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Developmental refinement of hair cell synapses tightens the coupling of Ca^{2+} influx to exocytosis

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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 24 June 2013

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

While referee #2 is fairly supportive of the work, referee #1 also raises many issues with the manuscript that I am afraid preclude publication here. Many of the issues raised concern the technical aspects of the paper. Referee #1 also finds that there is significant overlap between this paper and your Wong et al. J. of Neuroscience paper. I have looked at the Wong et al paper and while I do appreciate that this paper goes well beyond that paper, I also see the overlap as indicated by the referee. Given the technical concerns raised and the related Wong et al. paper, I am afraid that we can't offer to consider publication here.

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

REFEREE REPORTS

Referee #1

This is an interesting manuscript describing a potential pathway for synaptic maturation of ribbon synapses in auditory hair cells. It uses a variety of state of the art technology to explore the

molecular architecture of synaptic calcium channels and changes in the calcium dependence of release. It attempts to address questions regarding diversity of synaptic properties between hair cell afferent fibers as well as developmental changes in the molecular mechanisms of synaptic release. The figures are presented clearly and for the most part the manuscript is reasonably well written. There are a variety of issues that detract from overall enthusiasm and the likelihood of a broad readership. These include (in no particular order):

1- Calcium imaging is not well quantified. How are different ages compared, how many cells are included in the measurements? What were the conditions used for the measurements, ie external calcium concentration, voltage steps.

2- The table is not a particularly useful means of data presentation. Perhaps box plots that show individual measurements would be more informative.

3- There is considerable overlap between this work and a paper in press in Journal of Neuroscience. Data here appears in part to be what is left over from that work and doesn't often make a compelling story. The advance compared to published work is not that clear, other than the uncaging data which is interesting and important but not well quantified. A clear delineation between these works needs to be made.

4- Much of the writing is presented as if the authors have a knowledge base that is unavailable to the general audience, speculating about potential mechanisms as if they were either demonstrated in the manuscript or known to be true. Additionally referencing was biased toward identifying only those manuscripts supportive of the speculation and often only small parts of these manuscripts. Although this approach tells a good story, it detracts from the credibility of the presentation and makes evaluation more difficult.

5- The subtypes of identified ribbon synapses is interesting. Have the authors correlated these subtypes to location within the hair cell in order to infer something about function? Also are these data controlled for when (ie time of day) the animals were fixed. Changes in ribbon synapse morphology are known to occur diurnally, in photoreceptors but also in organotypic cochlea cultures, so that time of day for data collection may be relevant.

6- Were different ribbon morphologies identified in the same cells? Were they at particular cellular locations?

7- How do the authors separate between synapses that are forming, or degrading or are mature. In the snapshot that immunohistological data provides it seems quite difficult to know whether:
a. A floating ribbon has just detached and is on the pathway to degredation
b. A floating ribbon is about to attach and create a new presynaptic entity
c. A ribbonless synapse has any functional significance
d. A ribbonless synapse has just lost its ribbon and is in the process of degrading
e. A ribbonless synapse has just formed and is awaiting its ribbon
f. The morphologies presented for different ribbons are a function of fixation times
g. The ribbon morphologies are an indicator of forming and or degrading synapses

8- In the abstract, what does more connected mean? How was connectivity of the ribbon to the synapse measured? I could not find any real measurement of this in the manuscript.

9- Similarly in the abstract it states that stripe-like clusters were formed from smaller round clusters as if there were a causal link between these events. No link is demonstrated in the text. This needs to be clarified.

10- Similarly what specifically does 'tighten' mean in the abstract? It is stated that spatial coupling of influx to exocytosis is tightened. I assume that the authors are describing an increase in colocalization of calcium channels with Ctbp2 labeling, it is unclear how release is included in this argument or again what tightening specifically refers to.

11- In the introduction the authors refer to fusion competent vesicles tethered to ribbons and cite Frank et al. Where was fusion competency defined? It remains unclear, at least to me, how tethered

vesicles relate to nontethered vesicles relate to discrete pools of vesicles as estimated from capacitance measurements.

12- The description of calcium buffering also appears a bit oversimplified as published measurements range from micromolar to millimolar depending on reporting technique that is used yet the authors select high mobile buffer references as the accepted answer.

13- The description of figure 1 is quite confusing as it does not seem to match the data presented in the figure. Qualitatively there appears to be a consolidation of gutamate receptors to localize better with the ribeye labeling in adult as compared to immature synapses. However this needs to be quantified and there are many relatively simply algorithms for quantifying colocalization that could be used. In addition, the authors argue that the NaKATPase labeling supports a 1:1 correspondence between IHC synaptic structures and single neurites. This is not at all clear in Figure 1. The green labeling is quite diffuse, individual neurites cannot be distinguished and the colocalization with synaptic elements is at best poor. Some nonbiased means of quantifying this data is required. The circled region seems arbitrarily selected to make a point but many other circles could have been drawn.

14- It would be helpful for the authors to label synapses considered to be ribbon free.

15- Authors argue that the maximal intensity of the ribeye labeling confirms that the ribbons are doubling in size. How do the authors know that ribeye density is not changing? Given the EM images showing different shapes of ribbons, one might postulate an increase in density so that if the labeling were linearly related to number of molecules (a big if) then the change in intensity would still not be related to size but to density. The EM data appears more reliable for this level of analysis and it might be best to simply stick with that. Of course here, the argument would be an increase occurring only a week after hearing onset.

16- It should be made clear whether SEM or TEM was used for the measurments. I assume TEM but it is not always written that way.

17- Figure 3E, gold particles are not visible in image. 3F is also likely to not reproduce well as it is very small.

18- For both figure 3F and figure 4 the proprotion of the figure should be adjusted so that the zoomed in view is more prevalent. The larger lower power provides no useful information, even less when reproduced in a journal format. The higher resolution of the STED system is somewhat lost by how these data are presented. Zooming in on the individual synapses would be much more powerful presentation to demonstrate the STED resolution.

19- Quantifying the changes observed in figure 5 would greatly strengthen the authors' arguments. The qualitative assessment of colocalization is not convincing. This is particularly true for the calcium imaging data where no quantification, not even number of recorded cells, is provided. As shown the images are quite dark so arrows are visible but not much else. As presented the calcium imaging data is of little value. Quantification is required for interpretation.

20- The data in figure 6 suggest that the number of calcium channels between P14 and P20 are quite similar, however the immunofluorescence in Figure 5 does not really seem to support this argument. The spots are bigger and brighter at P14 then at P20? This is confusing and not addressed at all in the text.

21- How do the authors reconcile the difference in their data with preious reports from Marcotti group suggesting a change in maximal probability of opening with maturation? There I believe the authors used single channel measurements? Is the difference the associated with a noise analysis approach? This seems particularly critical especially when being used as an argument for nanodomains?

22- Figure 7 should be modified so that kinetics compartments can be seen in raw data.

23- How do the authors deal with a difference in available vesicles between ages when comparing

release rates? If fewer vesicles are avaliable or primed at one age then release rates may appear different but have no causal link to release mechanism. That is there could be a lot of misses where vesicles are either not present or not primed and so cannot be released. Similarly if release rates appear to be the same despite a difference in available vesicles, one might argue that there were intrinsic variations normalizing for the difference in vesicle number. Clearly the biggest difference in data presented is in number of vesicles available for release. And given the large number of vesicles needed to account for the large changes in capacitance, vesicles would need to be transported to the synapse. Perhaps it is differences in vesicle transport that the authors should be addressing here as it is unclear the data can reconcile changes in release rates?

24- Figure 8B could be made a little more clear if the 5mM Ca were shown at the start and if each segment matched the perfusion time, ie no gaps.

25- The discussion is highly speculative and little direct data is presented to support the authors arguments. Although some speculation is good, it typically is best to ensure the readership can tell the difference between what is known and what the authors think. There is no separation here. Alternate possibilities and a more eruditic approach to citations would help. As it stands the discussion does not due justice to the data presented or to the known literature.

26- Authors discuss rate constants and a changing of proportions in rate constant for figure 5 but provide no statistical analysis or summary box plots to illustrate their point.

27- The wilcoxin rank test requires data to be related, it is unclear how the data presented are related?

Referee #2

This manuscript very admirably describes the developmental progression of the mouse cochlear ribbon synapse, showing with outstanding light and electron microscopy that the synapse refines from multiple small ribbons to a single well-organized ribbon. These anatomical changes in the ribbon is correlated with a refinement of the CaV1.3 channels, as they are reduced in numbers and form into striped clusters near bassoon. Also correlated in time are alterations in synaptic physiology, including a reduced Ca current (correlating with the reduction of CaV1.3 channel number), an increase in evoked Cm, and linearization of the apparent Ca dependence.

In general, the data are outstanding and support the authors' conclusions very nicely. However, there are several presentation issues that need to be addressed, mostly with the light microscopy:

1. Fig. 1. While the immunocytochemistry with confocal and STED is outstanding, its presentation could improve greatly. The images are plagued by being too small and by using colors that don't allow clear visualization of the key molecules very well. The RGB combination, while the standard one used, has two problems: first, the blue is impossible to see, as its brightness appears to be very low, and second, red-green color blind individuals have a hard time with the images. Since the localization is a key part of this manuscript, these color and size issues should be addressed. For example, the lower mag panels of Fig. 1A and 1B could be done in green/magenta and thus would be much easier to comprehend. In addition, the magnified panels of 1A should be in grayscale for the individual panels (the blue is impossible to see when printed or on the monitor). Even though the blue is hard to see, Fig. 1C works because of the {plus minus} bassoon panels. Figs. 1D-E are OK but the blue is particularly hard to see in 1E; another triple-label color scheme might be better.

2. Fig. 3. Panel F should be in a green/magenta or similar color scheme that more readily pops out. In addition, the low mag panel of 1F will present no information in the journal, where it may well be smaller. The magnified panels in 1F should be at least 2x larger than they are now relative to the rest of the figure (maybe get rid of the low power view and magnify all the close-ups).

3. Fig. 4. This figure should be all grayscale (no value to color in the low-mag panels). Again, the value in the figure is in the close-up panels, which should be much larger.

4. Fig. 5A. Panels are OK but would probably be more easily understood if it was in green/magenta.

In addition, the scale should be the same for all panels and it would be better if the view was closer in.

5. Fig. 5B. There is no reason why the panels in B should be in color; grayscale would be much better (the gradations in intensity are far easier to see, particularly instead of red-the RIBEYE staining is impossible to see).

6. Fig. 6B. State in the legend that NCa comes from the nonstationary noise analysis (e.g., not from ICC).

Additional Correspondence 10 July 2013

As discussed, I did send your point-by-point response to referee #1. I have now heard back from the referee. The referee appreciates the proposed changes and finds that they would significantly improve the paper. I also like the inclusion of the biophysical model.

Given this, I would therefore like to invite you to submit a suitably revised manuscript that includes the proposed changes as outlined in your point-by-point response. When you resubmit your manuscript, please mention in the cover letter that this is an invited resubmission of MS 85547 and address it to me.

Resubmission 09 October 2013

Referee #1

This is an interesting manuscript describing a potential pathway for synaptic maturation of ribbon synapses in auditory hair cells. It uses a variety of state of the art technology to explore the molecular architecture of synaptic calcium channels and changes in the calcium dependence of release. It attempts to address questions regarding diversity of synaptic properties between hair cell afferent fibers as well as developmental changes in the molecular mechanisms of synaptic release. The figures are presented clearly and for the most part the manuscript is reasonably well written. There are a variety of issues that detract from overall enthusiasm and the likelihood of a broad readership. These include (in no particular order):

We would like to thank the reviewer for the overall appreciation of our work and for the efforts to provide a detailed feedback, based on which we further improved the MS. We have further strengthened the MS by providing further data to support our hypotheses, cautious interpretation where support by additional data could not be achieved within the scope of the study and more balanced discussion of the state of the art including reference to further studies.

On the aspect of new data we have included:
-additional experiments on the apparent Ca^{2+} dependence of exocytosis: we acquired a new data set for the isradipine block of Ca^{2+} current in p14-17 ("mature") IHCs in 5 mM $[Ca^{2+}]_e$. Figure 7B now focuses on p6-8 and p14-15 IHCs, recorded under identical conditions. The isradipine data on p9-10 and p14-15 IHCs acquired in 10 mM $[Ca^{2+}]$ are now provided in Fig. S8.
-biophysical modeling of presynaptic Ca^{2+} influx, Ca^{2+} buffering and diffusion as well as Ca^{2+} triggered exocytosis. This model uses estimates/assumptions on the number and topography of Ca^{2+} channels and readily releasable vesicles and other parameters (taken from the present study and previously published work). Modeling a realistic number and topography of channels and membrane proximal vesicles (which we and others interpret as likely representing the readily releasable pool) with the underlying intracellular Ca^{2+} dynamics and Ca^{2+} dependent vesicle fusion we could predict the outcome of biophysical experiment, which has been used to estimate the amount of overlap among the Ca^{2+} domains around each of the Ca^{2+} channels that contribute to the Ca^{2+} signal triggering exocytosis of a given vesicle in previous work on different synapses (Augustine *et al*, 1991; Mintz *et al*, 1995; Wu *et al*, 1999; Brandt *et al*, 2005; Fedchyshyn & Wang, 2005, Wang et al., 2008). We modeled various scenarios with different topography of Ca^{2+} channels and vesicular release sites as well as different positions of the Ca^{2+} sensor and different open probabilities. We

used the model to calculate time-averaged Ca^{2+} profiles and the effective number of channels contributing to the Ca^{2+} at the Ca^{2+} sensor. Most importantly, we also estimated the exponent *m* (or apparent cooperativity) describing the exocytosis vs. Ca^{2+} charge functions, when Ca^{2+} influx was manipulated by changes in either the number of open channels or in the single channel current. The various models typically predicted *m* near the experimentally observed estimates when simulating changes in the single channel current. The model of the mature active zone (AZ) indicates that in order to get a near linear apparent Ca^{2+} dependence (exponent *m* of 1.4 as observed in the experiments where more and more Ca^{2+} channels are pharmacologically blocked) a dominant contribution of the nearest channel needs to be implemented. Modeling exocytosis of immature IHCs was more challenging as there likely is also a contribution of extra-synaptic release and in addition not all cells showed the fast component of exocytosis upon Ca^{2+} uncaging, which we used to define the intrinsic Ca^{2+} dependence. Nonetheless, the models readily predicted *m* values close to the experimental estimates, which are more similar between the two manipulations (number of open channels and single channel current) than for the mature IHCs. For the sake of focus we selected 4 paradigmatic models (3 mature, 1 immature) for presentation in the main manuscript and provide additional models in the supplement.
In summary, biophysical modeling allowed us to better understand Ca^{2+} influx-exocytosis coupling in IHCs and lent support to our hypothesis that vesicles couple more tightly in space to Ca^{2+} channels at mature IHC active zones ("Ca^{2+} nanodomain").

1- Calcium imaging is not well quantified. How are different ages compared, how many cells are included in the measurements? What were the conditions used for the measurements, ie external calcium concentration, voltage steps.

We did provide the depolarization steps (to - 7 mV) in the legend to Fig. 5 (where we also stated the EGTA concentration in the pipette: 2 mM) and in the methods (where we also specified the $[Ca^{2+}]_{bath}$ (5 mM)). To satisfy the reviewer's request we now mentioned both, as well as the number of recorded cells for which the observed patterns are representative also in the results section.

In addition, we have performed additional analysis as advised by the reviewer and show quantitatively that Ca^{2+} signaling becomes confined to ribbon-type active zones upon maturation (results section and Fig. S5). Specifically, we find that the ratio of DF away from the ribbon over DF at the ribbon (indicative for the relative extrasynaptic Ca^{2+} signal) is larger in immature IHCs with "global change of Ca^{2+} indicator fluorescence" than in immature IHCs with "spot-like change of Ca^{2+} indicator fluorescence" and even more so after when compared to IHCs after the onset of hearing (Fig. S5).

Immunohistochemistry, confocal imaging of immunofluorescence and confocal Ca^{2+} imaging, each, were performed under identical conditions and in parallel (strictly in parallel for immunohistochemistry, in random succession for confocal Ca^{2+} imaging) for the specified age groups. As a response to the reviewer's comment this has now been more clearly stated in the MS (page 10-12), e.g.
"…, in experiments run in parallel under identical conditions for the different age groups." (page 10)

2- The table is not a particularly useful means of data presentation. Perhaps box plots that show individual measurements would be more informative.

Done, now included into Fig. 2

3- There is considerable overlap between this work and a paper in press in Journal of Neuroscience. Data here appears in part to be what is left over from that work and doesn't often make a compelling story. The advance compared to published work is not that clear, other than the uncaging data which is interesting and important but not well quantified. A clear delineation between these works needs to be made.

We regret that the reviewer thinks so. We were upfront, cited the paper *in press* and provided a preprint on our website also to enable comparison. The former looks at limited aspects of synaptic maturation in relation to development of *in vivo* sound encoding. The current MS employs various challenging and innovative approaches for comparison of changes in molecular nanoanatomy over development with changes in cellular biophysics. We very carefully avoided any overlap in data or

analysis with Wong et al., 2013. In our view the separation is at hand and the MS submitted to EMBO J is a novel and very substantial contribution to the field that advances the understanding of maturation of synaptic structure and function, and of stimulus-secretion coupling in particular, in hair cells but also more generally:

1) Wong et al., J Neurosci 2013 is a brief communication that is based primarily on physiology: imaging of synaptic Ca^{2+} signaling in IHCs and extracellular recordings from putative spiral ganglion neurons around the onset of hearing. This report makes the point that the emergence of synapses with more Ca^{2+} channels around the onset of hearing increases presynaptic heterogeneity and coincides with increased diversity of firing properties in the postsynaptic spiral ganglion neurons. This correlative argument is further supported by consideration of pre- and postsynaptic data from Bassoon mutant mice and a simple phenomenological model. In essence, this report argues for a link of the number of presynaptic Ca^{2+} channels and the functional properties of the postsynaptic spiral ganglion neurons.

2) The MS submitted to EMBO J, on the other hand, combines cell-biological (more than half of the figures) and biophysical approaches to study changes in structure and function during maturation of individual hair cell synapses with focus on exocytosis and its Ca^{2+} control.

The high-resolution morphological results obtained by standard (confocal, TEM, serial reconstruction) as well as innovative (STED, 2-color STED, EM-tomography) are novel and provide important insights e.g. into the spatial organization of Ca^{2+} channels and AMPA receptors before, during and after the onset of hearing as well as the maturation of the active zone scaffolds and ribbons. For an example, the findings of several spot-like presynaptic densities in EM and spot-like Ca^{2+} channel clusters at immature active zones, as well as the co-alignment of $Ca_V1.3$ Ca^{2+} channels and the presynaptic density protein bassoon are entirely novel. They represent important information for understanding active zone topography and the spatial coupling of Ca^{2+} influx to exocytosis during synaptic maturation, as now is even more evident due to inclusion of the model.
In terms of maturation of presynaptic function, we, for the first time used UV-laser photolysis of caged Ca^{2+} in immature IHCs to demonstrate that the intrinsic Ca^{2+} dependence of the fast component of exocytosis does not change over the developmental time window we observed. We found that about half of the immature IHCs lacked the fast component. We will follow this interesting finding with extending Ca^{2+} uncaging to several other stages of postnatal development in future experiments. When studying the apparent Ca^{2+} dependence of Ca^{2+} influx-driven exocytosis of the readily releasable pool (RRP) of vesicles (using 20 ms depolarizations, Moser and Beutner 2000, Beutner et al., 2001), we find a decrease of the apparent Ca^{2+} cooperativity (exponent *m*) when changing the number of open channels by dihydropyridine (isradipine) channel block from 2.6 to 1.4 between IHCs before and after the onset of hearing. At the same time *m* remained nearly constant over development (3.1 vs. 2.9) when changing the single-channel Ca^{2+} current, and was closer to the intrinsic Ca^{2+} cooperativity (4-5, Beutner et al., 2001). Focusing on RRP exocytosis with brief depolarizations allowed us to better isolate the Ca^{2+} dependence of fusion from the Ca^{2+} dependence of vesicle replenishment that is expected to affect the analysis when using longer depolarizations such as in Johnson et al., 2005, 2010 or Dulon et al., 2009. We then employed biophysical modeling (using realistic parameters) to test the impact of different topographies of Ca^{2+} channels and vesicular release sites at the active zones as well as different positions of the Ca^{2+} sensor. The biophysical model allowed us to better understand Ca^{2+} influx-exocytosis coupling in IHCs and lent support for our hypothesis that vesicles couple more tightly to Ca^{2+} channels at mature IHC active zones ("Ca^{2+} nanodomain control of exocytosis").

The nature of the marked increase in "Ca^{2+} efficiency" of exocytosis in IHCs over postnatal development of hearing and the precise coupling of Ca^{2+} influx to exocytosis at IHC active zones after the onset of hearing had remained two important question in the field. The loss of extrasynaptic Ca^{2+} channels, the morphological refinement of the AZs, the decrease of the Ca^{2+} cooperativity during dihydropyridine channel block together with the insight from biophysical modeling lead us the propose that changes in the **number of Ca^{2+} channels and the spatial coupling of Ca^{2+} channels and release sites rather than changes in the Ca^{2+} sensor** (as suggested by Johnson et al., 2010) underlie the greater efficiency of Ca^{2+} influx to drive exocytosis at mature synapses. In our view these findings provide a major advance of the understanding of hair cell development and function. Moreover, we are confident that the MS will have good impact also more generally, because the near-linear Ca^{2+} dependence of exocytosis found at the hair cell synapse for changes in

the number of channels and the good experimental accessibility of the hair cell synapse makes it an attractive model for studying the Ca^{2+} nanodomain control of exocytosis.

4- Much of the writing is presented as if the authors have a knowledge base that is unavailable to the general audience, speculating about potential mechanisms as if they were either demonstrated in the manuscript or known to be true. Additionally referencing was biased toward identifying only those manuscripts supportive of the speculation and often only small parts of these manuscripts. Although this approach tells a good story, it detracts from the credibility of the presentation and makes evaluation more difficult.

We understand that the reviewer found the introduction of the analysis of Ca^{2+} influx-exocytosis coupling, for which we build upon previous communications of the conceptual and experimental framework (e.g. Augustine et al., 1991; Roberts 1993; Brandt et al., 2005; Goutman and Glowatzki, 2007; Wang et al., 2008; Moser et al., J Physiol 2006; Matveev et al., 2011; Eggermann et al., 2012; Pangrsic et al., 2012), to be insufficient. We have performed a major revision of the entire MS addressing the reviewers request for broader and open introduction and discussion of concepts on functional and morphological vesicle pools, mode of vesicle release and Ca^{2+} influx-exocytosis coupling including improved referencing and have cut on speculation. We strongly believe that the biophysical model enhances the credibility of the Ca^{2+} nanodomain hypothesis of exocytosis in mature IHCs.

5- The subtypes of identified ribbon synapses is interesting. Have the authors correlated these subtypes to location within the hair cell in order to infer something about function?
Also are these data controlled for when (ie time of day) the animals were fixed. Changes in ribbon synapse morphology are known to occur diurnally, in photoreceptors but also in organotypic cochlea cultures, so that time of day for data collection may be relevant.

We assume that the reviewer alludes to ribbon morphology analyzed in Fig. 2 of the submitted MS and the work of the Liberman lab on the cat (Merchan-Perez and Liberman, 1996), recently followed up by the Sewell lab (Kantardzhieva et al., 2013), which described large and complex active zones facing low spontaneous rate fibers at the modiolar face of the IHC and smaller, simple active zones opposite the postsynaptic boutons of high spontaneous rate fibers at the pillar face of the IHC. We agree with the reviewer that this is a very attractive research question. We have started such work, are developing reliable and generalizable estimation of synapse position and are establishing further techniques to address this question appropriately at the light and electron microscopical level. However, this work is clearly beyond the scope of our present study and will fill another whole MS. In the present data set, we did not distinguish the location of a given synapse within the IHC and have included a statement on this.

<u>time of day for data collection:</u>
We have dissected and processed the apical cochlear coils of the different age groups around noon. We have now stated the time of collecting the tissue in the methods section.

6- Were different ribbon morphologies identified in the same cells?
Yes, now stated in the MS page 8
"Different ribbon shapes were observed in the same IHCs but we did not analyze their subcellular distribution."

Were they at particular cellular locations?
please see our response to 5

7- How do the authors separate between synapses that are forming, or degrading or are mature. In the snapshot that immunohistological data provides it seems quite difficult to know whether:
a. A floating ribbon has just detached and is on the pathway to degredation
b. A floating ribbon is about to attach and create a new presynaptic entity
c. A ribbonless synapse has any functional significance
d. A ribbonless synapse has just lost its ribbon and is in the process of degrading
e. A ribbonless synapse has just formed and is awaiting its ribbon
f. The morphologies presented for different ribbons are a function of fixation times
g. The ribbon morphologies are an indicator of forming and or degrading synapses

These are great questions and several of them are very hard to test with currently available methods. In order to address the reviewers concern, we have further stressed the limitations of our current experimental approach for distinguishing these hypotheses. Moreover, we have much reduced the discussion on the potential mechanisms of refinement, cut on speculation and identified remaining speculation as such and suggest possible future experimental strategies.
To us questions (a), (d) and (g) are representative for AZ degradation or pruning, while (b) and (e) belong to synaptogenesis. More generally, we agree that, based on a rough temporal sampling of morphology it is impossible to unambiguously decide on the fate of any observed structure. For example, we cannot quite distinguish pruning and fusion as the underlying mechanism for the AZ refinement. In fact, they may coexist and therefore we discuss those two as hypotheses. To address the main question of the reviewer how to separate between synapses that are forming or degrading live STED microscopy of tagged active zone components would be required beyond what is already provided in the paper. This is not in reach for several years it seems because:

i) we cannot observe hair cells within the living cochlea by STED microscopy

ii) viral transduction of hair cells with cDNAs of this large size is barely possible and no transgenic mouse line is currently available

iii) trying to study synaptic maturation in culture will arguably reflect the physiological process.

Therefore addressing the main question raised by the reviewer is beyond the scope of this study. However, we argue that is reasonable to characterize p6 synapses taken from pre-hearing mice as immature and p20/21 synapses from taken from hearing mice as mature. In our hands, neither the Ca^{2+} influx and exocytosis of IHCs *in vitro* nor the sound encoding of spiral ganglion neurons *in vivo* differ between 3 and 4-10 week old C57bl/6 mice. Therefore, we consider the ultrastructure and molecular organization of p20/21 synapses to be predominantly mature and suggest that it represents the result of a developmental refinement, of which we took snapshots at 3 developmental stages: p6, p9, p14-15.

We feel comfortable addressing the question suggested by the reviewer (7c) about if a ribbonless synapse has any functional significance. Previous work on bassoon mouse mutants showed their active zones mostly lacked ribbons and had reduced release rates primarily due to fewer Ca^{2+} channels and functional vesicular release sites (e.g. Buran et al., 2010; Frank et al., 2010). However, they otherwise seem functionally normal in many respects.
Option 7f has not been systematically addressed. However because ribbonless active zones were found in immature IHCs with mild and short fixation for immunohistochemistry, no fixation for high pressure freezing followed by electron microscopy and strong and long aldehyde fixation followed by electron microscopy we suggest that fixation is not critical for this observation. On the other hand, the time of day at which the preparations were fixed is relatively restricted in our data (see response #5), and should not contribute significantly to the variability in shapes. However, we did not explore the possibility of circadian changes of ribbon morphology, which we consider another project in its own right.

8- In the abstract, what does more connected mean?

We have removed this notion from the abstract and now discuss the anchoring of the ribbon in the results section (page 9; Figs. 2 and 3 as well as Fig. S3).
“Immature ribbons were anchored to the AZ membrane via up to two rootlets (presynaptic densities, Figs. 2A and 3A-B). In contrast, a single continuous density containing bassoon, as shown by the anti-bassoon immuno-gold EM (Fig. 3F), attached the entire base of the mature ribbon to the AZ (Fig. 3C-D). This notion of anchor consolidation was supported by the observation of fewer electron-dense connections between membrane and ribbon in random sections of ribbon-occupied AZs from p6-p20 (Fig. S3).”

How was connectivity of the ribbon to the synapse measured? I could not find any real measurement of this in the manuscript.:

To address this request, we estimated the number of electron-dense connections from the membrane of the active zone to the synaptic ribbon observed in random synaptic sections along development (see Fig. S3). The transition is from 2-3 rootlets that anchor the immature, typically spherical ribbon to a single extended anchor at the mature synapse.

9- Similarly in the abstract it states that stripe-like clusters were formed from smaller round clusters as if there were a causal link between these events. No link is demonstrated in the text. This needs to be clarified.

We thank the reviewer for calling attention to this mistake. Currently, technical limitations prevent us from testing causality (please see our response to comment #7).
We revised the abstract to now state: "After the onset of hearing (1) IHCs had fewer and larger ribbons; (2) $Ca_V1.3$-channels formed stripe-like clusters rather than the smaller and round clusters at immature AZs;…"

10- Similarly what specifically does 'tighten' mean in the abstract? It is stated that spatial coupling of influx to exocytosis is tightened. I assume that the authors are describing an increase in colocalization of calcium channels with Ctbp2 labeling, it is unclear how release is included in this argument or again what tightening specifically refers to.

In our work we provide evidence for two mechanisms that contribute to an increased efficiency of Ca^{2+} influx in triggering exocytosis: 1.) loss of extrasynaptic channels that likely contribute less to driving exocytosis at the vesicular release site and 2.) a spatial reorganization of the topography of Ca^{2+} channels and membrane proximal vesicles resulting in tighter spatial coupling of channels and release sites. Mechanism 1 is evident from data shown in Fig. 5 (maturational confinement of $Ca_V1.3$ immunofluoresence and Ca^{2+} signaling to ribbons after the onset of hearing), data of Fig. 7 (parallel shift of the DC_m/Q_{Ca} relationship to lower Q_{Ca} values with maturation) and the loss of channels is evident also from the fluctuation analysis (former Fig. 6, now Fig. S6D-E).
Mechanism 2 was primarily indicated by the developmental reduction of the apparent Ca^{2+} cooperativity *m*, estimated as the slope in the double-logarithmic plot of DC_m vs Q_{Ca}, when reducing the number of active Ca^{2+} channels by isradipine block. Focusing on RRP exocytosis we show that *m* is >2 in immature IHCs, no matter whether the number of active channels or their single channel current was manipulated. In mature IHCs *m* was 2.9 for changes of the single channel current, but close to unity (1.4) when changing the number of active channels. Using biophysical modeling we show that this "linearization" of *m* indicates an increased dominance of the nearest Ca^{2+} channel. Such "Ca^{2+} nanodomain control" may indicate a molecular coupling between one or few Ca^{2+} channels and a given vesicular release site, as discussed for other synapses (Eggermann *et al*, 2012). We are confident that the biophysical model (Fig. 8 and Supplementary Material 9) substantiates our argument for mechanism 2 and we have revised the MS to still better convey and discuss these concepts.

We have now clarified this throughout the manuscript:
Abstract: "After the onset of hearing (1) IHCs had fewer and larger ribbons; (2) $Ca_V1.3$-channels formed stripe-like clusters rather than the smaller and round clusters at immature AZs; (3) extrasynaptic $Ca_V1.3$-channels were selectively reduced, (4) the intrinsic Ca^{2+} dependence of fast exocytosis probed by Ca^{2+} uncaging remained unchanged but (5) the apparent Ca^{2+}-dependence of exocytosis linearized, when assessed by progressive dihydropyridine block of Ca^{2+}-influx. Biophysical modeling of exocytosis at AZs with mature and immature topographies suggests that Ca^{2+} influx through an individual channel dominates the $[Ca^{2+}]$ driving exocytosis at each mature release site. We conclude that IHC synapses undergo major developmental refinements, resulting in tighter spatial coupling between Ca^{2+}-influx and exocytosis."
Introduction:
"We find the intrinsic Ca^{2+} dependence of fast exocytosis to be similar in immature and mature IHCs, arguing against hypothesis 3. On the other hand, we find a diminution of extrasynaptic Ca^{2+} channels (supporting hypothesis 1) and indicate a developmental switch from Ca^{2+}-microdomain to Ca^{2+}-nanodomain control of vesicle fusion around hearing onset (supporting hypothesis 2)" (page 6-7)
Results: from page 14-19
Discussion:

"Biophysical experiments and modeling indicated tighter spatial coupling between Ca^{2+} influx and RRP exocytosis after the onset of hearing. In agreement with our own and other published work, *m* was near unity for manipulation of (N_{Ca} x P_{open}) in mature IHCs (Fig. 7D; Brandt *et al*, 2005). This means that release scaled linearly with the number of open channels, suggesting either Ca^{2+}-nanodomain coupling or a linear intrinsic Ca^{2+}-dependence of exocytosis."
and detailed discussion: page 23-25

11- In the introduction the authors refer to fusion competent vesicles tethered to ribbons and cite Frank et al. Where was fusion competency defined? It remains unclear, at least to me, how tethered vesicles relate to nontethered vesicles relate to discrete pools of vesicles as estimated from capacitance measurements.

We realize that the statement was misunderstood. We referred to membrane proximal vesicles at the base of the ribbon (which can also be tethered to the ribbon), which are often interpreted as being fusion competent (e.g. in retinal bipolar cells: von Gersdorff et al., 1996, Zenisek et al., 2000; frog saccular hair cells: Lenzi et al., 1999; 2002; Rutherford and Roberts, 2006; mouse inner hair cells: Khimich et al., 2005, Frank et al., 2010). In Frank et al., 2010 we found that many of these vesicles are tethered to the active zone membrane similar to what was described for membrane proximal and tethered vesicles at conventional synapses for which a correlation of tether-number and length with fusion competence has been suggested (Fernandez-Busnadiego, 2011 and 2013). In agreement with classical synaptic literature (Liley and North, 1953 and Elmqvist and Quastel, 1965) and common reasoning (Neher, Neuron 1998) we have interpreted the fastest and size-limited component of Ca^{2+} triggered exocytosis as fusion of a finite readily releasable pool of vesicles (Moser and Beutner, 2000), which seems related in vesicle number to the population of membrane proximal vesicles (Zenisek et al., 2000;Khimich et al., 2005; Rutherford and Roberts, 2006; Pangrsic et al., 2010; Frank et al., 2010). Other authors go a step further in the interpretation of the exocytosis data and also argue for a second limited vesicle population, that they attribute to the other ribbon-associated vesicles.

In order address #11 we revised the introduction to now state: "One to two dozen synaptic vesicles are tethered at the plasma membrane within few tens of nanometer distance of the Ca^{2+}-channel cluster and are thought to represent the putative readily releasable pool (RRP) of vesicles for exocytosis (Zenisek *et al*, 2000; Moser & Beutner, 2000; Lenzi *et al*, 2002; Rutherford & Roberts, 2006; Frank *et al*, 2010). "

12- The description of calcium buffering also appears a bit oversimplified as published measurements range from micromolar to millimolar depending on reporting technique that is used yet the authors select high mobile buffer references as the accepted answer.

We presumed the reviewer was referring to the following statement in Introduction, p4:
"This Ca^{2+}-nanodomain hypothesis is further supported by…, high concentration of mobile "buffering" Ca^{2+}-binding sites in hair cells (Roberts, 1993; Edmonds *et al*, 2000; Hackney *et al*, 2005; Johnson *et al*, 2008)…".

Although the amount of individual mobile Ca^{2+} protein buffers (e.g. calbindin, calretinin, parvalbumin) were often reported to tens of micromolar concentration in hair cells from different preparation (e.g. Hackney et al, 2003, 2005), considering the number of binding sites per protein and the presence of multiple Ca^{2+} buffers lead to a **high micromolar to millimolar concentration of Ca^{2+} binding sites**. The references we cited encompass Ca^{2+}-buffer concentration ranges from ~0.5 mM (total binding site at P16, calculated from 60 μM calbindin-D28k, 20 μM calretinin and 90 μM parvalbumin-α, Hackney *et al*., 2003), to around 1 mM (Roberts, 1993; Johnson *et al*, 2008), and to a few millimolar (~ 6 mM total binding site, from 1.2 mM calretinin, Edmonds *et al*, 2000). These measurements also are representative of different reporting techniques including quantitative immuno-electron microscopy (Hackney, 2005) and buffer replacement experiments on activation of a Ca^{2+}-gated K^+-channel (Roberts, 1993; Edmonds et al, 2000), and exocytosis (Johnson et al, 2008). Unpublished data from our lab studying the effects of exogenously added buffers on exocytosis in IHCs of a knockout line lacking calbindin, parvalbumin-alpha and calretinin also suggest the presence of around 1 mM equivalent of mobile Ca^{2+} binding sites in mature IHCs. We believe these are representative examples of investigations on buffer concentration in hair cells.

Most other published measurements fall within this range, except one study by Ricci et al. (1998), which proposed a 0.1 mM to 0.4 mM BAPTA equivalent for hair bundle adaptation in turtle hair cells. However, conflicting results were obtained from the same preparation (~1 mM BAPTA equivalent, Tucker & Fettiplace, 1996; ~1-3 mM total binding site, from 129-627 μM calbindin-D28k, 223-256 μM parvalbumin-3, Hackney et al., 2003) using different methodologies.

Within the word-limit of the revised manuscript, in the introduction we have given priority to

1) General introduction of the hair cell synapse
2) general introduction of Ca^{2+} influx-exocytosis coupling: nanodomain vs. microdomain
3) other concepts on the function of the hair cell synapse: multivesicular release relate it to Ca^{2+} influx-exocytosis coupling
4) development of Ca^{2+} influx-exocytosis coupling

We now introduce and discuss the determinants of Ca^{2+} influx-exocytosis coupling within the framework of the biophysical model, and list the parameters used in the model in the corresponding supplementary material (Supplementary Material 9).
Moreover, we also devoted more space to the discussion of Ca^{2+} influx-exocytosis coupling in the discussion section (and extended in Supplementary Material 9), whereby the modeling served to test hypotheses and the impact of parameters.

13- The description of figure 1 is quite confusing as it does not seem to match the data presented in the figure. Qualitatively there appears to be a consolidation of gutamate receptors to localize better with the ribeye labeling in adult as compared to immature synapses. However this needs to be quantified and there are many relatively simply algorithms for quantifying colocalization that could be used. In addition, the authors argue that the NaKATPase labeling supports a 1:1 correspondence between IHC synaptic structures and single neurites. This is not at all clear in Figure 1. The green labeling is quite diffuse, individual neurites cannot be distinguished and the colocalization with synaptic elements is at best poor. Some nonbiased means of quantifying this data is required. The circled region seems arbitrarily selected to make a point but many other circles could have been drawn.

In response to the reviewer's comment we have performed a major revision on Fig. 1 and 2 and the corresponding results. We now present projections of fewer confocal sections for more clarity. We made the description of figures and the figures (by adding graphical aids) more intelligible. In addition, we consider the possibility that the resolution of figure and/or the brightness of the blue channel may have limited the appreciation by the reviewer. We have toned down the interpretation of the Na/K-ATPase labeling, but would like to keep it, because in our view it membrane staining of postsynaptic boutons provides good orientation. We included a quantitative analysis on number and intensity of RIBEYE and GluA2/3 puncta (Fig S2), where an increase in ribbon:GluA2/3 puncta number ratio from ~0.6 at p6 to nearly 1 for p20 IHCs (Fig. S2D) was observed. This is consistent with our observations of ribbonless AZs in immature IHCs and with the idea of a 1:1 correspondence between ribbons and receptor clusters per SGN bouton upon maturation.
Quantification of immunfluorescence is presented in Fig. 2 and Fig. S2.

14- It would be helpful for the authors to label synapses considered to be ribbon free.

Done (arrowheads in Fig. 1B)

15- Authors argue that the maximal intensity of the ribeye labeling confirms that the ribbons are doubling in size. How do the authors know that ribeye density is not changing? Given the EM images showing different shapes of ribbons, one might postulate an increase in density so that if the labeling were linearly related to number of molecules (a big if) then the change in intensity would still not be related to size but to density. The EM data appears more reliable for this level of analysis and it might be best to simply stick with that. Of course here, the argument would be an increase occurring only a week after hearing onset.

We followed the reviewer's advice and present the EM quantification of ribbon and PSD size as well as the number of ribbon-associated vesicles as bar plots in Figure 2. Only after presenting EM data we provide the immunofluorescence analysis as corroborating data set, clearly notifying potential caveats of the analysis. We would prefer to keep the analysis of the immunofluorescence data as this second line of evidence. We quantified the amount of CtBP2 immunoreactivity by

integrating the fluorescence intensity over the area of each spot. While we cannot confirm that the increase in integrated intensity was solely due to an increase in the size of the ribbon (and state this), the FWHM of both the long and short axes of the fluorescence puncta significantly increased from P6 to P14 (long axis: 0.37 vs 0.39 μm, $p < 0.001$; short axis: 0.28 vs 0.32 μm, $p < 0.001$) further supporting the notion of larger ribbon size upon maturation.

Page 8-9:
"Comparisons of confocal RIBEYE immunofluorescence between p6 and p20 revealed an approximate halving of ribbon number per IHC and an approximate doubling of the summed pixel intensities per ribbon (Fig. 2F, G and Fig. S2). An increase in full-width-at-half-maximum (FWHM, Fig. 2F) of these RIBEYE spots is consistent with the increase in ribbon-size observed in TEM. For GluA2/3, both the reduction in puncta number and the increase in intensity per punctum were larger than for ribbons (Fig. S2C,E). We note that confocal measurements provide only a semi-quantitative comparison because immunolabeling may not be linear or equally efficient amongst puncta and because some ribbons or GluA2/3 puncta can be smaller than the resolution limit of confocal microscopy, especially in immature IHCs."

16- It should be made clear whether SEM or TEM was used for the measurments. I assume TEM but it is not always written that way.

Transmission electron microscopy was used in this study, this has now been explicitly stated in the method and results sections.

17- Figure 3E, gold particles are not visible in image. 3F is also likely to not reproduce well as it is very small.

We have now replaced the panel with two examples with better contrast and included arrowheads emphasizing on the silver enhanced gold particles labeling the anchorage of the ribbon. It is worth to note that the silver enhancement creates dark electron-dense label that is less well-defined in shape than simple gold particles. We also increased the size of the panel so that the labeling can be better appreciated.

18- For both figure 3F and figure 4 the proprotion of the figure should be adjusted so that the zoomed in view is more prevalent. The larger lower power provides no useful information, even less when reproduced in a journal format. The higher resolution of the STED system is somewhat lost by how these data are presented. Zooming in on the individual synapses would be much more powerful presentation to demonstrate the STED resolution.

done

19- Quantifying the changes observed in figure 5 would greatly strengthen the authors' arguments. The qualitative assessment of colocalization is not convincing. This is particularly true for the calcium imaging data where no quantification, not even number of recorded cells, is provided. As shown the images are quite dark so arrows are visible but not much else. As presented the calcium imaging data is of little value. Quantification is required for interpretation.

We followed the request as detailed in our response to comment #1: results and Fig. S5

20- The data in figure 6 suggest that the number of calcium channels between P14 and P20 are quite similar, however the immunofluorescence in Figure 5 does not really seem to support this argument. The spots are bigger and brighter at P14 then at P20? This is confusing and not addressed at all in the text.

In response to the request we provide the integrated intensity of the ribbon-associated $Ca_V1.3$ immunofluorescence spots (as done for the analysis for CtBP2/Ribeye immunofluorescence so far shown in Table I). We did not find significant difference in the integrated intensity (p14: 7721 ± 265 a.u. vs p20: 7424 ± 196 a.u., $p = 0.87$) nor the FWHM of the short axis (p14: 0.246 ± 0.002 μm. vs p20: 0.249 ± 0.002 μm, $p = 0.97$). The FWHM of the long axis increased slightly (p14: 0.336 ± 0.004 μm. vs p20: 0.350 ± 0.003 μm, $p = 0.005$). This result has now been integrated into the results section. (page 10-11)

21- How do the authors reconcile the difference in their data with preious reports from Marcotti group suggesting a change in maximal probability of opening with maturation? There I believe the authors used single channel measurements? Is the difference the associated with a noise analysis approach? This seems particularly critical especially when being used as an argument for nanodomains?

This point is now noted in the results section of the revised version of the MS (page 12, line 15-22) and discussed in detail in the supplement along with Fig. S6. The reviewer is very correct in the notion that open probability affects the extent of domain overlap among neighboring channels. However, the higher open probabilities assumed here (based on fluctuation analysis) actually impede the dominance of the nearest channel for controlling Ca^{2+} at a given vesicle, and, hence Ca^{2+} nanodomain control, that we favor for the mature AZ, would even be more likely if assuming the lower open probabilities reported from single channel recordings.

Results section:

"The single channel current (i_{Ca}) estimate did not change significantly ($p = 0.1$, ANOVA, Fig. S6F) and the estimated maximal open probability ($P_{open, max}$) decreased slightly ($p < 0.001$, ANOVA; Fig. S6F), whereas single channel recordings indicated a reduction in i_{Ca} and an increase in P_{open} over development (Zampini et al, 2010, 2013). While both approaches agree on the notion of much higher total Ca^{2+} channel numbers in immature cells, they differ in their estimates of i_{Ca} and P_{open}, likely for technical reasons, and also report opposite trends over development (see text accompanying Fig. S6 for discussion)." (page 12)

Text along with Fig. S6:

Comparing the estimation of microscopic channel properties by non-stationary fluctuation analysis and direct cell-attached single channel recordings

The main point of doing the fluctuation analysis was to seek confirmation of the hypothesis that the number of Ca^{2+} channels (N_{Ca}) declines around the onset of hearing. This was, indeed, observed and also agrees with previously published work based on single channel recordings (Zampini *et al*, 2010). However, we note that both approaches differed in their estimates of single channel current i_{Ca} and open probability (P_{open}), which we believe reflects technical differences that are discussed below.

Generally, fluctuation analysis tends to underestimate the i_{Ca} due to the limited bandwidth of recording (8.5 kHz), and this seems to be the case here, too. Our estimates of approximately -0.6 pA at -68 mV compare to approximately -1.0 pA measured in cell-attached at -68 mV after hearing onset (Zampini *et al*, 2013). For this reason we relied on the single channel recording estimates for i_{Ca} for our biophysical model.

We believe that likely methodological differences gave rise to the differences between the P_{open} estimates from single channel experiments (0.15 at -20 mV for immature IHCs (Zampini et al., 2010) and 0.21 for mature IHCs (Zampini et al., 2013) of the gerbil) and fluctuation analysis (around 0.8 at approximately +60 mV in estimates from mature IHCs (Meyer et al., 2009; Frank et al., 2010 and the present study for mature IHCs, and slightly higher (0.88) for immature IHCs). Using BayK8644 and strong depolarization we aim to maximize the open probability (greater than 0.5) as required for faithful estimation of the channel properties by binomial fitting. As stated in the main manuscript we aim to estimate the total number of Ca^{2+} channels and identify our open probability estimates as "maximal open probability" to make these points. While the single channel work by the Zampini et al., 2010 and 2013 also used BayK8644, they used lower depolarizations and longer pulses (mostly 500 ms). We consider it likely that the lower depolarization level (via less activation) and potentially also Ca^{2+} dependent inactivation lead to lower estimates of open probability. Since Ca^{2+} dependent inactivation is greater in immature than in mature IHCs (Grant and Fuchs, 2008) one would then expect lower open probability estimates in immature IHCs, which seems to be the case (see above). Further observations of Zampini et al., 2010 and 2013 fitting this hypothesis are that the bursts (mode 2 of gating) were preferentially observed at the beginning of the sweep and that inactivation is evident in the ensemble currents. For this reason in our biophysical model we relied on the P_{open} estimate of the fluctuation analysis. We used P_{open} of 0.4, which we derived from the measured 0.8 and divided it by the factor of 2 by which BayK8644 enhances the whole cell Ca^{2+} current at the peak Ca^{2+} current potential ((Brandt *et al*, 2005; Frank *et al*, 2010). We note that this likely reflects an upper boundary, which actually impedes the dominance of the nearest

channel for controlling Ca^{2+} at a given vesicle, and, hence Ca^{2+} nanodomain control that we favor for the mature AZ, would even be more likely if assuming the lower open probabilities reported from single channel recordings.

22- Figure 7 should be modified so that kinetics compartments can be seen in raw data.

Done. We have completely re-organized Figure 7 (now Fig. 6) and panel A now shows the fast C_m rise of representative examples with the components of the fits in better resolution.

Figure 5:

23- How do the authors deal with a difference in available vesicles between ages when comparing release rates? If fewer vesicles are avaliable or primed at one age then release rates may appear different but have no causal link to release mechanism. That is there could be a lot of misses where vesicles are either not present or not primed and so cannot be released. Similarly if release rates appear to be the same despite a difference in available vesicles, one might argue that there were intrinsic variations normalizing for the difference in vesicle number. Clearly the biggest difference in data presented is in number of vesicles available for release. And given the large number of vesicles needed to account for the large changes in capacitance, vesicles would need to be transported to the synapse. Perhaps it is differences in vesicle transport that the authors should be addressing here as it is unclear the data can reconcile changes in release rates?

Also for this reason we separately compared rate constants (as an amplitude-independent measure of kinetics) and amplitudes of the C_m rise. Fig. 7D (now Fig. 6C) clearly states "rate constants" and so do text and figure legends.
And yes, there is a difference in the amplitude, which we discussed (page 13, line 18-21). We also offer justification of relating the fast component of the Ca^{2+} uncaging-evoked C_m rise to kinetics vesicle fusion during Ca^{2+} influx and discuss potential caveats (page 22, line 16-21).

24- Figure 8B could be made a little more clear if the 5mM Ca were shown at the start and if each segment matched the perfusion time, ie no gaps.

Done, Figure 8B was modified as suggested, now Fig. 7B.

25- The discussion is highly speculative and little direct data is presented to support the authors arguments. Although some speculation is good, it typically is best to ensure the readership can tell the difference between what is known and what the authors think. There is no separation here. Alternate possibilities and a more eruditic approach to citations would help. As it stands the discussion does not due justice to the data presented or to the known literature.

We have performed a major revision of the discussion for less speculation on the maturation of molecular nanoanatomy and for more treatment of Ca^{2+} influx-exocytosis coupling at hair cell active. In particular, we now also discuss insights from the modeling, which, we think, clarifies several issues of Ca^{2+} influx-exocytosis coupling that remain hard to decipher solely by experiments. We also added discussion of previous studies by various groups. Throughout, we have provided experimental evidence wherever possible and have identified speculations as such.

26- Authors discuss rate constants and a changing of proportions in rate constant for figure 5 but provide no statistical analysis or summary box plots to illustrate their point.

We are uncertain whether perhaps results of Fig. 7 (now Fig 6) were meant and whether the reviewer primarily referred to the comparison of rate constants and the contributions of the kinetic components of the C_m rise. Assuming that this is the case, we note that given the steep Ca^{2+} dependence of the rate constant statistical comparisons are somewhat difficult. In the revised manuscript we now added a comparison of the rate constants of the fast component and the exocytic delays for p6-8 and p14-17 IHCs for a narrow $[Ca^{2+}]$ range of 15-25 μM for which we found the best comparable representation of $[Ca^{2+}]$ changes. To address the reviewer's request we also quantified the contributions of the kinetic components to the C_m rise, which added as Figure S7.

27- The wilcoxin rank test requires data to be related, it is unclear how the data presented are related?

We used the Wilcoxon Rank Sum test implemented in IgorPro software, which is also known as Mann-Whitney U-test and appropriate for unpaired samples. Potentially the reviewer referred to the Wilcoxon Signed Rank test, which requires paired sample. We have stated the equivalence of the Wilcoxon Rank Sum and Mann-Whitney U-test in the methods section.

Referee #2

This manuscript very admirably describes the developmental progression of the mouse cochlear ribbon synapse, showing with outstanding light and electron microscopy that the synapse refines from multiple small ribbons to a single well-organized ribbon. These anatomical change in the ribbon is correlated with a refinement of the CaV1.3 channels, as they are reduced in numbers and form into striped clusters near bassoon. Also correlated in time are alterations in synaptic physiology, including a reduced Ca current (correlating with the reduction of CaV1.3 channel number), an increase in evoked Cm, and linearization of the apparent Ca dependence.
In general, the data are outstanding and support the authors' conclusions very nicely. However, there are several presentation issues that need to be addressed, mostly with the light microscopy:

We would like to thank the reviewer for the overall appreciation of our work and for the criticism that helped us to improve the MS.

1. Fig. 1. While the immunocytochemistry with confocal and STED is outstanding, its presentation could improve greatly. The images are plagued by being too small and by using colors that don't allow clear visualization of the key molecules very well. The RGB combination, while the standard one used, has two problems: first, the blue is impossible to see, as its brightness appears to be very low, and second, red-green color blind individuals have a hard time with the images. Since the localization is a key part of this manuscript, these color and size issues should be addressed.

We have revised Figure 1 for better visibility and clarity of take home message.

For example, the lower mag panels of Fig. 1A and 1B could be done in green/magenta and thus would be much easier to comprehend.

done

In addition, the magnified panels of 1A should be in grayscale for the individual panels (the blue is impossible to see when printed or on the monitor).

done

Even though the blue is hard to see, Fig. 1C works because of the {plus minus} bassoon panels. Figs. 1D-E are OK but the blue is particularly hard to see in 1E; another triple-label color scheme might be better.

Increased the brightness of blue in all panels. Individual fluorescence channels in Fig 1C (now Fig 1B) are now also displayed as separate grayscale images.

2. Fig. 3. Panel F should be in a green/magenta or similar color scheme that more readily pops out. In addition, the low mag panel of 1F will present no information in the journal, where it may well be smaller. The magnified panels in 1F should be at least 2x larger than they are now relative to the rest of the figure (maybe get rid of the low power view and magnify all the close-ups).

done

3. Fig. 4. This figure should be all grayscale (no value to color in the low-mag panels). Again, the

value in the figure is in the close-up panels, which should be much larger.

done

4. Fig. 5A. Panels are OK but would probably be more easily understood if it was in green/magenta. In addition, the scale should be the same for all panels and it would be better if the view was closer in.

done

5. Fig. 5B. There is no reason why the panels in B should be in color; grayscale would be much better (the gradations in intensity are far easier to see, particularly instead of red-the RIBEYE staining is impossible to see).

done

6. Fig. 6B. State in the legend that NCa comes from the nonstationary noise analysis (e.g., not from ICC).

done, now figure S6.

2nd Editorial Decision 18 November 2013

Thank you for submitting your manuscript to The EMBO journal. This is an invited resubmission of MS 85547. I asked the original two referees to review the paper and I have now received their comments.

I hope that you will be pleased to see that both referees are very positive about your work. Referee #1 finds that the manuscript should be presented in a more focused manner and offers a number of suggestions for how to do so. No new experiments are needed it is just a matter of getting the paper in perfect shape. Referee # 1 suggests removing the modeling data. I would rather prefer leaving it in as I like how it fits in the paper. We have no space restriction and so you can expand your discussion.

So please consider the comments of referee #1 and modify as you see best fit. Once we received the revised version, I will accept the paper for publication here.

Thank you for the opportunity to consider your work for publication. I look forward to seeing the final version.

REFEREE REPORTS

Referee #1:

Investigators provide a large body of data to support the hypothesis that the coupling between calcium influx and exocytosis is enhanced during hair cell synapse maturation. Data including immunohistochemistry using confocal or super-resolution imaging, transmission electron microscopy,calcium imaging, calcium uncaging, electrophysiological monitoring of cell capacitance and mathematical modeling are incorporated into this body of work to create a story addressing synaptic maturation. In general the data is of a high quality.

The presentation is reasonably clear, though often very focused on a particular interpretation. 8 complex figures and 14 additional supplementary figures is a lot to present and I think a lot to evaluate or take in at the necessary level of detail. Questions arise with each of the figures at some level but to list all of these or really to expect the authors to address every question arising form these 22 multipaneled figures would likely require more figures and simply propagate the problem.

For example the TEM of bassoon is quite nice but is not quantified and likely there are particles in other places as well, more reflecting the STED imaging than what is shown in TEM. The conclusions drawn from the data as presented seem overstated. Another example is ribbon shapes, are these really developmental changes or perhaps like in other ribbon synapses a reflection of a circadian effect?

Should the vesicles associated with each ribbon type be compared separately? Are the locations of these different ribbons within the cell specialized? It is hard to figure what to make of this data on its own and it is difficult to see how it relates to the fundamental question of the paper which seems to be more to do with calcium channel refinement. In an overburdened paper, these data could be eliminated and used as the basis for a more complete assessment of ribbon development. This would also allow more detail to be placed on the main point of the paper.

Another example is that the vesicle populations being assessed with the uncaging experiments are likely different than those assessed with depolarization, simply based on the capacitance responses being pF vs fF. The uncaging data must be more biased toward vesicle recruitment and replenishment and the short depolarizations toward existing local pools of vesicles. These differences are not clearly addressed, likely due to space restrictions but are pretty fundamental toward understanding what is happening.

Also the argument for a lack of change in calcium dependence of release is a bit undermined by the significant variance between measurements and although the authors are likely correct the interpretations presented and which the modeling is based seem a bit strong. And finally the modeling data as presented is not well served, a single figure with a short description does not allow for careful evaluation and really undervalues what could be a very useful model. I would strongly suggest removing the model from this paper, expanding it and submitting separately. The model is really presented to argue for a nanodomain vs microdomain model for vesicle release (nomenclature is a bit jargony and likely should be changed to better reflect significance), the data presented throughout this manuscript do not directly address this issue, rather they strongly support a consolidation of calcium signaling to better more directly regulate mature synaptic transmission. As this is an important enough topic to address in one paper, it seems the model, similar to the ribbon size and shape could be removed for separate publications.

Streamlining this work will in my opinion make it much more readable to a general audience and will likely increase its significance as it will be more focused on the primary findings. It will also allow both the ribbon shapes and the modeling to be thoroughly flushed out, thus increasing their significance as well. Overall this is a very good piece of work but it needs to be better focused. By removing the model and the ribbon shapes data, more attention can be placed onto the more critical aspects of this work addressing synaptic maturation.

Referee #2:

The authors did a good job in improving the manuscript, both in the upgrades to the figures I suggested and the more substantive changes they made in response to the other reviewer.

1st Revision - authors' response 24 November 2013

Referee #1:

We would like to thank the reviewer for her/his appreciation of our work and the continued help on improving the MS. In response to the new comments we have further worked on improving the readability of the MS for a general audience and addressed the specific points raised.

1. For example the TEM of bassoon is quite nice but is not quantified and likely there are particles in other places as well, more reflecting the STED imaging than what is shown in TEM. The conclusions drawn from the data as presented seem overstated.

Done:

"In contrast, a single continuous density attached the entire base of the mature ribbon to the AZ (Fig. 3C-D). Using anti-bassoon immuno-EM (Fig. 3F) we indicate that bassoon localizes to the presynaptic density underneath the ribbon, where the majority of immuno-gold was found (on average 1.4 ± 0.3 particles at the base of the ribbon and 0.3 ± 0.1 elsewhere at the ribbon, $p < 0.001$, $n = 21$ labeled synapses). This is consistent with a previous immuno-EM study on photoreceptor ribbon synapses (Dick *et al*, 2001) and also supported by present STED microscopy of hair cell synapses (see Fig. 3G and below). The notion of ribbon-anchor consolidation was supported by the observation of fewer electron-dense connections between membrane and ribbon in random sections of ribbon-occupied AZs from p6-p20 (Fig. S3)."

2. Another example is ribbon shapes, are these really developmental changes or perhaps like in other ribbon synapses a reflection of a circadian effect?

As mentioned in the manuscript (previously in Methods, in response to the reviewer's comment now also in the results section) all TEM preparations were fixed and processed at around noon. While we acknowledge the circadian changes of ribbon morphology in hair cells as an avenue worthy of future exploration, we argue that our short preparation time window would favor detection of changes over development.

3. Should the vesicles associated with each ribbon type be compared separately? Are the locations of these different ribbons within the cell specialized? It is hard to figure what to make of this data on its own and it is difficult to see how it relates to the fundamental question of the paper which seems to be more to do with calcium channel refinement. In an overburdened paper, these data could be eliminated and used as the basis for a more complete assessment of ribbon development. This would also allow more detail to be placed on the main point of the paper.

Analyzing the ribbon-associated vesicle for each ribbon-shape and age group in separation would reduce the statistical power required for the comparison of developmental stages and would further increase the amount of detail beyond that the reviewer asked for in the previous round and that s/he now worries about.

We respectfully disagree with the reviewer in that we think the overall developmental changes in ribbon shapes and size constitute, together with bassoon and $Ca_V1.3$ clusters, an integral part of presynaptic ultrastructural changes during development. The size and shape of the ribbon is highly relevant for the Ca^{2+} signaling at the AZ as the Ca^{2+} channel complement of the AZ correlates with the size of the ribbon during development (Fig. 2E, F and $Ca_V1.3$-immunofluorescence, end of page 10) and also after maturation reflecting correlated AZ-size and Ca^{2+} channel complement heterogeneity (Frank *et al*, 2009). Moreover, previous findings in our lab (Frank *et al*, 2010; Jing *et al*, 2013) and other labs (Sheets *et al*, 2011, 2012) indicate that ribbon and $Ca_V1.3$ clusters can influence one another. In addition, the developmental changes in the number of ribbon-associated vesicles across development constitute part of our basis for interpreting the changes in the amplitude of Ca^{2+}-uncaging responses.

The positional difference in ribbon size and shape is an important question we currently address in another study, where, again, we combine functional and morphological approaches for a comprehensive analysis of the matter.

4. Another example is that the vesicle populations being assessed with the uncaging experiments are likely different than those assessed with depolarization, simply based on the capacitance responses being pF vs fF. The uncaging data must be more biased toward vesicle recruitment and replenishment and the short depolarizations toward existing local pools of vesicles. These differences are not clearly addressed, likely due to space restrictions but are pretty fundamental toward understanding what is happening.

Done: We have enhanced the discussion of the possible caveats (end of page 23 and beginning of page 24).

5. Also the argument for a lack of change in calcium dependence of release is a bit undermined by the significant variance between measurements and although the authors are likely correct the interpretations presented and which the modeling is based seem a bit strong. And finally the modeling data as presented is not well served, a single figure with a short description does not

allow for careful evaluation and really undervalues what could be a very useful model. I would strongly suggest removing the model from this paper, expanding it and submitting separately. The model is really presented to argue for a nanodomain vs microdomain model for vesicle release (nomenclature is a bit jargony and likely should be changed to better reflect significance), the data presented throughout this manuscript do not directly address this issue, rather they strongly support a consolidation of calcium signaling to better more directly regulate mature synaptic transmission. As this is an importantenough topic to address in one paper, it seems the model, similar to the ribbon size and shape could be removed for separate publications. Streamlining this work will in my opinion make it much more readable to a general audience and will likely increase its significance as it will be more focused on the primary findings. It will also allow both the ribbon shapes and the modeling to be thoroughly flushed out, thus increasing their significance as well. Overall this is a very good piece of work but it needs to be better focused. By removing the model and the ribbon shapes data, more attention can be placed onto the more critical aspects of this work addressing synaptic maturation.

The referee has correctly identified that the refinement of presynaptic Ca^{2+} signaling and its regulation of synaptic transmission is one of the major message in this manuscript. The framework of controlling synaptic vesicle exocytosis by a Ca^{2+} nanodomain or a Ca^{2+} microdomain is established in the field of synaptic transmission (Neher, 1998; Matveev *et al*, 2011; Eggermann *et al*, 2012; Tarr *et al*, 2013) and this study combines structural and functional data with modeling that reconciles the experimental data on the hair cell synapse showing how this control can change along with synapse maturation. We believe strongly that the model helps to better understand how changes in Ca^{2+} channel clustering and positioning can influence the control of vesicle fusion. In this revised version, we have improved the presentation of the model to make it more accessible and aimed to further harmonize the nomenclature among the different part of the manuscript (morphology, physiology, modeling) to allow the reader to associate the concepts running through as a whole, as we intended to convey.

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