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Disruption of adaptor protein 2μ (AP-2μ) in cochlear hair cells impairs vesicle reloading of synaptic release sites and hearing

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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 09 June 2015

Thank you for submitting your manuscript to The EMBO Journal. Your manuscript has now been seen by three referees and their comments are provided below.

As you can see from the comments, the referees find the analysis interesting and insightful. They raise a number of different concerns that need to be sorted out. There is the issue of only using 2 animals in the rescue experiments in Fig 2D - ideally more animals are needed to support the findings. Referee #3 also raises the point if the vesicle ribbon phenotype observed might be due to lack of otoferlin expression, I don't know how to best address this point, but happy to discuss this and others points further.

I have provided the link below to submit your revised manuscript.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

This manuscript examines synaptic transmission defects in mouse hair cells deficient for the endocytic adaptor protein 2μ (AP-2μ). Postsynaptic responses to sound were reduced and interspike intervals were increased. strong stimulation led to the accumulation of abnormal organelles on the presynaptic side. Finally the authors show that AP-2μ is required for sorting of the essential presynaptic protein otoferlin. The authors conclude that AP-2 is required for release site clearance and reloading, synaptic vesicle reformation and sorting of otoferlin.

This is a really impressive multidisciplinary study with an intimidating combination of the best possible techniques, often in intact preparations (ex vivo, in vivo) and a highly advanced analysis including mathematical modeling to interpret physiology data. The genetics are excellent, using cell specific full knock out and in vivo (partial) rescue using transuterine AAV injection. The physiology is 3excellent too, with ex vivo perforated patch-clamp capacitance recordings and in vivo analysis at single active zones. The electronmicroscopy is state of the art incl. 3D reconstructions, and superresolution imaging of vesicle recycling using STED imaging of a novel fixable recycling reporter. All these methods produce convincing, interesting and novel data and the paper is well written.

Conceptually, the current data also allow conclusions that reach beyond previous papers on AP-2 function and AP-2μ mutant mice, which have been described by two other groups before (Kononenko et al 2014, Mitsunari et al 2005). While the vesicle reformation aspect has been established already by Kononenko et al., otoferlin sorting aspect and the site clearance proposal are novel. The observation that AP-2 defects arise exceptionally fast, within 20ms after start of the stimulus, is an important and intriguing finding.

The main limitations of this study are primarily in the interpretation. The only experimental weakness is that among all the sophisticated methodology, 15 min high K+ is a questionable approach to probe biologically relevant vesicle recycling. It is difficult to connect physiological phenotypes in hair cell synaptic transmission on the msec timescale to cell biological phenotypes obtained with 15 min permanent depolarization (and other osmotic and pH-effects that this stimulus might produce).

The rescue using transuterine AAV injection is rather anecdotal. There are only 2 rescued animals, which show partial rescue (Fig 2D) and no statistically significant rescue. The statement "transgenic expression of AP-2μ greatly improved the ABR thresholds" seems not substantiated. Maybe these data placed in the supplemental data section and the conclusions toned down (or more experiments should be performed).

The main text, the figure legend and the methods section contain no information on the (complex) statistics in Fig 6F-G. How many cells were quantified from how many animals? Were cells or animals used as independent variables in the statistical tests? Maybe the quantification of clathrin coated structures can be printed on a different scale so the differences are better eligible.

Figures 7A-D and 9E contain hand drawn lines, probably to indicate the cell limits, but it is not clear how that was detected. Moreover, a lot of the mCling appears to be taken up outside the cell in a stimulation dependent manner and the spatial distribution of this accumulation differs from otoferlin distribution. The authors should explain this better and provide explanations. Furthermore, the black lines in the "stimulation average" images in Fig 7 and 9 appear to make no sense. If these images are averaged over 10-15 ribbons, how can a boundary be drawn??

The conclusion that AP-2 mediates active zone clearance is not convincing and the discussion is 'leading'. In the Discussion, the authors only consider impaired synaptic vesicle regeneration or active zone clearance to explain the observed defects in synaptic transmission. The 4 arguments

listed in favor are not convincing. The fact that exocytosis was impaired in AP-2µ-deficient hair cells as early as 20 ms after stimulus is only an argument against vesicle reformation not other possible defects. Moreover, requirements for efficient fusion might also be lost already before the onset of stimulation, but only be noticed during. Any defect in the still poorly understood events that take place between a vesicle arriving at the active zone and fusion pore opening can explain the observed phenotype, without any 'site clearance'. The authors should discuss such alternatives, for instance impaired sorting of important proteins/lipids or some rate limiting biochemical reaction that fails or AP-2 might be required for otoferlin function (in addition to stability/localization). The conclusions on site clearance should be toned down.

Some discussion on the fact that he slow, linear component of release endocytic Cm decline is unaltered by AP-2 loss (Figure 3C, D) should be discussed in the Discussion. This seems inconsistent with previous studies.

Minor points:
Fig 3E: provide statistics for panel E and conclusion that amplitude is reduced
Fig 7-9: Explain how the black dot was placed in the legend.
Fig 9D suggests mitochondrial depletion in the mutant. Is that phenotypic?
Fig 9F is not clear and the legend insufficiently explains what is depicted

Referee #2:

Jung et al examined the complex biological machinery involved in rapid reset of membrane areas after synaptic vesicle fusion. The authors provide evidence revealing a critical role of the AP-2 complex in resetting proteins and vesicle recycling. They show that the absence of AP-2µ results in a reduction in exocytosis, and that this defect occurs both prior to significant endocytic retrieval and while membrane-proximal vesicle remain a non-limiting factor. Additionally, a statistical analysis shows that the changes in the rates of vesicle release are compatible with AP-2µ playing a role in active zone clearance during exocytosis. The findings are important and interesting. Writing is in general very clear. Techniques used are strong.

Major concerns
1. Suggested action of AP-2 in dissociation of otoferlin from prior binding partners should be strengthened by in vitro binding assays, or, if prior work provides this information, then such references should be included.
2. The number of animals used in the work presented in Fig. 2D was 2, and while the error bars were small, the confidence in the difference would be improved by the use of additional mice.
3. The coomassie gel in Fig. 8D does not show AP-2σ. A gel that used radiolabels would show all the involved proteins.

Minor concerns
1. The terms Gamma Process, Gamma Distribution, and Poisson Distribution are relevant to the introduction of this paper, but the introduction section would benefit from a 1 or 2 sentence description of these terms for unfamiliar readers.
2. The abstract defines AZ and IHC, the introduction defines AZ but not IHC. Please ensure consistency in the use of abbreviations.

3. Majority of figures suffer from the lack or poor use of color. Please make better use of color to allow easy visual interpretation. In particular, the black and 3 colors of gray in Fig. 3C and the multiple shades of green in Fig 6F stick out as graphs in which more readily distinguishable colors would facilitate communication.
4. In Fig. 3E, is there no statistical difference between the bars on the left graph, and if there is, why isn't it marked with an asterisk?
5. For Fig. 6A, B, the opening sentence is over 60 words long, not counting the three parenthetical sections. Please break into multiple sentences to improve readability, such as at the colon or semicolon.
6. Regarding Fig. 6, are the virtual slices from the reconstructed tomograms? If so, please mark the relevant vesicles (magenta arrows) shown in Fig. 6E in Fig. 6E'.

7. The description for Fig. 8C, E, is heavy on methods that may be better presented in the Methods section.

8. What is the purpose of the green arrow in Fig. 9B

Referee #3:

Manuscript # EMBOJ-2015-91885

- General summary and opinion
To study the role of the assembly polypeptide 2 (AP-2) complex in the reloading process of synaptic vesicle at the release sites of cochlear inner hair cells (IHCs), the authors have genetically deleted the AP-2µ subunit in the mouse. The AP-2 subunit deletion was made under the control of the Vglut3-promoter, a promoter strongly active in IHCs. The results show that the mice lacking AP-2µ are profoundly deaf due a defect of the IHC synapses. High resolution confocal imaging and cellular electrophysiology experiments bring solid arguments in favor of an impaired clearance of the synaptic vesicles at the active zones. Binding of AP-2 to its cargo otoferlin is proposed to facilitate the clearance of the active zones and the vesicle-recharging of the synaptic ribbons. This study presents interesting novel information on the role of the AP-2 complex and otoferlin in the synaptic vesicle life cycle and turnover at the hair cell ribbon synapses.

- Specific major concerns
1) VGlut-3 is known to also be strongly expressed in neurons of the peripheral and central auditory pathway, in particular in the type I spiral ganglion neurons (SNGs). Therefore the deletion of AP-2 in the present study is not hair cell specific and I wonder whether a deletion of AP-2 in SGNs may have also directly impacted on the development and the responses of the afferent auditory nerve fibers.
2) In absence of AP-2µ, the otoferlin expression level in IHCs is strongly decreased (Fig.9). Therefore, it is questionable whether the vesicle ribbon phenotype is not essentially coming from a lack of otoferlin? Ideally, a rescue of otoferlin in the AP-2 KO mouse could clarify this.
3) I think it would have been very informative to study the exocytotic-endocytotic defect during development, i.e. in early pre-hearing IHCs at P2-3, an age at which otoferlin is not yet fully engaged (see Beurg et al., 2010).
4) To further characterize the vesicle traffic congestion at the active zones, I think it is essential to study exocytosis under a repetitive train of stimulations or during a paired-pulse protocol in figure 3.

- Minor concerns that should be addressed
1) The authors should give us some reasons why deleting and studying the µ subunit instead of the other subunits of the AP-2 complex.
2) Figure 1 does not give much information on the morphology of the hair bundle, as stated in page 6 (line 18). Scanning electron microscopy of the hair bundle is required. Also it is difficult to conclude anything on the synaptic base of the IHCs in this figure. For example, NF-200 labeling of the bundle of afferent fibers contacting the base of the IHCs would have been more instructive and convincing.
3) I have a strong concern on the DPOAEs measurements shown in Fig 2C. They show a rather high threshold above 50-60 dB SPL. Usually, DPOAEs in mice display thresholds well below 30 dB SPL.
4) In Fig 2D, the untreated ear seems to show some ABRs recovery as compared to 1A. Some explanation should be given. Do not give error bars with n= 2, show individual mouse data points.
5) Figure 7 is very confusing and not convincing. It should be re-orgainzed with 9E.
6) Why looking for AP-2 interactions with the otoferlin N-terminal C2A-B-C domains? Some reason should be given.
7) In fig 9C, it does not look like the level of otoferlin is reduced? In Fig 9D: I don't see the ribbons? At left, what are the numerous grey circles, vesicles or mitochondria? I have a hard time to see and understand the otoferlin lateral diffusion in Fig9E, I only see an internal diffusion of otoferlin.

1st Revision - authors' response 03 August 2015

Referee #1:

We would like to thank the reviewer for her/his appreciation of our work and the help with improving the MS. Below please find our point-by-point response.

1. The only experimental weakness is that among all the sophisticated methodology, 15 min high K+ is a questionable approach to probe biologically relevant vesicle recycling. It is difficult to connect physiological phenotypes in hair cell synaptic transmission on the msec timescale to cell biological phenotypes obtained with 15 min permanent depolarization (and other osmotic and pH-effects that this stimulus might produce).

With regard to the question of physiological relevance of and insights from 15 min depolarization with high K^+, we certainly agree with the reviewer that future experiments should also employ shorter depolarizations close to the ones used in patch-clamp or single auditory nerve fiber recordings. In fact, we are working hard to establish this by combining optogenetic stimulation with high pressure freezing. However, we consider this type of experiment as beyond the scope of the present MS, since we will likely need more than one year to establish the technique and obtain a baseline in wild-type IHCs.

Nevertheless, we would like to stress that also the long depolarization is of biological relevance. Tonic release of glutamate is a key feature of ribbon synapses in the ear and eye justifying prolonged stimulation protocols. In fact, protocols with comparable K^+ concentrations and stimulation durations are the current state of the art in research on the ultrastructure of functional states of the hair cell synapse (Lenzi *et al*, 2002; Pangrsic *et al*, 2010; Vogl *et al*, 2015). Also, while the current study mostly involved pulsatile electric or acoustic stimulation, these stimuli were repeated many times, and this requires efficient vesicle replenishment to recover the readily releasable pool of vesicles. Other highly relevant protocols to assess the auditory system even more implicitly rely on the tonic transmitter release than our protocol. As an example we refer to the recordings of auditory steady-state responses (Picton *et al*, 2003; Pauli-Magnus *et al*, 2007).

Secondly, we were aiming at driving the synapse to limiting performance in order to uncover function(s) of AP-2µ beyond the postulated role in AZ clearance. In fact, while we suggest that impaired site clearance is the primary mechanism underlying the auditory deficit described in our study, the data acquired with prolonged and strong depolarization is important in revealing a depletion of ribbon-associated vesicles at the membrane-distal part of the ribbon. This finding supports the notion of a role of AP-2µ in clathrin-mediated vesicle regeneration from endosome-like vacuoles. Moreover, this finding is important for our understanding of ribbon function: it supports the conveyor belt hypothesis of ribbon function by indicating that new vesicles are recruited to the top of the ribbon and then likely make their way to the release sites at the base of the ribbon. Finally, the finding of budding of vesicles without obvious involvement of clathrin in stimulated *AP-2µ$^{fl/fl}$:Cre* IHCs is supportive of a role of the synaptic ribbon and potentially of RIBEYE (Schwarz *et al*, 2011) in vesicle regeneration from endocytosed membranes. The latter point, however, needs further work in order to validate the hypothesis that RIBEYE through its membrane curvature promoting lysophosphatidyl-transferase activity (Schwarz *et al*, 2011) facilitates the budding of synaptic vesicles from endocytosed membranes and, hence, is not discussed in this MS.

In response to the comment we have extended the introduction of the method and referenced previous work using such stimulation (top paragraph of page 13). There, in addition, we now state explicitly that this long stimulation differs from the other stimuli used in the study and why we chose it.

2. The rescue using transuterine AAV injection is rather anecdotal. There are only 2 rescued animals, which show partial rescue (Fig 2D) and no statistically significant rescue. The statement "transgenic expression of AP-2µ greatly improved the ABR thresholds" seems not substantiated. Maybe these data placed in the supplemental data section and the conclusions toned down (or more experiments should be performed)."

Done, we have successfully performed additional rescue experiments in order to strengthen the study:

By injecting AAV at postnatal day 10 via the round window into scala tympani of the left ear we have obtained near complete rescue of auditory brainstem response threshold and amplitudes in the

injected ear of four mice (Figure 2 C-E). The non-injected ear of the same mice was as deaf as the non-treated mice and served as an internal control of the rescue (Figure S2C).
Moreover, we are really excited to now also report the restoration of IHC function based on patch-clamp recordings of exocytosis from transduced IHCs (Figure 3A, B). Indeed, transgenic expression of AP-2µ restored sustained exocytosis of *AP-2µ$^{fl/fl}$:Cre* IHCs to wild-type levels. The fact that AP-2µ expression driven by the CMV enhanced human ß-actin promoter, which likely surpasses AP-2µ wild-type levels, did not enhance exocytosis beyond wild-type levels suggests that AP-2µ is not rate-limiting for exocytosis beyond the AP-2µ copy number present in wild-type IHCs. Finally, the AAV-rescue also increased otoferlin levels in IHCs of *AP-2µ$^{fl/fl}$:Cre* mice (Figure S10).
Apart from their value for understanding AP-2µ function in sound encoding at the IHC synapse, our rescue experiments add to recent work on establishing AAV-mediated gene transfer for therapeutical purposes (Akil *et al*, 2012; Askew *et al*, 2015). While genetic deafness due to mutations of AP-2µ has not yet been reported, mutations in other synaptic genes such as *OTOF* and *VGLUT3* cause deafness. The extent of rescue obtained by AAV-mediated transgenic expression indicates that early postnatal gene replacement, indeed, can restore function in a complex structure such as the inner ear to enable near normal hearing. This is now briefly stated in the last section of discussion.
We have now included the rescue data in the appropriate Figures (Figs. 2 and 3 as well as Fig. S10) and the accompanying results/methods/supplement sections. We now entirely focus on the postnatal AAV-rescue as it is still more interesting from a gene-therapy perspective and have removed the data obtained from transuterine AAV injections from the MS.

3. The main text, the figure legend and the methods section contain no information on the (complex) statistics in Fig 6F-G. How many cells were quantified from how many animals? Were cells or animals used as independent variables in the statistical tests? Maybe the quantification of clathrin coated structures can be printed on a different scale so the differences are better eligible?
Following the reviewer's advice we now present the quantification of clathrin-coated structures on a different scale for improved visualization.
The statistical analysis was performed based on the random sections deriving from several IHCs of 1-2 mice (see below).
Wt rest: N = 1; n (random sections) = 27 (deriving from several IHCs)
Wt stim: N = 1; n (random sections) = 25
AP2 rest: N = 1; n (random section) = 30
AP2 stim: N = 2, two independent embeddings; n (random sections) = 56
The numbers of sections are now provided in the legend to Figure 6:
"Statistical analysis was based on random sections of ribbon-occupied AZs containing from several IHCs per genotype: *AP-2µ* control rest $n_{random\ section}$ = 27; *AP-2µ* control stim n = 25; *AP-2µ$^{fl/fl}$:Cre:GFP* rest n = 25; *AP-2µ$^{fl/fl}$:Cre:GFP* stim n = 56. 1-2 mice were used per condition."

4. Figures 7A-D and 9E contain hand drawn lines, probably to indicate the cell limits, but it is not clear how that was detected.
We have originally obtained 2-color STED images for both mCLING and otoferlin. Figure 7, however, only shows the mCLING channel. The cellular borders were drawn onto the otoferlin images, in which they are quite evident (as in Figure 1 of this response letter, also compare Figure 9 of MS). They were then copied to the mCLING images. We have now also used the re-analyzed data for revised Figure 7.

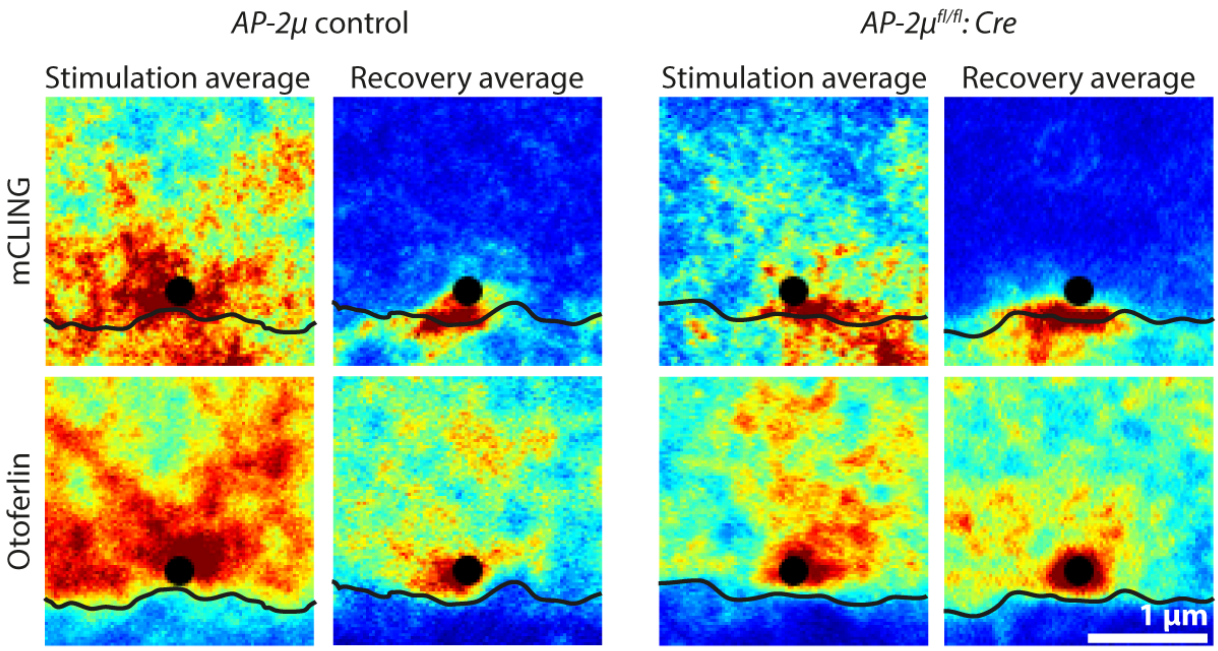

Figure 1
2-color STED imaging of 200 nm sections of wild-type and mutant IHC ribbon synapses labeled with mCLING and fixed during stimulation or after recovery from stimulation followed by immunolabeling for otoferlin.

We now state in the figure legend what the lines indicate and what they are based on:
"White lines indicate the location of the plasma membrane, which was approximated in STED images of otoferlin fluorescence in the same sections (where the cell borders are quite evident, see Figure 9) and copied onto the mCLING images. Scale bar, 1 µm.

5. Moreover, a lot of the mCling appears to be taken up outside the cell in a stimulation dependent manner and the spatial distribution of this accumulation differs from otoferlin distribution. The authors should explain this better and provide explanations.??
mCLING, as a non-specific membrane marker, is taken up not only by the IHCs, but by other cells as well. In addition, mCLING decorates the extracellular leaflets of all plasma membranes, including those of supporting cells and of the afferent and efferent synapses that are positioned below the active zones of the IHCs. This results in a relatively strong mCLING signal below the IHCs, as shown in Figure 2 of this response letter. Finally, the stimulation-dependent uptake outside IHCs is most likely due to the increase in vesicle trafficking in the efferent synapses, as we have shown in the past using electron microscopy following FM1-43 uptake and photo-oxidation (Kamin et al., PLoS One, 2014).

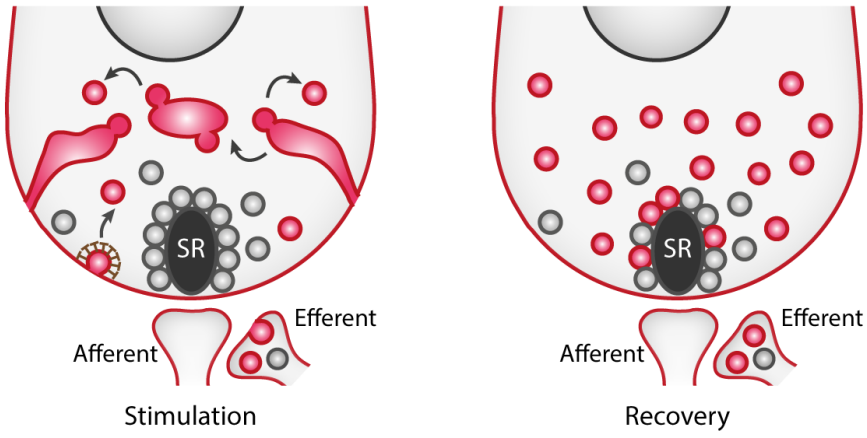

Figure 2 Schematic summary of current knowledge on mCLING labeling in IHCs (Revelo *et al*, 2014). During stimulation mCLING is taken up by endocytosis (clathrin-mediated: illustrated as coated pit and bulk retrieval: invaginated membrane budding of small vesicles) into the IHC also near the synaptic ribbon (SR) and presynaptic efferent terminals ("efferent"). After recovery from stimulation mCLING resides in regenerated synaptic vesicles.

We have now mentioned the mCLING membrane labeling/uptake of/into cells other than IHCs in the legend to Figure 7.
"Note that mCLING, as a non-specific membrane marker, is taken up not only by the IHCs, but by other cells as well. In addition, mCLING decorates the extracellular leaflets of all plasma membranes, including those of supporting cells and of the afferent and efferent synapses that are positioned below the AZs of the IHCs, which likely explains the fluorescence outside the IHCs."

6. Furthermore, the black lines in the "stimulation average" images in Fig 7 and 9 appear to make no sense. If these images are averaged over 10-15 ribbons, how can a boundary be drawn?

Images shown in Figures 7 and 9 of the MS and in Figure 1 of this response letter are averages of 10-15 ribbons containing sections that have been rotated to provide maximal overlap prior to averaging as described in the methods section. Accordingly, the boundaries like the colour-coded labeling intensities are not indicative of a single obtained image, but represent the average outline of the cell. We hope that the reviewer agrees that a rough outline of the cell can be visualized according to the averaged otoferlin channel. As indicated in the previous paragraphs, we used this outline to delineate the border of the IHCs.

7. The conclusion that AP-2 mediates active zone clearance is not convincing and the discussion is 'leading'. In the Discussion, the authors only consider impaired synaptic vesicle regeneration or active zone clearance to explain the observed defects in synaptic transmission. The 4 arguments listed in favor are not convincing. The fact that exocytosis was impaired in AP-2**µ**-deficient hair cells as early as 20 ms after stimulus is only an argument against vesicle reformation not other possible defects. Moreover, requirements for efficient fusion might also be lost already before the onset of stimulation, but only be noticed during. Any defect in the still poorly understood events that take place between a vesicle arriving at the active zone and fusion pore opening can explain the observed phenotype, without any 'site clearance'. The authors should discuss such alternatives, for instance impaired sorting of important proteins/lipids or some rate limiting biochemical reaction that fails or AP-2 might be required for otoferlin function (in addition to stability/localization). The conclusions on site clearance should be toned down..

In response to the reviewer's comment we have broadened the discussion, which now also states the otoferlin deficiency and a possibly otoferlin-related deficit in vesicle priming as possible explanations for the impairment of exocytosis. Moreover, we have weakened our statements on impaired site-clearance as the main mechanism underlying the AP-2 related phenotype in abstract (last sentence), introduction (top paragraph of page 6), results (end of page 17 and page 18) and discussion (starting page 18).
However, given the now clear phrasing as hypothesis, we prefer to still present impaired site-clearance as our favorite model for explaining the slowed vesicle reloading of release sites. We think that it is critical for the field to postulate and test molecular hypotheses that go beyond stating an important function of otoferlin in vesicle fusion and replenishment. While we fully agree with the reviewer on a central role of otoferlin in these processes, the question is how and through which protein-protein and protein-lipid interactions otoferlin exerts its function. The interaction of otoferlin with the endocytic adapter protein AP-2 offers entry points for further analysis of efficient vesicle replenishment of release sites and of otoferlin's function therein. We sought to investigate whether this interaction might fuel site-clearance, given that IHC release sites turn over vesicles at high pace and that a role of AP-2 in site-clearance has been proposed for the calyx of Held (Hosoi *et al*, 2009). We think that the impairment of sustained exocytosis and the increased contribution of the Gamma process to the interspike interval statistics are consistent with the hypothesis that AP-2 is required for site-clearance at the IHC active zone and that the interaction of AP-2 and otoferlin might contribute to this process. This is as far as we could get within the scope of this first study on the function of AP-2µ in IHCs. However, it will now be important for future studies to further test the site-clearance hypothesis by employing additional approaches such as functional super-resolution fluorescence imaging of exocytosis reporters at the active and periactive zone and investigations on

the role of other endocytic proteins that might similarly contribute to this process. We have now mentioned this as perspectives in the MS and have clearly stated the speculative nature of the molecular model (results: from end of page 17; discussion: from page 18).

8. Some discussion on the fact that he slow, linear component of release endocytic Cm decline is unaltered by AP-2 loss (Figure 3C, D) should be discussed in the Discussion. This seems inconsistent with previous studies.
Done, we now include the following paragraph in our
Results section, page 9, second paragraph:

"This finding is surprising given that AP-2 is a key clathrin adaptor and might indicate a function of residual AP-2 complex (e.g. comprising a,b2,s) in clathrin-mediated endocytosis (CME) of AP-2µ deficient IHCs among other possibilities."

and discussion to address this point:
"Finding that the linear endocytic decline of IHC capacitance was unaltered despite AP-2µ disruption was puzzling as this component of endocytosis was previously impaired upon pharmacological inhibition of clathrin and, hence, was assumed to reflect CME (Neef *et al*, 2014), which involves AP-2 as the key clathrin adaptor. These seemingly inconsistent findings might be reconciled when considering a potential function of a µ-deficient residual AP-2 complex in CME. Indeed, coated vesicles were observed, albeit at much lower number (Figure 6)."

9. Fig 3E: provide statistics for panel E and conclusion that amplitude is reduced
Done, comparisons in E and F revealed no statistically significant differences, there is only a non-significant trend towards smaller amplitudes in mutant IHCs. This now stated in results and figure and p values are stated in the legend.

10. Fig 7-9: Explain how the black dot was placed in the legend.
The black dot represents the position of the ribbons. All images were centered on the respective immunofluorescently labeled ribbons, and then averaged by rotating until the best overlay was obtained (see Methods). This procedure results in an average image of the ribbon as well, in the image center. We placed a black dot on its position in the mCLING frames.

11. Fig 9D suggests mitochondrial depletion in the mutant. Is that phenotypic?
We used low magnification transmission electron microscopy to further address this point. As you will see from the examples of the appendix to the letter, there is no obvious difference in the abundance of mitochondria in the basolateral compartment between IHCs of *AP-2µ*$^{fl/fl}$*:Cre* and *AP-2µ* control.

12. Fig 9F is not clear and the legend insufficiently explains what is depicted
We have enhanced the explanation of the figure panel in the results section and legend to figure 9:
Results, page 18:
"We speculate that following vesicle exocytosis AZ-localized otoferlin binds to perisynaptic AP-2 along an opposing gradient of these two proteins, which might speed lateral diffusion (Figure 5E, Figure S9) of otoferlin and associated exocytosed proteolipid away from the fusion zone into the perisynaptic area ("clearance zone", illustrated in Figure 9F). In this hypothetical model we assume that otoferlin localizes to both synaptic vesicles (Roux *et al*, 2006; Revelo *et al*, 2014) and plasma membrane (Roux *et al*, 2006; Pangrsic *et al*, 2010; Vogl *et al*, 2015) of IHCs at rest and becomes enriched at AZ during exocytosis, while AP-2 resides in the cytosol and is bound to the plasma membrane via phosphatidylinositol 4,5-bisphosphate (Gaidarov & Keen, 1999). We note that this hypothesis will require further testing e.g. by superresolution life microscopy."

Legend:
"F, Schematic of the proposed role of AP-2 in AZ clearance. The presence of otoferlin in synaptic vesicles and at the plasma membrane is indicated in red, the presumed distribution of AP-2 is shown in green. Our model proposes that AP-2 binds vesicular otoferlin, potentially only after vesicle fusion, and speeds its lateralization to a periactive clearance zone."

Referee #2:

We would like to thank the reviewer for her/his appreciation of our work and the help with improving the MS. Below please find our point-by-point response.

1. Suggested action of AP-2 in dissociation of otoferlin from prior binding partners should be strengthened by in vitro binding assays, or, if prior work provides this information, then such references should be included.

The suggested action of AP-2 in dissociating otoferlin from prior binding partners was a speculation on our part in order to envision mechanisms that might underlie site clearance. At this point it is not possible to address this hypothesis experimentally, as the potential prior binding partners are not characterized in detail. We now place more emphasis in our discussion on the speculative nature of this suggested action of AP-2 (starting end of page 18).

2. The number of animals used in the work presented in Fig. 2D was 2, and while the error bars were small, the confidence in the difference would be improved by the use of additional mice.

Done, we have successfully performed additional rescue experiments in order to strengthen the study:

By injecting AAV at postnatal day 10 via the round window into scala tympani of the left ear we have obtained near complete rescue of auditory brainstem response threshold and amplitudes in the injected ear of four mice (Figure 2 C-E). The non-injected ear of the same mice was as deaf as the non-treated mice and served as an internal control of the rescue (Figure S2C).

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We have now included the rescue data in the appropriate Figures (Figs. 2 and 3 as well as Fig. S10) and the accompanying results/methods/supplement sections. We now entirely focus on the postnatal AAV-rescue as it is still more interesting from a gene-therapy perspective and have removed the data obtained from transuterine AAV injections from the MS.

3. The coomassie gel in Fig. 8D does not show AP-2σ. A gel that used radiolabels would show all the involved proteins.

Done, In an attempt not to overload the gel with the higher molecular weight components of the AP-2 complex we had loaded too little protein onto the previously depicted gel to visualize the much smaller AP-2σ subunit as well. To show all subunits we now rerun the gel after loading substantially higher protein amounts, which leads to a clear band for the AP-2σ subunit. We have now replaced the image of the previous Coomassie gel with the new one (compare Figure 8D) and indicate the purified proteins by arrow heads.

4. The terms Gamma Process, Gamma Distribution, and Poisson Distribution are relevant to the introduction of this paper, but the introduction section would benefit from a 1 or 2 sentence description of these terms for unfamiliar readers.

Done, we now include some additional introductory sentences to familiarize the readers with these concepts.

5. The abstract defines AZ and IHC, the introduction defines AZ but not IHC. Please ensure consistency in the use of abbreviations.

Done, we checked the consistency of all our abbreviations.

6. Majority of figures suffer from the lack or poor use of color. Please make better use of color to allow easy visual interpretation. In particular, the black and 3 colors of gray in Fig. 3C and the multiple shades of green in Fig 6F stick out as graphs in which more readily distinguishable colors would facilitate communication.
Done, in response we introduced color to figures 2-5 and improved the color layout of figure 6.

7. In Fig. 3E, is there no statistical difference between the bars on the left graph, and if there is, why isn't it marked with an
Done, there is no statistically significant difference between the IHCs of both genotypes for data of panels E and F. This now noted in the results section and the graph and p values are stated in the legend.

8. For Fig. 6A, B, the opening sentence is over 60 words long, not counting the three parenthetical sections. Please break into multiple sentences to improve readability, such as at the colon or semicolon.
Done, we have improved the overall readability by using shorter sentences.

9. Regarding Fig. 6, are the virtual slices from the reconstructed tomograms? If so, please mark the relevant vesicles (magenta arrows) shown in Fig. 6E in Fig. 6E'.
Done, we marked all relevant vesicles.

10. The description for Fig. 8C, E, is heavy on methods that may be better presented in the Methods section.
Done, we agree and have transferred details to the Methods section.

11. What is the purpose of the green arrow in Fig. 9B
It points to the interesting peak of otoferlin fluorescence at the plasma membrane of IHCs of *AP-2μ$^{fl/fl}$:Cre*, which is not observed in IHCs of the otoferlin missense mutant *Otof$^{pga/pga}$*. This now stated in the legend.

Referee 3:

We would like to thank the reviewer for her/his appreciation of our work and the help with improving the MS. Below please find our point-by-point response.
1) VGlut-3 is known to also be strongly expressed in neurons of the peripheral and central auditory pathway, in particular in the type I spiral ganglion neurons (SNGs). Therefore the deletion of AP-2 in the present study is not hair cell specific and I wonder whether a deletion of AP-2 in SGNs may have also directly impacted on the development and the responses of the afferent auditory nerve fibers.

We have carefully re-examined this point: we do not find any GFP-reporter fluorescence in the SGNs of Vglut3-Cre mice and have previously also published absence of Vglut3 immunolabeling in the SGNs (Figure 1B of (Reisinger *et al*, 2011)) and specifically in their postsynaptic terminals (Figure 3 of (Neef *et al*, 2014)). These findings as well as our present study (Figure 1A, and now in addition Figure S1A) are consistent with previous knock-out-controlled studies (Seal *et al*, 2008; Akil *et al*, 2012). As pointed out by the reviewer there are some studies that report Vglut3 expression in cochleovestibular neurons a cycling mouse mutant (Lee *et al*, 2015) and in rat (Peng *et al*, 2013), but these studies did not use knock-out controls.

2) In absence of AP-2μ, the otoferlin expression level in IHCs is strongly decreased (Fig.9). Therefore, it is questionable whether the vesicle ribbon phenotype is not essentially coming from a lack of otoferlin? Ideally, a rescue of otoferlin in the AP-2 KO mouse could clarify this.
Unfortunately, virus-mediated expression of full-length otoferlin is currently not yet feasible due to the large size of the cDNA. So far, we failed in spite of all our efforts to overexpress otoferlin using various viral constructs (unpublished data). Moreover, the group of Dr. Petit has recently demonstrated that AAV-mediated expression of a C-terminal otoferlin fragment containing the C_2D-F, in contrast to a rescue experiment in zebrafish (Chatterjee *et al*, 2015), cannot restore hearing in otoferlin knock-out mice (ARO midwinter meeting abstract 2015).

Therefore, in response to the reviewer's comment, we have broadened the discussion, which now also states the otoferlin deficiency and a possibly otoferlin-related deficit in vesicle priming as possible explanations of the impairment of exocytosis. Moreover, we have weakened our conclusion that impaired site-clearance is the main mechanism underlying the AP-2 related phenotype, but prefer to still present it as our favorite interpretation. We think it is critical for the field to postulate and test molecular hypotheses that go beyond stating an important function of otoferlin in vesicle fusion and replenishment. While we fully agree with the reviewer on a central role of otoferlin in these processes, the question is how and through which protein-protein and protein-lipid interactions otoferlin exerts its function. The interaction of otoferlin with the endocytic adapter protein AP-2 offers entry points for such analysis. We sought to investigate whether this interaction might fuel site-clearance, given that IHC release sites turn over vesicles at high pace and that a role of AP-2 in site-clearance has been proposed for the calyx of Held (Hosoi *et al*, 2009).

3) I think it would have been very informative to study the exocytotic-endocytotic defect during development, i.e. in early pre-hearing IHCs at P2-3, an age at which otoferlin is not yet fully engaged (see Beurg et al., 2010).
We thank the reviewer for this advice. However, we do consider such an entirely new data set beyond the scope of the present study that we purposely performed after the onset of hearing, because only at this time point we could perform the in-depth analysis of sound encoding including the systems level. Moreover, it is likely that AP-2µ is not yet sufficiently depleted at the advised early time point (postnatal day 2-3) in our current mouse model, as Cre expression, controlled by the Vglut3 promoter, supposedly sets in only at around birth. Hence, we would like to refrain from attempting these important experiments at the present point in time and would take on the challenge in a future study by then switching to another Cre line (e.g. Math1-Cre^{Er}) to achieve AP-2µ deletion at the late embryonal/early postnatal time window.
4) To further characterize the vesicle traffic congestion at the active zones, I think it is essential to study exocytosis under a repetitive train of stimulations or during a paired-pulse protocol in figure 3.
We take this comment to indicate that the reviewer would like us to apply even stronger stimuli than the 200 ms of step depolarizations to further provoke the site replenishment phenotype. Therefore, in response to this request, we have analyzed the exocytic responses to longer depolarizations (500 ms). These data were acquired in the presence of 5 mM [Ca^{2+}] and show a significant reduction of exocytosis in IHCs of *AP-2µ$^{fl/fl}$:Cre:GFP* mice: 386.8 ± 72.8 fF (n=10) vs. 800.9 ± 97.5 fF (n=14), p = 0.006, Wilcoxon Rank Sum Test. Ca^{2+} current integrals were not significantly different (0.36, Wilcoxon Rank Sum Test).
While these responses to 500 ms depolarizations were more variable in amplitude in both genotypes than for shorter depolarizations and may also include additional extrasynaptic exocytosis (Pangršič *et al*, 2015), this data corroborate our notion that AP-2µ disruption reduces sustained exocytosis.
In addition, we would like to refer the reviewer to our *in vivo* recordings from single SGNs that readout release from individual IHC active zones and directly address the point raised by the reviewer. Figure 4 shows how the response to tone bursts runs down at higher rates of stimulation (going from 2 to 10 Hz repetition of 50 ms tone burst stimulation at 30 dB relative to threshold) due to impaired replenishment of the release sites. Figure 5 directly measures the extent of short-term depression and the recovery from it, and demonstrates the impaired vesicle replensihment.

5) The authors should give us some reasons why deleting and studying the µ subunit instead of the other subunits of the AP-2 complex.
The AP-2 complex comprises four subunits encoded by five distinct genes in mouse. The alpha subunit is encoded by two distinct, yet, functionally overlapping genes (termed a_A and a_C), and, hence would require the generation of double knockout strains. The b2 gene is highly similar to b1 and when deleted has been reported to result in a genetic hypomorph (Li *et al*, 2010) due to misincorporation of b1 into AP-2 complexes. Finally, while loss of function of s2 remains uncharacterized it is known that loss of AP-2m largely abrogates AP-2 function and leads to early embroynic lethality (Mitsunari *et al*, 2005). Hence, we decided to target AP-2µ for conditional deletion in IHCs.

6) Figure 1 does not give much information on the morphology of the hair bundle, as stated in page 6 (line 18). Scanning electron microscopy of the hair bundle is required. Also it is difficult to conclude anything on the synaptic base of the IHCs in this figure. For example, NF-200 labeling of the bundle of afferent fibers contacting the base of the IHCs would have been more instructive and convincing.

We now show apparently normal hair bundle morphology by phalloidin labeling as Figure S1C.

7) I have a strong concern on the DPOAEs measurements shown in Fig 2C. They show a rather high threshold above 50-60 dB SPL. Usually, DPOAEs in mice display thresholds well below 30 dB SPL.
Thank you for detecting this mistake. Indeed the calculation of DPOAE intensity in dB SPL had been incorrect. The analysis has now been redone. The best DPOAE thresholds were at an F2 intensity of 20dB SPL. The DPOAE graphs have been moved to Supplementary Figure 2 to make space for the new ABR data from virus-mediated rescue of AP2-expression.

8) In Fig 2D, the untreated ear seems to show some ABRs recovery as compared to 1A. Some explanation should be given. Do not give error bars with n= 2, show individual mouse data points.
Done, we have successfully performed additional rescue experiments in order to strengthen the study:
By injecting AAV at postnatal day 10 via the round window into scala tympani of the left ear we have obtained near complete rescue of auditory brainstem response threshold and amplitudes in the injected ear of four mice (Figure 2 C-E). The non-injected ear of the same mice was as deaf as the non-treated mice and served as an internal control of the rescue (Figure S2C).
Moreover, we are really excited to now also report the restoration of IHC function based on patch-clamp recordings of exocytosis from transduced IHCs (Figure 3A, B). Indeed, transgenic expression of AP-2µ restored sustained exocytosis of *AP-2µ$^{fl/fl}$:Cre* IHCs to wild-type levels. The fact that AP-2µ expression driven by the CMV enhanced human ß-actin promoter, which likely surpasses AP-2µ wild-type levels, did not enhance exocytosis beyond wild-type levels suggests that AP-2µ is not rate-limiting for exocytosis beyond the AP-2µ copy number present in wild-type IHCs. Finally, the AAV-rescue also increased otoferlin levels in IHCs of *AP-2µ$^{fl/fl}$:Cre* mice (Figure S10).
Apart from their value for understanding AP-2µ function in sound encoding at the IHC synapse, our rescue experiments add to recent work on establishing AAV-mediated gene transfer for therapeutical purposes (Akil *et al*, 2012; Askew *et al*, 2015). While genetic deafness due to mutations of AP-2µ has not yet been reported, mutations in other synaptic genes such as *OTOF* and *VGLUT3* cause deafness. The extent of rescue obtained by AAV-mediated transgenic expression indicates that early postnatal gene replacement, indeed, can restore function in a complex structure such as the inner ear to enable near normal hearing. This is now briefly stated in the last section of discussion.
We have now included the rescue data in the appropriate Figures (Figs. 2 and 3 as well as Fig. S10) and the accompanying results/methods/supplement sections. We now entirely focus on the postnatal AAV-rescue as it is still more interesting from a gene-therapy perspective and have removed the data obtained from transuterine AAV injections from the MS.

9) Figure 7 is very confusing and not convincing. It should be re-orgainzed with 9E
Done, we have worked on the figure legend and the results section to clarify the message.

10) Why looking for AP-2 interactions with the otoferlin N-terminal C2A-B-C domains? Some reason should be given.
It is due to technical difficulties with the expression and detection of the tagged C-terminal C2D-E-F fragment that we cannot provide an interaction analysis for this part of otoferlin as well at this point. Unfortunately the available otoferlin antibody is directed against the N-terminal fragment, so that we could not resort to this alternative detection means. This has now been added to the results section (top paragraph, page 16).
Moreover, in an independently performed experiment we used otoferlin C2A-C to pulldown interaction partners from brain lysate and found several subunits of the AP-2 complex to interact with the N-terminal part of otoferlin (unpublished observation), thus indicating a substantial interaction of this otoferlin fragment with the AP-2 complex.
In the future we will intensify our attempts to dissect the structural details of complex formation between full-length otoferlin and the AP-2 complex by performing cross-linking experiments.

11) In fig 9C, it does not look like the level of otoferlin is reduced? In Fig 9D: I don't see the ribbons? At left, what are the numerous grey circles, vesicles or mitochondria? I have a hard time to see and understand the otoferlin lateral diffusion in Fig9E, I only see an internal diffusion of otoferlin.

The focus of Figure 9C is on the subcellular distribution of otoferlin and not on the semiquantitative comparison of protein levels (this is done in Figure 9B). Images presented in Fig 9C come from 200 nm-thick melamine sections. Hence, they represent an extremely small fraction of the total volume of each IHC. Therefore, such images cannot be used to judge overall protein abundance, but rather protein distribution in respect to membranes (labeled with mCLING). For quantitative purposes, tens of sections would need to be pooled together to make a clear estimate of protein abundance differences throughout the IHC anatomy. This is especially difficult, considering that otoferlin distribution changes from the cell top to bottom. That is the reason why quantification from confocal projections is more reliable in this case (see Figure 9A, B).
Figure 9D does not depict ribbons, the grey "circles" indeed are mitochondria. We have now added a graphical aid to mark one mitochondrium per image.
Figure 9E shows STED imaging of the otoferlin-immunofluorescence distribution at ribbon synapses of wild-type IHCs obtained after fixation of organs of Corti during steady-states during rest, K^+ stimulation and after recovery from stimulation,-no life imaging was performed. We agree with the reviewer, that one clear effect of stimulation is an increased intracellular label which likely reflects otoferlin on membranes internalized during exocytosis, as it appears to colocalize with the membrane tracer mCLING. We did /do not claim that Figure 9E proofs lateral diffusion of otoferlin. However, we hope reviewer agrees that there is a reduction of the ribbon-associated otoferlin immunofluorescence from "stimulated" to "recovered". We consider this as consistent with a redistribution of otoferlin from the center of the active zone.

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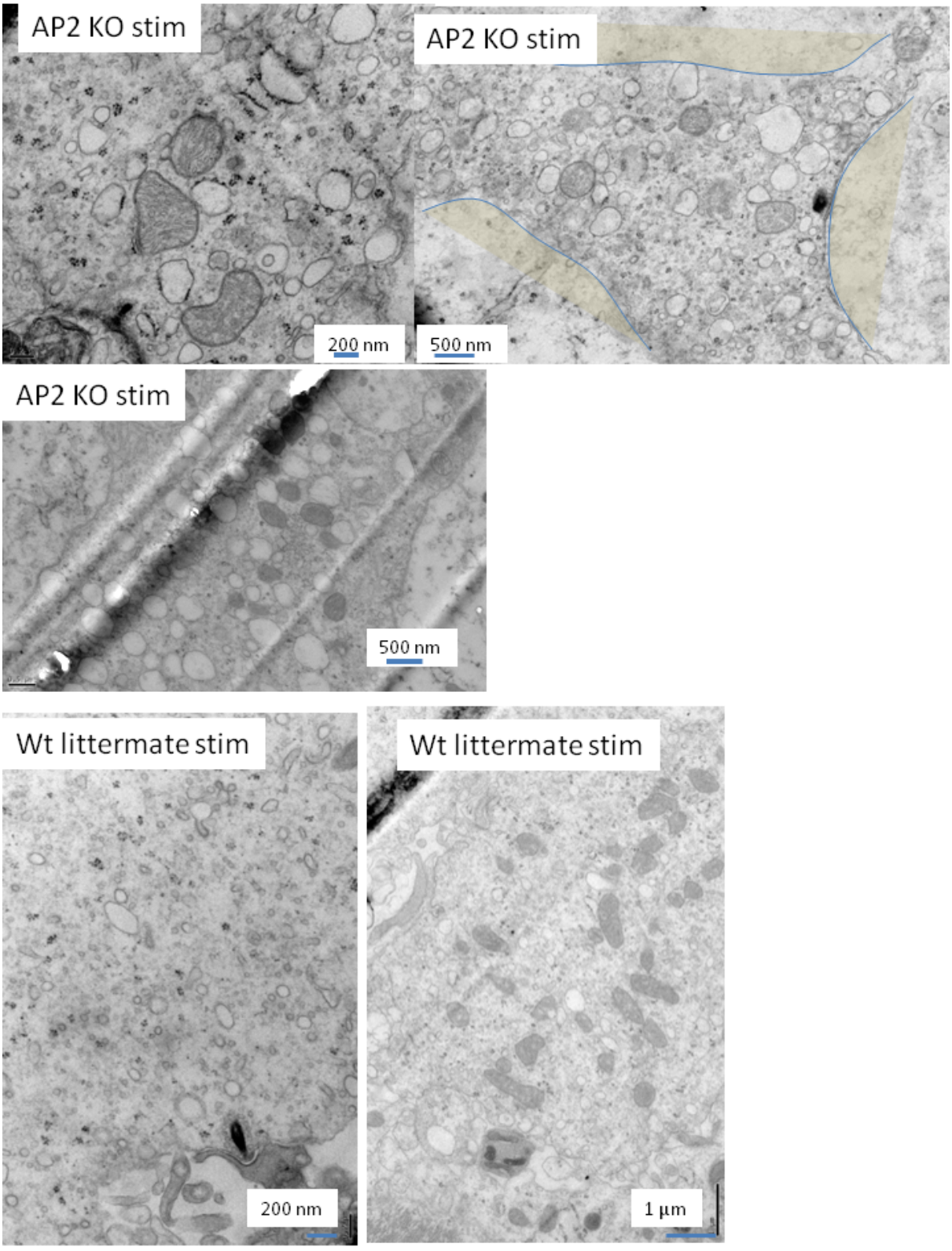
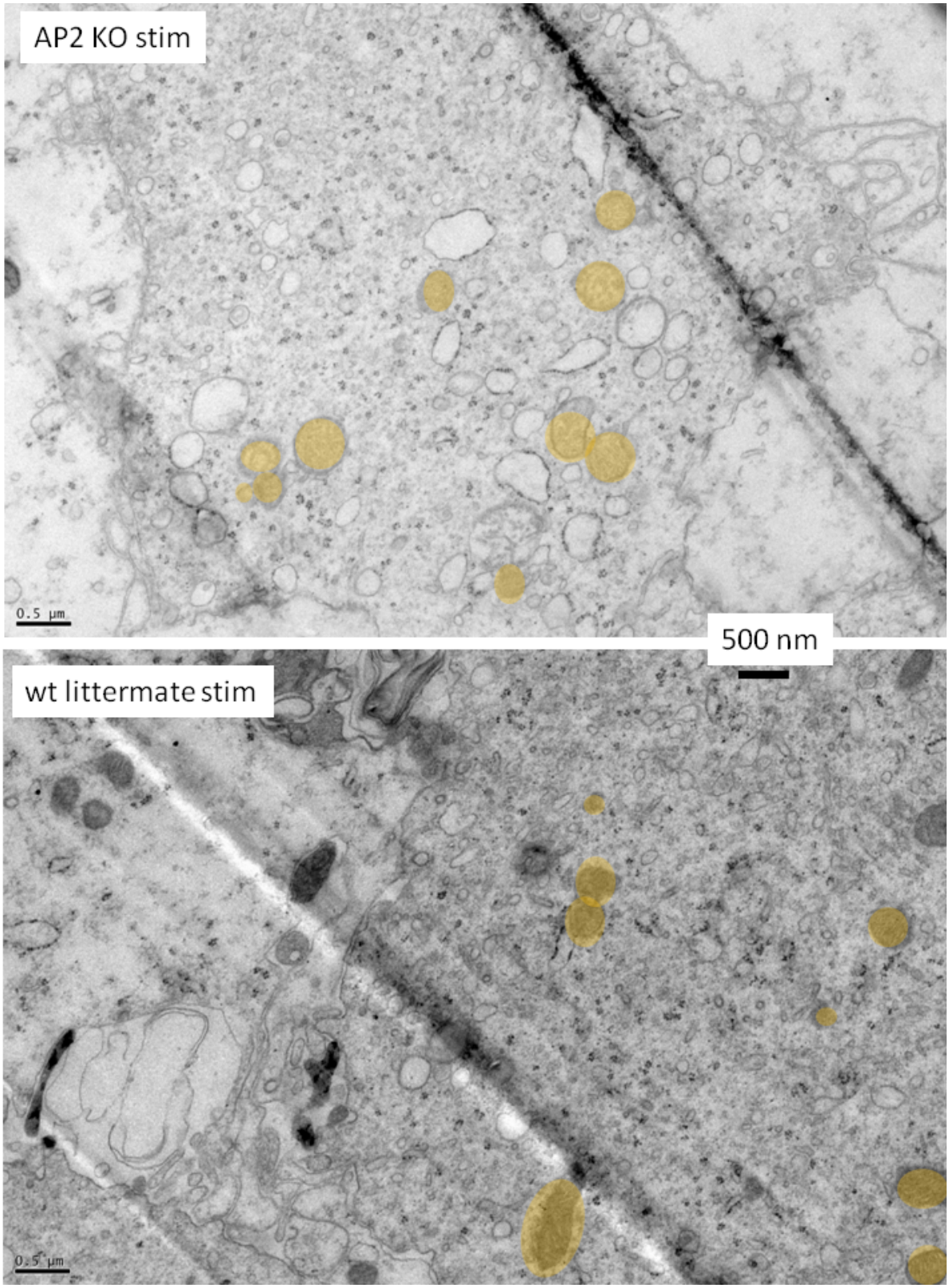
Appendix Sample low magnification TEMs of basolateral wild-type and mutant IHCs: no obvious difference in the number of mitochondria between IHCs of both genotypes. We apologize for the low quality of some of the images (cutting artifacts).





2nd Editorial Decision 25 August 2015

Thanks for sending us your revised version. The manuscript has now ben re-reviewed by referees #1 and 3. As you can see below, both referees appreciate the introduced changes. I am therefore very pleased to accept the manuscript for publication here.

REFEREE REPORTS

Referee #1:

The authors have adequately dealt with all points raised, in fact more than I proposed (e.g., in vivo rescue). The only issue that I consider not fully satisfactory is the fact that the ultrastructural morphometry is still based on too few independent observations (my major point 3). Variation between animals remains a confounding factor. However, since it would require months to correct this, I think it can be left like this. The text clearly states the fact that only 1-2 animals were used.

Referee #3:

The authors have have satisfactorily answered the raised criticisms.

