact less Abeta but the ratio shifts to longer Abeta.

Response: We have changed the text in lines 32-33.

Line 107 to 112: Reference to fig.2 is wrong and should be Fig. S2 (and appropriate panels), no? In this section the authors suggest that the 40% loss after 60 min chase is in agreement with APP degradation in the lysosome. As supported by several papers, I do not dispute that APP is not degraded in this route, but I am less convinced that this occurs via classical lysosomal degradation. There are namely g-secretases in this route as well (see also Sannerud et al., 2016, but also earlier proteomic studies on isolated lysosomes (Pasternak et al)), and hence also this pool could follow the canonical degradation by dual processing. In fact, no real study exists that makes a balance sheet between the pool degraded through beta/alpha-gamma-secretase or lysosomal enzymes and the latter pool might be rather small.

Response: We double-checked the references that we found to be correct for Figure 2 but indeed there was a reference missing to panel 2d which we have now corrected in line 128.

About APP degradation in the lysosome: Since 1992, when Christian Haass and Dennis Selkoe showed that membrane APP increases when the lysosome is inhibited, several groups, as the reviewer agrees, have shown that APP is degraded in the lysosome. To clarify reviewer 1's point and per suggestion of reviewer 2 (major critique point 2) we inhibited lysosome function with leupeptin (inhibitor of lysosomal hydrolases) and performed APP pulse-chase experiments (Fig. EV3C). We observed upon inhibition of the lysosome that only 15% of membrane APP was degraded as compared to 49% in control cells, thus indicating that substantial part of membrane APP is degraded by lysosomal hydrolases. the lysosomal degradation of a substantial part of the membrane pool of APP (Fig. EV3C). Moreover, we now show that total APP levels increase by 53 % when the lysosome is inhibited (Fig. EV3D). Nevertheless, we agree with the reviewer that our data does not fully exclude a canonical degradation by non-amyloidogenic dual processing along the route to the lysosome instead of a classical lysosomal degradation. We also fully agree with the reviewer that the contribution of the activity of g-secretase in the lysosomes to APP lysosomal degradation should be determined, but is outside the scope of this paper. For example, it would be interesting to perform these assays in PS2 KO cells given its recently described lysosomal localization to determine the amount of APP degraded by g-activity in the lysosome, since in PS1 KO cells APP degradation was unaltered(De Strooper et al., 1998).

Line 163. Here an alternative explanation could be that CD2AP depletion results as well in halting cargo delivery from maturing endosomes in dendrites to mature lysosomes in cell bodies. This could explain the increase 22C11 in dendrites and decrease in cell bodies.

Response: This is a very interesting point that reviewer #1 raises that we have now included in lines 181-183.

Line 225: '... recapitulating the early endosome enlargement that occurs early in AD.' This is too speculative here, and should be rather included in the discussion (see major comment).

Response: We have moved this to the discussion (line 421).

Line 237: the authors state here that downregulation of Bin1 as well as CD2AP results in an increased colocalization of APP with BACE1. If so, why are opposite effects observed for APP-CTF (Fig. 1h-i)?

Response: To clarify, we would like to point out all our results: In Fig. 1H, we observed upon CD2AP KD a reduction in the levels of APP CTFs by western blot, however in the presence of DAPT, inhibiting APP processing, the levels were restored (Fig. EV2E). By immunofluorescence, and by detecting both APP and APP CTFS with the antibody anti-C-terminal APP (Y188), we observed a consistent decrease in APP/APP CTFs at early endosomes upon CD2AP KD (Fig. 7A). However, in the presence of DAPT (Fig. 7A), the APP/APP CTFs levels increased at early

endosomes. The increased colocalization of APP with BACE1 that the reviewer refers to was observed in the presence of DAPT (Fig. 5E and 5G). Therefore, CD2AP KD reduces APP/APP-CTFs in absence of DAPT, in contrast when DAPT is present the levels of APP/APP-CTFs increase at early endosomes thus explaining the increased colocalization of APP with BACE1. The increase in APP/APP-CTFs in early endosomes is likely due to the retention of APP/APP-CTFs in the endosomal limiting membrane, as shown in Fig. 7. This is also supported by our observation that DAPT treatment of siControl cells does not result in increased APP/APP-CTFs at the endosomal limiting membrane (Fig 7). The increased colocalization between APP and BACE1 upon Bin1 KD likely has a different underlying mechanism. When Bin1 is downregulated colocalization increases likely because there is more BACE1 in early endosomes as result of impaired recycling and since there is no alteration of APP endocytic trafficking.

Line 254 and following: The authors measure here circularity and length of each carrer and conclude that they are increased in Bin1-depleted axons. However, I find this set of data a bit far-fetched given the low resolution of classic confocal microscopy. I would limit it here by stating that endosomes appear more extended and enlarged (so measure volume) as the actual evidence of a failure of tubule scission is far more convincing with the live imaging data (fig 6e-f).

Response: We have now altered the text and now refer to BACE1 carriers as extended and enlarged instead of tubular (line 270) and on Fig. 6B legend.

Line 289 and following: the authors report here that upon inhibitor treatment or CD2AP combined with Rab5QL, APP is in the limiting membrane. This has been demonstrated already much earlier (then the by several other groups (Schneider, Rajendran et al 2006; Schneider, Rajendran et al 2008; Sannerud et al. 2011) and should be included. Secondly, it is an overstatement to say that APP localized to the lumen of enlarged Rab5 QL endosomes while in CD2AP it moved to the limiting membrane as the authors cannot distinguish between full-length APP and APP-CTF. Additional controls with BACE1 inhibitors (e.g. see Esselens et al 2012; Sannerud et al., 2011; Rajendran et al. 2006) might be considered.

Response: We have now added the indicated references in line 304. To clarify that APP-RFP identifies both APP full length and APP -CTFs we have extended the description of this construct (lines 249 and 325).

Line 313: the authors didn't study KO of Bin1 and CD2AP but KD. So 'absence' is an overstatement.

Response: We have deleted "absence" (line 337) and throughout the manuscript.

Line 319: the link to cellular propagation of seeds is too speculative and lacks support.

Response: We have removed the link to cellular propagation of amyloid seeds.

Line 369: 'We uncover the mechanisms by which APP and BACE1 segregate at early endosomes..." Although the authors have revealed novel sorting regulation, they ignore other mechanisms that also regulate the encounter/segregation of APP and BACE1. For instance, Sannerud et al (2011) demonstrated that APP and BACE1 follow distinct internalization routes in cells and neurons, which should be included in the discussion. Moreover, worth mentioning is the fact that the contribution of axonally produced Abeta is very low compared to dendritic Abeta: this is relevance in a discussion on the relative contributions of Abeta pools to the disease or to propagation.

Response: We now reference other previously reported mechanisms of segregation in the discussion paragraph that starts on line 434.

Following reviewer 1's suggestion we have mentioned the need of determining and discussed the relevance of axonal vs dendritic Aβ in the Discussion (lines 340 and 367). The reviewer mentions that the axonally produced Aβ is very low compared to dendritic Aβ. In our analysis we measured the average amount of Aβ per axonal and per dendritic segment and thus it is difficult to conclude if the total axonal amount is very inferior to the total dendritic amount. We agree that this is a very interesting point and we are currently working on determining the relative impact of axonal vs. dendritic Aβ accumulation to synapses.

<u>Specific remarks on the figures:</u>

For both Bin1 and CD2AP independent siRNA should be tested on a few basic readouts to be sure that the effects are not caused by off-targets.

Response: We understand the concern of reviewer #1. We would like to point out that we have made more clear in the text (line 85) that we used established and specific siRNA sequences previously validated in several publications (Falcone et al., 2014; Gauthier et al., 2007; Monzo et al., 2005; Tossidou et al., 2012) and have rescued the trafficking defects by re-expressing siRNA resistant Bin1 and CD2AP (Fig. EV3E and EV4D). Nevertheless, to reassure reviewer #1 we have now performed an additional rescue experiment of the increased Aβ production upon siRNA (Fig. EV2C), described in lines 86-88. These rescue experiments directly address the possibility of off-targets effects thus confirming the specificity of the siRNAs used in this study.

Fig. 1I shows a decrease in APP-CTF/APP while there is no effect on this ratio in FigS1d. Please explain.

Response: The decrease of APP -CTF/APP upon CD2AP KD could be caused by either increased degradation of APP CTFs or by increased processing. We found that inhibition of gamma-secretase rescued the defect as shown in Fig EV2E suggesting that increased processing is responsible for the decrease in CTFs.

It gives a better overview if panels S4d-e are included in Figure 3 (connected to panel I).

Response: We have now included the panel S4d in figure 3 as panel 3I and panel S4E in figure 4 as panel 4J.

I find the presentation of the data in figures S2 and S3 confusing. A better way might be to cluster the data on APP in S2 and on BACE1 in S3.

Response: Following reviewer 1's suggestion, we have clustered the data on APP and on BACE1 in former Fig. S2 and Fig. S3 of the manuscript in Fig. EV3 and Fig. EV4 of the revised manuscript.

Fig S4b: Immunostaining for Bin1/Ank is not convincing. It should demonstrate Bin1 in axons but most is in dendrites (inset).

Response: We agree with the reviewer that the visualization of Bin1 in axons was not straightforward. We have included a new immunofluorescence to substitute panel b. We believe that the visualization of Bin1 in axons is now clearer. Importantly Bin1 localization in axons is additionally evidenced by the 3-fold enrichment of Bin1 in axonal endosomes (see Fig 3I, 3J and 3K of the revised version).

Referee #2:

In this study, Ubelmann et al explore the role of Bin1 and CD2AP, two genes implicated as risk factors for late-onset Alzheimer's disease (AD). Both are regulators of endocytic trafficking, but their role in development of AD is yet unknown. The authors argue that both Bin1 and CD2AP depletion increase production of amyloid beta by increasing the interaction between APP and BACE1, though they act differently in the dendritic and axonal compartments. The authors report that Bin1 depletion prevents BACE1 recycling back to the plasma membrane in the axon, whereas CD2AP depletion prevents APP lysosomal degradation in the dendrite. The route of APP through the endo-lysosomal pathway and its points of regulation are of significant interest to the field of AD, both to understand how AD pathology develops and to identify potential targets for AD therapeutics.

Overall, the paper is well laid out and the experiments are clear. The authors extensively studied the differential regulation of APP processing in axons versus dendrites and provided mechanistic clues as to the potential role of Bin1 and CD2AP in AD pathology. However, there are a few elements which need to be addressed before publication and are as follows:

Major critiques:

In light of a recent manuscript that have come out regarding Bin1 and its role in BACE1 trafficking (Miyagawa et. al., 2016), the authors should discuss how their data supports or refutes these new findings.

Response: We fully agree with reviewer #2 that results from Miyagawa et al., published after we sent our paper to EMBO reports, should be discussed. In Miyagawa et al. 2016, Bin1 depletion induced BACE1 accumulation in early endosomes of primary neurons floxed out for Bin1 during 6 days by Cre incubation. We similarly observed BACE1 accumulation in early endosomes of primary neurons treated with siRNA for Bin1 for 3 days. We both hypothesized that BACE1 accumulation in early endosomes could result from a defect in recycling and/or degradation.

Miyagawa et al. concluded that there was a defect in BACE1 degradation, based on increased BACE1 total levels and decreased colocalization with LAMP1 (by fractionation). A shortcoming of their study is however that pulse-chase analysis of BACE1 degradation that would more rigorously analyse BACE1 degradation was not performed. Moreover, BACE1 recycling was not examined. We performed rigorous pulse-chase analysis of BACE1 recycling and degradation in n2a cells and primary neurons as well as analysis of BACE1 total levels. We observed a defect in BACE1 recycling in both cell types (Fig. 2I-L, 4C and 4D). However, pulse-chase analysis of BACE1 revealed no difference (Fig. EV4C) neither western blot analysis of BACE1 total levels (Fig. EV6C). Together our data are not consistent with a defect in BACE1 degradation. This could be due to differences in methodology and in the time of Bin1 depletion. Miyagawa et al. performed 6 days of complete Bin1 deletion while we did knockdown for 72h of transient siRNA transfection. Maybe the extra 3 days of Bin1 depletion are necessary for a defective BACE1 recycling to affect BACE1 total levels. Miyagawa et al. do not describe a mechanism for how Bin1 controls BACE1 degradation while we provide direct evidence for Bin1 to function in the scission of BACE1 tubules necessary for efficient recycling from early endosomes. We have now mentioned and discussed Miyagawa et al. results in lines 116 of the results and 379 of the discussion.

The authors interpret that the residual 22C11 signal seen in many of their siCD2AP experiments (for example, Figure 2d) is due to a lack of APP lysosomal degradation because APP is not sorted into intraluminal vesicles (ILVs). However, lysosomal degradation is not specifically tested by any kind of inhibitor (bafilomycin, NH4Cl) and in fact the retention of 22C11 signal could be due to an inhibition of cleavage of the N-terminal APP due to APP sequestration in ILVs. The authors should specifically test lysosomal degradation using both immunofluorescence ane biochemical techniques or adjust their interpretation accordingly.

Response: We thank reviewer 2 and reviewer 1 for raising this point. We have now specifically tested and found APP lysosomal degradation inhibited upon leupeptin treatment using both immunofluorescence and biochemical techniques (Fig. EV3C and EV3D). Please also see minor comments point 2 from reviewer 1. We would also like to point out that 22C11 retention was observed after leupeptin treatment similarly to treatment with siCD2AP, supporting that the residual 22C11 observed likely results from delayed lysosomal degradation. Moreover, please note that upon CD2AP depletion APP/APP CTFs was observed retained at the endosomal limiting membrane and not sequestered in ILVs (Fig. 7).

Other critiques:

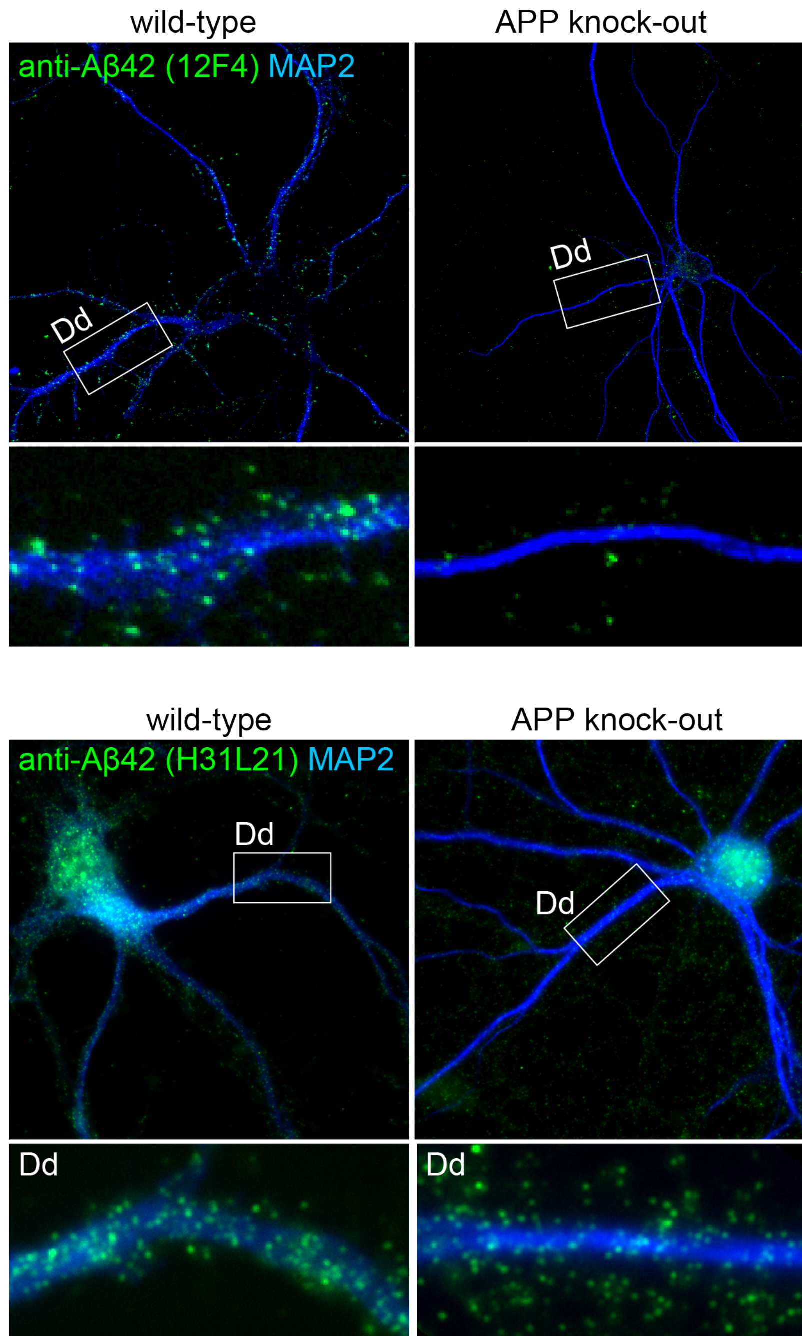
In figure 1, it is important to show the specificity of AB42 staining, perhaps by conducting experiments in an APP knockdown background or with BACE1 or gamma-secretase inhibitors.

Response: Both 12F4 and H31L21 are monoclonal antibodies raised against the c-terminus of Aβ42, that in their specifications refer to be specific and able to recognize Aβ42 from APP, APP-CTFs and Aβ40. We decided to use these two antibodies because they had been previously used in 246 publications for 12f4 and 18 for H31L21. We nevertheless acknowledge the points raised by reviewers 1 and 2 and thus have performed all the suggested experiments.

1. We performed Aβ42 immunofluorescence with both antibodies in the presence of prolonged treatment (48h) with DAPT (suggested by reviewer 1), and, as suggested by reviewers 1 and 2, with

BACE inhibitor (Fig EV1B). Following the advice of reviewer 1 we also performed immunostainings on cells overexpressing the beta-cleaved APP-CTF (C99) in presence or absence of DAPT to rule out any cross-reactivity of the antibodies with APP cleavage byproducts (Fig. EV1C and EV1D).

2. Additionally, Prof. Gunnar Gouras from Lund University, a renowned AD researcher, uses these antibodies and his post-doc Isak Martinsson had performed immunofluorescence experiments with APP knockout primary neurons. G. Gouras agreed to share with us and the reviewers some of his unpublished results. The figure is shown below. All these experiments show residual labelling with both antibodies, suggestive of background noise, confirming the high specificity of both these antibodies for Aβ42.



(Courtesy of Isak Martinsson and Gunnar K. Gouras, Lund University)

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In figure 1, what happens to intracellular levels of AB40? Since there are changes in extracellular secreted AB40, intracellular levels should be examined as well.

Response: Following this excellent suggestion by reviewer 2 we measured intracellular Aβ40 in N2a cells depleted for Bin1 or CD2AP by immunofluorescence using an antibody specific for Aβ40 (Fig EV2D). Aβ40 increased intracellularly upon Bin1 knockdown and remained unchanged upon CD2AP knockdown (line 96).

Additionally, the authors state that there is a tendency for higher secretion of AB42, which is not clear in figure (1g). How do they explain a decrease in extracellular AB40?

Response: We agree that a tendency for higher secretion of Aβ42 is not clear in Fig. 1G. We have re-written the sentence in line 94. The mechanisms of Aβ40 secretion are poorly characterized. As recently reviewed by Paul Gleeson, several recycling pathways can contribute to the secretion of Aβ40. Briefly we can speculate that once produced at early endosomes Aβ40 could be carried to the extracellular space by exocytosis of recycling carriers originating from early endosomes or by release of exosomes from MVBs where Aβ40 could accumulate (Rajendran et al., 2006; Toh and Gleeson, 2016). Bin1 depletion by inhibiting recycling to the plasma membrane would affect not only BACE1 but also Aβ40. CD2AP depletion by potentially inhibiting ILVs formation at MVBs would impair APP degradation, inhibit exosome formation and thus secretion of Aβ40-associated with exosomes. We have now discussed this (lines 374 and 403).

In figure 1 and S1, the method of normalization of APP-CTF levels is inconsistent, either to full-length APP (Fig. 1h) or tubulin (Fig. S1d). The authors should be consistent.

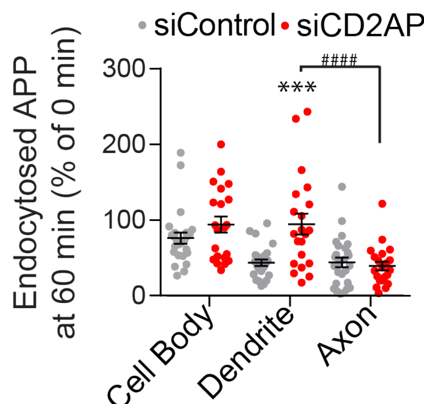
Response: We have now changed the method of normalization in Fig. EV1D to full length APP as in Fig. 1H to keep the results consistent as pointed out by reviewer 2.

In figure 2, the authors should include label of 22C11 antibody staining, and include time of chase for each experiment

Response: We have included the label 22C11 and M1, the anti-Flag-BACE1 antibody used and the respective times of pulse and chase for each experiment.

In figure 3d, should the y-axis label be % of time point 0?

Response: We recognize reviewer 2's point. Ideally we would normalize it to time zero as we did when we measured the total amount of endocytosis per N2a cells (Fig. 2E) or by biotinylation of primary neurons (Fig. 3F). However, there is a technical limitation inherent to the imaging of neurons at highest numerical aperture, necessary to best capture fluorescence intensity. The associated smaller field of view prevents us to acquire the whole cell given the large size of neurons. The solution was to measure the amount of endocytosis per 20 um of dendrite or axon that precludes the normalization to the total amount endocytosed per dendrite, per axon or per cell at time zero. We have nevertheless included below the quantification of APP levels after 60 min relative to time 0 but we think it is more correct to include in the paper the normalization to siControl levels.



In figures 6 & 7, the quantifications lack error bars. Even if they are % values from pooled experiments, the authors should express the variability from independent experiments. Data should be quantified such that the amount of variability between experiments can be expressed. Alternatively, they can use more appropriate statistical tests, such as chi-square.

Response: Fig. 6B, 7D and 7I now show the variability of the independent experiments analyzed.

Referee #3:

In this manuscript, authors examined the role of Bin1 and CD2AP in Abeta production and vesicular trafficking in neurons. They found that downregulation of Bin1 and CD2AP increased the production of Abeta by distinct mechanisms. Bin1 regulates BACE1 recycling from early endosome, and CD2AP controls the sorting of APP to the degradation pathway. Depletion of CD2AP and Bin1 increased the encounter of APP and BACE1 at early endosome, thereby increasing the Abeta production. The research design is adequate, the methods used for this study and data obtained are solid. I have several comments and suggestions for the authors to consider improving the quality of the paper:

1) They have shown that knockdown of CD2AP and Bin1 altered the levels of APP-CTFs (Fig. 1h). As gamma-secretase is also involved in the regulation of APP-CTF metabolism, the authors should show the levels of the gamma-secretase (e.g., nicastrin) in the lysates of siRNA-treated cells to exclude the possibility that the gamma-secretase activity was controlled.

Response: We thank the reviewer for raising this point. We have analyzed the levels of nicastrin as suggested in the lysates of siRNA-treated cells and found no significant alterations in the levels of nicastrin upon siRNA treatment (See Fig. EV2F), suggesting that gamma-secretase is unaltered upon Bin1 or CD2AP siRNA treatment (line 107).

2) It is general concern that tagging to intracellular region of the cargos affects their vesicular trafficking. Authors should examine the subcellular localization of endogenous APP as well as BACE1 in Bin1 or CD2AP-depleted N2a cells (or primary neurons) by immunocytochemistry.

Response: Reviewer 3's concern with the study of exogenous proteins is a valid point, that we have tried to address by analyzing endogenous proteins whenever possible. Concerning APP, we analysed endogenous APP processing (Fig. 1H, 1I, EV2E); endogenous APP degradation upon plasma membrane biotinylation (Fig. 3E and 3F); endogenous co-ip with CD2AP (Fig. 3G); and importantly, we analyzed the subcellular localization of endogenous APP to early endosomes of CD2AP-depleted primary neurons by immunofluorescence and spinning-disk confocal microscopy (Fig. 7A and 7B) and dSTORM super-resolution microscopy (Fig. 7H, 7I and 7J). These results were consistent with the ones obtained using exogenous expression of APP, indicating that tagging does not significantly affect APP vesicular trafficking. Regarding endogenous BACE1, we analyzed endogenous BACE1 total levels by western blot (Fig. S6C); and detected an endogenous co-ip with Bin1 (Fig. 4E). Despite working for western blot the polyclonal anti-BACE1 (Thermo Scientific PA1-757) used gave by immunofluorescence an ubiquitous signal inconsistent with the established BACE1 endocytic localization and BACE1 endocytic activity (Buggia-Prévot et al., 2013; John et al., 2014; Vassar et al., 1999; Vetrivel et al., 2009; Yan et al., 2001)(see below). To further try to address reviewer 3 suggestion we tested another antibody, a monoclonal against the ectodomain of BACE1 (R&D Systems clone 137612). Unfortunately, similar results were obtained (see below). Additionally, we pulsed live neurons with this antibody hoping to detect endogenous BACE1 endocytosis but again these experiments were unsuccessful since endocytosed fluorescent transferrin could be detected but not anti-BACE1 (shown below). It is possible that the antigens recognized by these antibodies are not exposed when the proteins are kept in their native conformation during immunofluorescence but not during western blot in denaturing conditions. We suspect that other researchers encountered similar difficulties because in all BACE1 trafficking papers, that we could find, the endogenous protein trafficking was not reported(Bauereiss et al., 2015; Buggia-Prévot et al., 2013; Chia et al., 2013; Das et al., 2013; Koh et al., 2005; Sannerud et al., 2011; Udayar et al., 2013; Vassar et al., 1999; Wu et al., 2011).

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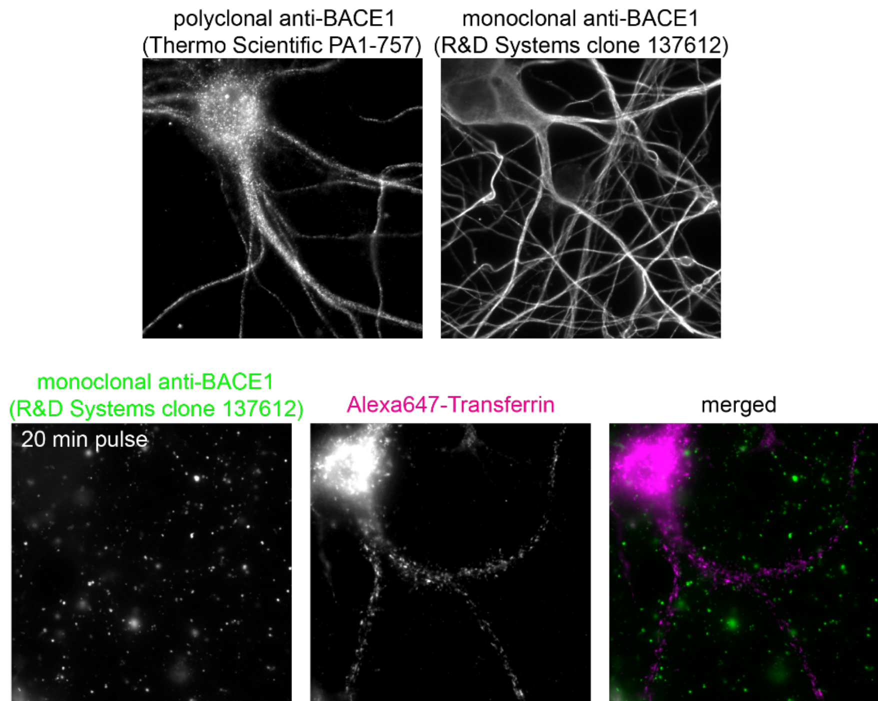

3) Authors indicated that knockdown of CD2AP inhibited the sorting of APP to the ILVs. However, the levels of APP holoprotein was now altered in siCD2AP treated cells. Why? Authors should discuss this issue.

Response: We assume the referee refers to the levels of APP holoprotein not being altered in siCD2AP-treated cells (Fig. 1H). To clarify since we used biotinylation of surface proteins that were subsequently chased for degradation we are in fact analyzing specifically the degradation of the membrane pool of APP. We have re-written this in the results (lines 188).
However, we found that CD2AP control of membrane APP degradation in dendrites was not sufficient to alter APP levels at steady state. This could be due to the abundance of APP in other cellular locations and/or to the incomplete block of APP degradation. Nevertheless, we could pinpoint CD2AP function to dendritic early endosomes identifying a novel dendritic specific control of APP degradation. To reinforce the specificity of CD2AP function, we have re-written the discussion (line 398).

4) Bin1 depletion inhibited the tubule scission for BACE1 in primary neurons. Is this phenomenon is specific to the BACE1?

Response: Bin1 function in scission has not been previously reported. In vitro, Bin1 alone can recruit dynamin to tubulated lipidic bilayers(Leprince et al., 1997; Nicot et al., 2007; Picas et al., 2014) and dynamin is known for its scission capacity(Roux et al., 2006). This evidence supports a role for Bin1 in scission of tubules independent of the presence of cargo. However, some cargo specificity exists in vivo since we observed that the recycling of APP is not altered by Bin1 depletion (Fig EV3B). Moreover, Bin1 depletion show transferrin recycling defects (Pant et al., 2009). The CLAP domain specific to neuronal Bin1 could also confer further specificity to Bin1 control of BACE1, since only the neuronal isoform rescued the BACE1 recycling defect observed upon Bin1 depletion (Fig EV4D). Together these data indicate that the tubules formed upon Bin1/dynamin scission likely contain BACE1 and transferrin but not APP. Regarding this point we have extended the discussion on Bin1 function (line 374).

5) Recently, similar result for Bin1 was reported (Miyagawa et al., Hum Mol Genet 2016 in press). Authors should refer this and discuss the functional aspect of Bin1 in BACE1 trafficking.

Response: Reviewer 2 also called our attention to this paper, can you please see our answer to reviewer 2 major point 1. In addition, Miyagawa et al. 2016 show a direct interaction of Bin1 with BACE1 further supporting Bin1 specificity as discussed in point 4. Although the identified

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interacting domain (BAR domain), present in all Bin1 isoforms, is inconsistent with the inability of ubiquitous Bin1 to rescue the BACE1 recycling phenotype (Fig. EV4D). Subsequent studies will be necessary to clarify this discrepancy.

We look forward to seeing our work accepted for publication in EMBO reports. We have a couple of figures that we will send in the next few days for you to consider for the cover. If you have any further questions, please do not hesitate to contact us.

2nd Editorial Decision 11 October 2016

We have finally received all referee comments on your revised manuscript that are included below.

While referees 2 and 3 did not raise concerns, referee 1 is still not convinced by the antibodies used and the coIP data. Upon cross-commenting, referee 3 agrees with referee 1 and suggests experiments to address the concerns. These comments are also pasted below. We usually only allow one round of revision. However, in this case, all referees indicate in the manuscript summary table that the findings are novel and interesting, and we would like to publish your study if the remaining concerns can be satisfactorily addressed. I would therefore like to give you the opportunity to do so, if you think this can be done in a reasonable timeframe.

It is our journal policy that manuscripts should be accepted at the latest 6 months after a first decision was made, which was in June in your case. We should therefore accept your manuscript before the end of December. Please let me know whether you think that the outstanding concerns can be addressed in the next few weeks/months.

Please note that we can only offer a maximum of 5 EV figures at the moment. Additional extra figures will need to be included in an Appendix file. Please see our guide to authors for more information. Please also add a scale bar to figure EV1c.

REFEREE REPORTS

Referee #1:

The authors have submitted a revised manuscript where they have addressed the critiques of the reviewers. Major concerns were related to the specificity of the antibodies used to detect Abeta42 by imaging, the co-IP data, additional controls to distinguish lysosomal degradation and the confrontation with Miyagawa et al (2016). The new version is indeed improved and the authors have satisfactorily many of the raised issues by including new controls and performing some additional experiments. There are however some remaining issues related to my major critiques that needs some more scrutiny.

With respect to the specificity of the Abeta42 antibodies, the first answer is not to the point: it is not because there are 246 papers using these antibodies that paper 247 is OK. The authors make the wrong conclusion that what is published is per definition correct: how many of these 246 papers provided proper controls for antibody specificity? There are a zillion antibodies actively used in the AD field directed against any risk factor or APP fragment. I can assure the authors that a dramatic high number of antibodies to for instance g-secretase subunits, APP, ADAM10 basically fail when checked in the respective KO backgrounds. Nevertheless, there are even more papers published using such antibodies. With respect to the two anti-Abeta42 antibodies, the authors support their claim for specificity by using g-secretase and bace1 inhibitors and providing data (via their collaborator) in APP KO neurons. The authors have to admit that the latter are not 100% conclusive or supportive. I can maybe live with the specificity of the 12F4 antibody as the APP KO shows little background. But the H31L21 shows on the contrary still (very) high backgrounds particularly in the cell body but as well in the dendrites: It is for me very difficult to distinguish major differences between the staining in the wt vs APP KO dendrites. In a way this is reflected in the quantifications using DAPT (but also Compount IV) where 24 to 48hrs inhibition only gives a moderate drop (30% to 50% for H31L21 and even less (25% to35%) for 12F4) in Abeta42: in such a time span these inhibitors should give drops up to 80%. So, to my opinion this is largely caused by the inherent high backgrounds these antibodies generate in ICC applications (similar analysis by ELISA on N2A would prove/disprove my case). The authors didn't make a stronger case by providing the APP KO

and DAPT data. However, I cannot deduce from the data and figures which experiments were done with the 12F4 or H31L21 antibody.

With respect to the co-IP data, the authors maintain them in the manuscript with only a minor change on interpretation (from 'interaction' to 'suggestive for interaction'). Again, the authors make the wrong argumentation that this might boost interest in the search for interaction domains. My point was that if co-IP data are not scrutinized with the proper controls, they are not reliable: it doesn't make any sense to use them to steer the community into further interaction studies. The only control in these experiments is the inclusion of control IgG. That is not sufficient to claim (suggestive) interaction. There should be at least additional controls, including beads+ antibody without lysate, additional control antigens that do not co-IP. For each IP the provided data have a problem. The co-IP with anti-APP antibodies shows a moderate enrichment for APP (while one would expect a dramatic higher amount in the IP-ed fraction compared to the input). On the contrary, the CD2AP seems to dramatically co-enrich: the levels in the input are barely detectable in contrast to the IP lane. In almost all cases one observes the reverse (high levels in input, low levels in the co-IP lane). With respect to the BACE1-Bin1 interaction: also here the BACE1 (co-ip antigen) levels in the IP lane are higher than in the input lane. Moreover, the IP lane contains 3 bands: impossible that all these bands are Bace1... If these co-IP blots are true, they can only be explained by a very strong interaction (in both cases), which is surprising as the IP is done even in more stringent conditions (TritonX100 extraction). And if these proteins interact so strongly, there should be a more evident colocalization at the immunofluorescence level: by stating that they see an increased 'overlap' the authors admit there is not much true colocalization. In fact, no single APP hot spot coincides with CD2AP immunoreactivity in dendrites (figure 3H). the same holds true for CD2AP-Rab5: they do not colocalize, but some organelles tend to overlap and it is the overlap the authors are measuring (for BIN1 vs BACE1, there is a better correlate). Nevertheless they use these readouts to further support interaction and localization in EE.

Minor comments:

- Most quantifications of imaging data are presented with individual data points combined with mean values and SEM. In these sets, the representative images correlate with the quantifications. In few cases, however, the authors use a line diagram (e.g. 2E, EV3C): in both cases, the representative images do not follow the quantification (although, if I read correctly, quantifications are similar and normalized). Based on the images, I would expect that the 'red line' (siCD2AP in 2E and Leupeptin in EV3C) would go higher than 100%, but it remains a straight horizontal line. Can the authors clarify this? Also related to the EV3C: the authors show that upon leupeptin treatment 22C11 accumulates and is not degraded. However, and this was the point of the critique, they do not show whether this represents full length APP or the shedded ectodomain that is accumulating.

- Related to the specificity of Bin1 recruiting BACE1 into recycling tubules: given that there are no conclusive data on the Bin1-Bace1 interaction, it should be of added value to show that besides Bace1 also TFR is recycled in a Bin1-dependent manner: in fact, repeat experiment 6E with internalized Tfr-488.

Referee #2:

The authors have addressed all the concerns raised by this referee, who now recommends publication in EMBO Reports.

Referee #3:

The authors fulfilled our requests. Regarding the comment (2), I would suggest the reviewers to refer a paper by Buggia-Prévot, V. et al., Mol. Neurodegener. 9, 1 (2014) to support the assumption that the trafficking of exogenous C-terminally tagged BACE1 is similar to that of endogenous BACE1.

Cross-comments from referee 3:
Regarding these specificity issues, I agree with both concerns raised by the reviewer. However, the

accumulation of intracellular Aβ was not a major focus in this manuscript. If they are able to show the increase in the intracellular Aβ by different method (e.g., western blot or ELISA) and omit fig. 1, that should be enough. Rather, this manuscript suffers the specificity of antibodies used in IP experiment, as the reviewer 1 indicated. I would suggest the authors to check the specificity of anti-APP, CD2AP and BACE1 antibodies using lysates obtained from RNAi-treated cells.

2nd Revision - authors' response 14 October 2016

Referee #1:

The authors have submitted a revised manuscript where they have addressed the critiques of the reviewers. Major concerns were related to the specificity of the antibodies used to detect Abeta42 by imaging, the co-IP data, additional controls to distinguish lysosomal degradation and the confrontation with Miyagawa et al (2016). The new version is indeed improved and the authors have satisfactorily many of the raised issues by including new controls and performing some additional experiments. There are however some remaining issues related to my major critiques that needs some more scrutiny.

1- With respect to the two anti-Abeta42 antibodies, the authors support their claim for specificity by using g-secretase and bace1 inhibitors and providing data (via their collaborator) in APP KO neurons. The authors have to admit that the latter are not 100% conclusive or supportive. I can maybe live with the specificity of the 12F4 antibody as the APP KO shows little background.

Response: We thank the reviewer for agreeing that antibody anti-ab42 12F4 is specific. Importantly we would like to point out that the main result of the manuscript regarding ab42 accumulation, fig. 1c, that shows ab42 polarization by KD of Bin1 and CD2AP was performed using the antibody 12F4 that gave the cleanest result using APP KO neurons and that reviewer #1 agreed was specific.

2- But the H31L21 shows on the contrary still (very) high backgrounds particularly in the cell body but as well in the dendrites: It is for me very difficult to distinguish major differences between the staining in the wt vs APP KO dendrites.

Response: We agree that H31L21 shows a higher background and some remaining labeling of dendrites of APP KO, in our experiments with wt neurons we did not observe such a high background, nevertheless, our quantifications of mean fluorescence of ab42 were always done upon background subtraction.

We used H31L21 to show an increase in ab42 fluorescence in N2a cells KD for Bin1 and CD2AP (fig.1a) and to show that re-expression of neuronal Bin1 and CD2AP restored ab42 levels to that of control, confirming the specificity of the increase in abeta42 observed (EV2C). We propose to remove fig1a to strengthen our finding as suggested by reviewer #1.

3- In a way this is reflected in the quantifications using DAPT (but also Compound IV) where 24 to 48hrs inhibition only gives a moderate drop (30% to 50% for H31L21 and even less (25% to35%) for 12F4) in Abeta42: in such a time span these inhibitors should give drops up to 80%. So, to my opinion this is largely caused by the inherent high backgrounds these antibodies generate in ICC applications (similar analysis by ELISA on N2A would prove/disprove my case). The authors didn't make a stronger case by providing the APP KO and DAPT data. However, I cannot deduce from the data and figures which experiments were done with the 12F4 or H31L21 antibody.

Response: We acknowledge that there are limitations with using immunofluorescence quantifications for absolute measurements of protein amount. We agree with the reviewer that theoretically we should observe a higher effect. However we think it is important to point out that, despite the limitations of this technique, we are able to observe the expected reduction of ab42 levels after the treatment with DAPT and compound IV (Fig EV1b) and increase of ab42 levels upon overexpression of APP and BACE1 (Fig. EV1a). Importantly, we are able to observe changes in the levels of ab42 between the axon and dendrites in a single neuron, which is not possible to achieve using other techniques.

Regarding which antibodies (12F4 or H31L2) where used in data and figures we appreciate this point from the reviewer since we missed that information in several cases. We have now added that information in the figures and figure legends and expect that it will be more clear to the readers to understand our findings.

In summary, we and others (Liao et al., 2015; Miyagawa et al., 2016) have shown that Bin1 and CD2AP loss of function can increase ab42 production. However, in the current manuscript we provide for the first time evidence for a polarization of the increased production of ab42 upon Bin1 and CD2AP loss of function: ab42 increases in dendrites when CD2AP is KD but in axons when Bin1 is KD. Such polarization of ab42 production can only be measured in the same cell by immunofluorescence. We think that with the controls provided as suggested by the reviewer #1, we demonstrate that we can measure relative changes in ab42 levels within a single neuron.

4-With respect to the co-IP data, the authors maintain them in the manuscript with only a minor change on interpretation (from 'interaction' to 'suggestive for interaction'). Again, the authors make the wrong argumentation that this might boost interest in the search for interaction domains. My point was that if co-IP data are not scrutinized with the proper controls, they are not reliable: it doesn't make any sense to use them to steer the community into further interaction studies. The only control in these experiments is the inclusion of control IgG. That is not sufficient to claim (suggestive) interaction. There should be at least additional controls, including beads+ antibody without lysate, additional control antigens that do not co-IP. For each IP the provided data have a problem. The co-IP with anti-APP antibodies shows a moderate enrichment for APP (while one would expect a dramatic higher amount in the IP-ed fraction compared to the input). On the contrary, the CD2AP seems to dramatically co-enrich: the levels in the input are barely detectable in contrast to the IP lane. In almost all cases one observes the reverse (high levels in input, low levels in the co-IP lane). With respect to the BACE1-Bin1 interaction: also here the BACE1 (co-ip antigen) levels in the IP lane are higher than in the input lane. Moreover, the IP lane contains 3 bands: impossible that all these bands are Bace1... If these co-IP blots are true, they can only be explained by a very strong interaction (in both cases), which is surprising as the IP is done even in more stringent conditions (TritonX100 extraction). And if these proteins interact so strongly, there should be a more evident colocalization at the immunofluorescence level: by stating that they see an increased 'overlap' the authors admit there is not much true colocalization. In fact, no single APP hot spot coincides with CD2AP immunoreactivity in dendrites (figure 3H). the same holds true for CD2AP-Rab5: they do not colocalize, but some organelles tend to overlap and it is the overlap the authors are measuring (for BIN1 vs BACE1, there is a better correlate). Nevertheless they use these readouts to further support interaction and localization in EE.

Response: With respect to the co-ip data we now understand the reviewer concerns and agree to remove it from the paper as it was suggested by reviewer 1 point 2 in the first revision.

Minor comments:

- Most quantifications of imaging data are presented with individual data points combined with mean values and SEM. In these sets, the representative images correlate with the quantifications. In few cases, however, the authors use a line diagram (e.g. 2E, EV3C): in both cases, the representative images do not follow the quantification (although, if I read correctly, quantifications are similar and normalized). Based on the images, I would expect that the 'red line' (siCD2AP in 2E and Leupeptin in EV3C) would go higher than 100%, but it remains a straight horizontal line. Can the authors clarify this?

Response: I understand the reviewer expectation when looking at the images the 22C11 signal upon the 60 min chase appears brighter. This is due to the concentration of the signal in a smaller area that sums up and result in the brighter spots observed upon 60min in siCD2AP in 2E and Leupeptin in EV3C. So to our eyes, the representative image of 60 min chase looks brighter than 10 min pulse. However, when we quantify the total fluorescence of the cell, 60 min chase has the same or lower fluorescence than upon 10 min pulse. By other words, our quantification of the intensity is at both time points divided per the whole cell area. In the experiments shown in fig. 2E and EV3C, we performed a pulse of 22C11 that binds APP and is endocytosed, we consider the amount after this 10min pulse to be 100%. Upon extensive washing this finite amount of 22C11 bound to APP traffics until the lysosome and part of it gets degraded resulting in a decrease of the initial amount of 22C11

pulsed. In absence of degradation, the end point should have an amount of 22C11 equal to the initial one, which is close to 100%. Therefore, in these pulse-chase experiments, it is not expected to observe values above 100%. Indeed both upon CD2AP KD and leupeptin treatment we observed that the level of 22C11 was close to 100%.

Also related to the EV3C: the authors show that upon leupeptin treatment 22C11 accumulates and is not degraded. However, and this was the point of the critique, they do not show whether this represents full length APP or the shedded ectodomain that is accumulating.

Response: We agree with the reviewer that the pulse-chase with 22C11 does not exclude a contribution of the shedded domain of APP to the build up of 22C11. To circumvent this we analysed APP full length levels upon leupeptin treatment by western blot with anti-APP C-terminal domain (APP Y188) in fig. EV3D. We observed an increase in APP full length levels upon leupeptin inhibition of the lysosome suggesting that in the pulse chase experiments, 22C11 build-up includes full length APP.

- Related to the specificity of Bin1 recruiting BACE1 into recycling tubules: given that there are no conclusive data on the Bin1-Bace1 interaction, it should be of added value to show that besides Bace1 also TFR is recycled in a Bin1-dependent manner: in fact, repeat experiment 6E with internalized Tfr-488.

Response: We appreciate that the suggestion of the reviewer will show that TfR recycles with BACE1. However, transferrin receptor recycling has been shown upon Bin1 KD (Pant, 2009), Transferrin receptor recycling occurs only in the somatodendritic domain and not in axons (West, AE; 1997) where we found BACE1 recycling to be dependent on Bin1, moreover Miyagawa 2016 recently showed evidence for a direct interaction between Bin1 and BACE1. Thus although this experiment would confirm the results described in these publications, at least for N2a cells, would not provide further mechanistic evidence for the findings presented in the current manuscript. So we would propose not to perform these experiments.

Referee #2: The authors have addressed all the concerns raised by this referee, who now recommends publication in EMBO Reports.

Referee #3: The authors fulfilled our requests.

Regarding the comment (2), I would suggest the reviewers to refer a paper by Buggia-Prévot, V. et al., Mol. Neurodegener. 9, 1 (2014) to support the assumption that the trafficking of exogenous C-terminally tagged BACE1 is similar to that of endogenous BACE1**.**

Response: We have now added the suggested ref in line 134 of the results section.

3rd Editorial Decision 19 October 2016

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Journal Submitted to: EMBO Reports
Manuscript Number: EMBO-2016-42738

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Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
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- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
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2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was determined based on pilot studies. (p.44)
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/a
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Only samples judged of insuficient technical quality were excluded. (p.38)
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Most quantifications were made using automatic tools on randomly aquired images. In the few instance that we quantified data based on visual inspection complementary automatic quantifications were performed. (p.43)
For animal studies, include a statement about randomization even if no randomization was used.	N/a
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	N/a
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5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Normal distribution of the data was tested using the normality test D'Agostino-Pearson omnibus (p. 44)
Is there an estimate of variation within each group of data?	No
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	yes see p.38
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N2a cells were originally from ATCC. Only mycoplasm-free cells were used. (p.37)

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	wild-type Balbc mice were used to at E16-18 for the preparation of primary neurons. (p.37)
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/a
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	N/a

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