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Bassoon-disruption slows vesicle replenishment and induces homeostatic plasticity at a CNS synapse

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(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 July 2013

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

As you can see, the referees appreciate the findings reported although referee #2 is more hesitant if the paper is a good fit for publication here. Given the overall comments provided, I would like to invite you to submit a suitably revised manuscript for our consideration. Referees #1 and 3 raise minor issues, whereas referee #2 brings up more significant ones that I would like to ask you to address in a revised version. I should add that it is EMBO Journal policy to allow only a single round of revision and that it is important to address the raised concerns at this stage.

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://www.nature.com/emboj/about/process.html

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

REFEREE REPORTS

Referee #1

The manuscript by Schulz et al. analyzes the structural and functional changes at the endbulb-bushy cell connection in the antero-ventral cochlear nucleus upon partial disruption of the presynaptic active zone protein bassoon. The authors show that BsnΔEx4/5 endbulbs have increased quantal size and release probability, a reduced RRP size and a reduced vesicle replenishment rate. Despite these defects, mutant synapses were almost as reliable as wild-type and auditory signaling in vivo was maintained most likely via homeostatic adaptations in PSD size and bushy cell excitability. The manuscript is a technically sound, combining highly advanced in vitro and in vivo electrophysiological recording with various (LM and EM) morphological measures. The conclusions are justified.

The BsnΔEx4/5 mouse model has been well described; showing major structural defects in ribbon synapses (Altrock et al, 2003; Dick et al, 2003; tom Dieck et al, 2005; Frank et al, 2010) and enhanced short-term depression in central glutamatergic synapses (Hallermann et al, 2010). A common observation is the upregulation of piccolo, which may compensate for some of bassoon's functions (Waites., et al 2013). Therefore, the main strength of the manuscript lies in elegantly revealing the homeostatic compensation resulting from partial activity deprivation in the auditory system perhaps more than advancing our understanding of bassoon's function. The unique in vivo recordings show that auditory signaling is hardly impaired in BsnΔEx4/5 mice despite the transmission defects showing the robustness of this connection in vivo and highlighting the importance of in vivo follow up of in vitro observations. These findings would certainly interest a broad audience.

Minor comments:

The authors propose several mechanisms to explain the increased quantal size. In addition to enlarged PSD and changed AMPA receptor-type composition, enhanced expression of presynaptic vGlut is a plausible mechanism that the authors should test by measuring vGlut intensities in Figure 2.

The term "vesicle reloading" in the ms title not optimal. Probably "vesicle pool replenishment" is better.

The running title should be reconsidered too; it does not convey the main message of the ms

Referee #2

The study by Schulz et al. investigates the consequence of a Bassoon (Bsn) hypomorph mutation on synaptic signaling at the endbulb synapse in the mouse auditory system. Consequences of the Bsn KO have previously been described in cultured neurons (Altrock et al. 2003), in other CNS synapses studied in slice preparations (Hallermann et al. 2010), as well as in detail at the ribbon-type synapses of inner hair cells, by this lab (Kimich et al 2005, Frank et al. 2010, Jing et al. 2013). In light of the previous studies, the design, and the main aim of the present study was not well explained. The authors find changes in the pool of vesicles and recovery speed which largely re-capitulate previous work. In addition, there were small, but significant increases in PSD length and in the quantal amplitude, and there was a putative increase in p-rel (but see point 3). These latter changes are interpreted as a "homeostatic" plasticity, but the molecular mechanisms of this putative homeostatic change are not explored, nor was the physiological background well explained (is this due to a decreased sound-driven activity in Bsn KO mice?). It was also difficult to follow the experimental design of re-adjusted sound intensities in Fig. S7.

The paper contains a lot of potentially interesting data, but its main thrust remains somewhat fuzzy.

Further specific points:

1) The description of the IHC results in Figs 1, 2 should be improved, and more to the point. It should be explained at the outset which hypothesis was tested, and the main results should be highlighted. Initially, images of the various individual channels should be shown separately side-by-side. The labeling "sap7f" should best be changed to "Bassoon". Also, what was the sap7f signal

outside synapses (see Figs 1D, E green smear on the sides -- axonal signals?).
Why were there only few Munc13 punctae (Fig. 2D) as compared to other active zone markers (e.g. Rim2-Fig. 2C).

2) It remained unclear whether Bsn KO mice had an outright docking phenotype, or not (the two different measure gave different results).

3) The authors claim to have found an increased p-rel but inspection of Table 1 shows that this parameter was significantly increased only for the 300 Hz train stimuli, but not with 100 or 200 Hz trains. The reasons for this should be discussed.

4) Throughout, the number of independent repetitions should be stated in the Results text. In all bar graphs which show average value plus/minus SEM; the data points from individual cells could be overlaid (e.g. in Fig. 4B - F).

5) The authors claim that Bsn KO cells "fire more APs for small current injections" (p. 12 top), but the corresponding Figure only shows a set of example traces for a WT cell (Fig. 8A). This example seems to show only a single AP at the onset even with the largest I-injection, whereas the average data for WT suggests about 3-4 APs (Fig. 8C, black symbols). Thus, a more representative example, in which all APs all clearly visible, and example traces for a Bsn KO cell should be shown. Also, it appears that FigS6A contains the same set of traces as Fig. 8A.

Referee #3

This is an excellent manuscript designed to assess the contribution of the presynaptic active zone protein Bassoon on synaptic function and plasticity within the auditory system. Specifically, the authors describe a set of morphological and electrophysiological experiments assessing functional changes at the endbulb of held forming synapses on bushy cells within the anteroventral cochlear nucleus. Their results show that in the absence of Bassoon, Held synapses are formed normally, in both size and number. While vesicle replenishment and the standing pool of readily releasable pool vesicles is reduced, they noted that postsynaptic densities are larger as these synapses exhibit increases in quantal size and vesicular release probability. Intriguingly, in vivo homoestatic mechanisms appear to compensate for these changes allowing for improved synchronous signaling of the dysfunctional auditory pathway in BsnΔEx4/5 mice.
This is a beautifully designed and executed study. The data are of very high quality and the manuscript is clearly and logically written. I have no concerns.

1st Revision - authors' response 07 October 2013

Referee #1

The manuscript by Schulz et al. analyzes the structural and functional changes at the endbulb-bushy cell connection in the antero-ventral cochlear nucleus upon partial disruption of the presynaptic active zone protein bassoon. The authors show that BsnΔEx4/5 endbulbs have increased quantal size and release probability, a reduced RRP size and a reduced vesicle replenishment rate. Despite these defects, mutant synapses were almost as reliable as wild-type and auditory signaling in vivo was maintained most likely via homeostatic adaptations in PSD size and bushy cell excitability. The manuscript is a technically sound, combining highly advanced in vitro and in vivo electrophysiological recording with various (LM and EM) morphological measures. The conclusions are justified.

The BsnΔEx4/5 mouse model has been well described; showing major structural defects in ribbon synapses (Altrock et al, 2003; Dick et al, 2003; tom Dieck et al, 2005; Frank et al, 2010) and enhanced short-term depression in central glutamatergic synapses (Hallermann et al, 2010). A common observation is the upregulation of piccolo, which may compensate for some of bassoon's functions (Waites., et al 2013). Therefore, the main strength of the manuscript lies in elegantly revealing the homeostatic compensation resulting from partial activity deprivation in the auditory system perhaps more than advancing our understanding of bassoon's function. The unique in vivo

recordings show that auditory signaling is hardly impaired in BsnΔEx4/5 mice despite the transmission defects showing the robustness of this connection in vivo and highlighting the importance of in vivo follow up of in vitro observations. These findings would certainly interest a broad audience.

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

Minor comments:

1.) The authors propose several mechanisms to explain the increased quantal size. In addition to enlarged PSD and changed AMPA receptor-type composition, enhanced expression of presynaptic vGlut is a plausible mechanism that the authors should test by measuring vGlut intensities in Figure 2.

Done:
1. **VGLUT1 immunofluorescence intensity** was measured wt and $Bsn^{\Delta Ex4/5}$ terminals around bushy cells and were **not significantly different** from each other. We present this data in Figure S1.

2. In addition to the analysis of VGLUT1 immunofluorescence intensity, we also checked for an increased **vesicle size** as a potential mechanism for the observed increased quantal size. We analyzed the synaptic vesicle diameter (inner SV diameter) and found it **unchanged**. This data is now presented as Figure S2C.

2.) The term "vesicle reloading" in the ms title not optimal. Probably "vesicle pool replenishment" is better.
Since we run into character restriction issues for the title, we propose the following title as a compromise:

"Bassoon-disruption slows **vesicle replenishment** and induces homeostatic plasticity at a CNS synapse"

Old title:
Bassoon-disruption slows vesicle reloading and induces homeostatic plasticity at a large CNS synapse

3.) The running title should be reconsidered too; it does not convey the main message of the ms
The proposed new running title is
Bassoon disruption induces homeostatic plasticity - 49 characters

Old title: *(max allowed characters with spaces: 50)*
Role of bassoon at large central auditory synapse

Referee #2

The study by Schulz et al. investigates the consequence of a Bassoon (Bsn) hypomorph mutation on synaptic signaling at the endbulb synapse in the mouse auditory system. Consequences of the Bsn KO have previously been described in cultured neurons (Altrock et al. 2003), in other CNS synapses studied in slice preparations (Hallermann et al. 2010), as well as in detail at the ribbon-type synapses of inner hair cells, by this lab (Kimich et al 2005, Frank et al. 2010, Jing et al. 2013).

We would like to thank the reviewer for the criticism that helped us to further improve our manuscript. We have addressed all comments of the reviewer and performed additional experiments towards elucidating the molecular mechanisms underlying the changes of vesicular release probability, vesicle replenishment and quantal size. In brief, we find by immunohistochemistry that the recently identified, bassoon interacting and synaptic-vesicle-associated protein Mover is downregulated in $Bsn^{\Delta Ex4/5}$ endbulbs. As Mover may contribute to regulate vesicle replenishment and may negatively regulate release probability, we consider its reduced abundance a very interesting candidate mechanism for the increased vesicular release probability and reduced vesicle

replenishment observed in $Bsn^{\Delta Ex4/5}$ endbulbs. In addition, using semi-quantitative immunofluorescence analysis we show that the abundance of the two priming factors Munc13-1 and RIM2a at the AZs and the synaptic glutamate transporter VGLUT1 in $Bsn^{\Delta Ex4/5}$ endbulbs is unaltered, which argues against their implication in the increased release probability and for a general preservation of the molecular composition of the AZs. Finding that the VGLUT1 immunofluorescence and synaptic vesicle diameter are unaltered in endbulbs of $Bsn^{\Delta Ex4/5}$ mutants, we suggest that the quantal size is increased because of changes in postsynaptic GluA receptors in bushy cells (BCs).
In addition, we provide additional analysis that further corroborates our conclusion: e.g. increased release probability supported by decreased paired pulse ratio $Bsn^{\Delta Ex4/5}$ endbulb across three different stimulus intervals (Table 2), BC volume reduced based on immunohistochemistry, and unchanged synaptic vesicle diameter arguing against a presynaptic origin of increased quantal size.
Finally, we rewrote a substantial part of the manuscript to better convey the aims, design and outcome of the study.

1.) In light of the previous studies, the design, and the main aim of the present study was not well explained. The authors find changes in the pool of vesicles and recovery speed which largely re-capitulate previous work.

We agree with the reviewer that our finding of a role of bassoon for efficient vesicular replenishment is consistent with the report of Hallermann et al. (2010) on cerebellar high throughput synapse. However, our study goes far beyond by:

- characterizing the molecular composition of the active zone and the synaptic ultrastructure
- demonstrating an increased quantal size, reduced size of the readily releasable pool and increased release probability
- decreased soma size of the postsynaptic neuron.

We also agree that the cochlear phenotype and specifically the defect of sound coding at the hair cell ribbon synapse has been characterized thoroughly in previous work. However, we actually see this as an advantage that enabled us to explain our observations (ultrastructural and functional synapse changes) as homeostatic adaptation, whereas, for example, no information on the firing rate of the presynaptic neuron was available in the study of Hallermann et al. Finally, we advance the understanding of the signaling deficit in the auditory pathway of Bassoon mouse mutants by comparing sound-driven spiking in the presynaptic and postsynaptic neurons of the cochlear nucleus, demonstrating a near normal transmission due to homeostatic plasticity. This opportunity of studying synaptic transmission in vivo and in vitro with good control on the presynaptic activity makes the system under study, the early auditory pathway, quite unique and highly valuable.

In order to address this concern of the reviewer in the revised MS we now better explain the rationale of the present study and state in:
Introduction, page 4,5
"Working on a cerebellar synapse with high transmission rate an impairment of vesicle replenishment was found in the absence of bassoon (Hallermann et al., 2010). However, potential accompanying ultrastructural and molecular changes remain to be investigated. Robust morphological and functional phenotypes were observed at bassoon-deficient ribbon synapses of retinal photoreceptors (Dick et al., 2003) and cochlear inner hair cells (Buran et al., 2010; Frank et al., 2010; Jing et al., 2013; Khimich et al., 2005) including detached or missing ribbons and a reduced rate of transmitter release. In hair cells, fewer membrane-tethered vesicles and Ca^{2+} channels lead to a reduced number of readily releasable vesicles and their replenishment is impaired in addition (Frank et al., 2010). However, it remained challenging to distinguish direct effects of bassoon disruption from those of impaired ribbon anchorage (Jing et al., 2013). Consistent with the reduction of exocytosis from inner hair cells, the auditory nerve fibers (ANFs) exhibited approximately halved sound-evoked spiking rates (Buran et al., 2010; Jing et al., 2013) and the compound action potential of the spiral ganglion was much reduced (Buran et al., 2010; Jing et al., 2013; Khimich et al., 2005). However, the subsequent auditory brainstem responses were better maintained, suggesting some compensatory mechanism in the cochlear nucleus."

and

"We combined quantitative light and electron microscopy, *in vitro* electrophysiology in acute brainstem slices and *in vivo* extracellular recordings of sound driven activity to study the role of

Bassoon in synaptic transmission from ANFs to BCs and potential compensatory mechanisms in hearing impaired $Bsn^{\Delta Ex4/5}$ mice."

2.) In addition, there were small, but significant increases in PSD length and in the quantal amplitude, and there was a putative increase in p-rel (but see point 3). These latter changes are interpreted as a "homeostatic" plasticity, but the molecular mechanisms of this putative homeostatic change are not explored nor was the physiological background well explained (is this due to a decreased sound-driven activity in Bsn KO mice?).

Yes, $Bsn^{\Delta Ex4/5}$ mutants show an activity deprivation of the AVCN due to the reduction of spontaneous and evoked rates of single auditory nerve fibers by a factor of 4 and 2.5, respectively (Buran *et al*, 2010; Jing *et al*, 2013). We postulate based on our previous and the present work, that this partial activity deprivation induces an increase of synaptic strength and of intrinsic excitability. We assume that the observed changes reflect homeostatic plasticity also because increased quantal size has not yet been reported for other synapses of bassoon mutants. We agree with the reviewer that we do not directly address the molecular regulation mediating the observed homeostatic plasticity, which is hard to do in a system as complex and small as the AVCN and beyond the scope of this study.
However, in order to address this concern of the reviewer we have:

1. clarified the rationale, i.e. the activity deprivation of the AVCN in the $Bsn^{\Delta Ex4/5}$ mutants due to the synaptic defect of inner hair cells
2. performed further experiments and analysis to better elucidate the molecular mechanisms underlying the functional synaptic phenotype. We now show that:
    a. VGLUT1 immunofluorescence is unaltered in endbulbs of $Bsn^{\Delta Ex4/5}$ mutants
    b. the synaptic vesicle diameter is unaltered in endbulbs of $Bsn^{\Delta Ex4/5}$ mutants
    c. immunofluorescence estimates for Munc13-1 and RIM2 at excitatory AZs around BCs are largely unaltered
    d. presynaptic protein Mover is down-regulated in endbulbs of $Bsn^{\Delta Ex4/5}$ mutants, which serves as a molecular candidate mechanism for the increased release probability (since Mover siRNA increased release probability in the calyx of Held synapse, Körber 2011) and reduced vesicle replenishment
    e. BC (bushy cell) somata are smaller also by immunofluorescence analysis

Despite numerous attempts and successful use of the same antibodies for GluA3 and GluA4 on other tissue we had great difficulty to reproducibly obtain specific GluA labeling in the BCs. We now provide careful discussion of the increased quantal size, favoring the increased postsynaptic GluA receptor number.

3.) It was also difficult to follow the experimental design of re-adjusted sound intensities in Fig. S7.
We realize that we did not sufficiently communicate the rationale of the experiment now presented in Figure S8. As mentioned in the response to comment 2, the spontaneous and evoked rates of single auditory nerve fibers (ANFs) of $Bsn^{\Delta Ex4/5}$ mutants are reduced. We have revised the corresponding section of the results section to now state:
"In order to elucidate how bassoon disruption at the endbulb synapses affects transmission of auditory information in the AVCN, we compared BC firing in $Bsn^{\Delta Ex4/5}$ and Bsn^{wt} mice while matching ANF activity between both genotypes. We reduced the sound pressure levels for Bsn^{wt} mice so that their ANF adapted spike rates matched the maximal adapted firing rate of $Bsn^{\Delta Ex4/5}$ ANF (Figure S8). We found comparable rates in BCs of $Bsn^{\Delta Ex4/5}$ and Bsn^{wt} mice (Figure S8) suggesting no further impairment in afferent auditory transmission at endbulb synapses due to bassoon disruption. In summary, these *in vivo* findings corroborate our hypothesis that due to homeostatic plasticity afferent auditory transmission from ANFs to BCs is intact at least at the firing rates of $Bsn^{\Delta Ex4/5}$ ANF amenable to sound stimulation. We propose that homeostatic plasticity and convergence of inputs likely underlie the improved sound onset firing of BCs and the better preserved wave 2 of the auditory brainstem response."

4. The paper contains a lot of potentially interesting data, but its main thrust remains somewhat fuzzy.

As mentioned in our general response we have revised the manuscript for better explanation and flow.

Further specific points:

5. The description of the IHC results in Figs 1, 2 should be improved, and more to the point. It should be explained at the outset, which hypothesis was tested, and the main results should be highlighted.

Done, we provide better motivation and guidance and have added the results of additional experiments and analysis.

Initially, images of the various individual channels should be shown separately side-by-side. The labeling "sap7f" should best be changed to "Bassoon".

We show the individual channels separately next to the merged figure. We would like to refrain from changing sap7f to bassoon, as we need to distinguish another (c-terminal) anti-bassoon antibody. As a compromise we have renamed it to Bsn-sap7f and Bsn-c-term and have used this notation throughout.

6.) Also, what was the sap7f signal outside synapses (see Figs 1D, E green smear on the sides -- axonal signals?).

This immunofluorescence is certainly unspecific as it also observed in the mutant tissue, where we do not find any noticeable sap7f immunofluorescence in endbulbs around the BC (Fig. 1E). We suspect that the stained structures represent capillaries and not axons, which are much thinner (see supplementary Fig. S4). We now mention this in the results section.

7.) Why were there only few Munc13 punctae (Fig. 2D) as compared to other active zone markers (e.g. Rim2-Fig. 2C).

In order to address this comment and the overall concern that the molecular mechanisms underlying the functional changes should be further investigated, we analyzed the Munc13-1 expression in presumptive endbulb AZs around 10 Bsn^{wt} and 5 $Bsn^{\Delta Ex4/5}$ BCs. We did not find fewer, but in fact, more Munc13-1 positive AZs in both genotypes. The higher count of excitatory AZs may have technical reasons, because the gephyrin immunofluorescence was stronger in these stainings, which may have caused underestimation of inhibitory AZs by confluence of immunofluroescent spots. We found a small but significant difference in the number of Munc13-1 positive AZs in $Bsn^{\Delta Ex4/5}$ endbulbs, but comparable Munc13-1 immunofluorescence per AZ between Bsn^{wt} and $Bsn^{\Delta Ex4/5}$ endbulbs (Figure 2).

8.) It remained unclear whether Bsn KO mice had an outright docking phenotype, or not (the two different measure gave different results).

Currently the view of morphological substrates of functional vesicle states is being revolutionized by the use of high pressure freezing and EM-tomography. While previously docking (physical contact between vesicle and plasma membranes) were considered a prerequisite for fusion competence of vesicles, it has become clear now that vesicles tethered to the membrane but not "contacting" may be fusion competent (Siksou *et al*, 2009; Fernández-Busnadiego *et al*, 2013). In the light of these developments and given that we used random section EM we revised our statement to:

Results:

"We then compared the vesicle distribution by counting synaptic vesicles in five 40 nm bins from the presynaptic membrane into the cytosol of the presynaptic terminal (Figure S2 and data not shown). We observed a trend towards fewer membrane-proximal vesicles in $Bsn^{\Delta Ex4/5}$ excitatory synapses (first bin), which became significant when related to the PSD length (Figure 3D). The number of vesicles in direct contact with the presynaptic plasma membrane (Figure 3D) was unaltered, as were the vesicle counts for the other bins and the vesicle size (Figure S2). "

Discussion:

"The integration technique revealed a significant reduction of the RRP size for all stimulation frequencies. The morphological correlate of the physiologically defined RRP is subject to active research. It may not only include "membrane contacting" or "docked" vesicles, which were not changed in number at AZs of $Bsn^{\Delta Ex4/5}$ endbulbs, but potentially also membrane proximal tethered

vesicles (Siksou et al., 2009). We found a significant reduction of membrane proximal vesicles per μm PSD, which seems consistent with the functional finding of a reduced RRP, but did not employ EM tomography that is required to resolve tethers. In addition, there may be fewer functional release sites due to fewer presynaptic Ca^{2+} channels. A reduction in the number of AZ Ca^{2+} channels was indeed reported for bassoon-deficient synapses of photoreceptors and hair cells (tom Dieck et al., 2005; Frank et al., 2010), but a role of the synaptic ribbon to promote clustering of Ca^{2+} channels was postulated (Frank et al., 2010; Jing et al., 2013). Finally, the standing RRP, resulting from the balance of release and replenishment (Frank et al., 2010; Oesch and Diamond, 2011; Pangršič et al., 2010), may be lower due to impaired vesicle replenishment. Indeed, we found evidence for impaired replenishment during and after stimulation."

9.) The authors claim to have found an increased p-rel but inspection of Table 1 shows that this parameter was significantly increased only for the 300 Hz train stimuli, but not with 100 or 200 Hz trains. The reasons for this should be discussed.

In response to this comment we have additionally calculated the paired-pulse ratio of the responses to the first two pulses as an additional metric related to release probability and find it decreased in $Bsn^{\Delta Ex4/5}$ endbulbs for all three frequencies (now included in Table 2), indicating enhanced release probability in the absence of functional bassoon. We argue that the release probability obtained from EPSC integration likely underestimate it in the $Bsn^{\Delta Ex4/5}$ endbulbs, because the impaired vesicle replenishment is expected to bias the extrapolated RRP estimate to larger values. Since the difference in steady state EPSCs, likely reflecting the balance of vesicle replenishment and subsequent fusion is smallest for 333 Hz, which also drives the most rapid RRP depletion, we trust the release probability estimate obtained at 333 Hz the most.
We have revised the corresponding results and discussion sections accordingly.

Results:
"Responses of $Bsn^{\Delta Ex4/5}$ endbulbs showed faster and stronger depression with lower steady-state values (Figure 5A, B, D, E; Table 2). Stronger depression was also evident from the lower paired-pulse ratio for the responses to the first two pulses at all stimulus frequencies (Table 2). The lower paired-pulse ratio and faster decline of responses during the train indicates an increased release probability in the $Bsn^{\Delta Ex4/5}$ endbulb synapses (see below). We estimated release probability, size of the readily releasable pool of vesicles (RRP) and vesicle replenishment by applying the method of integration (Schneggenburger et al., 1999) to the EPSC trains (Figure 5F). Line fitting to the last 10 of the 20 data points was used to estimate the number of readily releasable vesicles (the ordinate crossing of the extrapolated line-fit divided by the mean mEPSC amplitude) and to approximate vesicle replenishment (slope of fit), which are summarized in Table 2. In brief, we found a decreased RRP size for all stimulus frequencies and an increased release probability for 333 Hz stimulation."
Discussion:
"An increase in release probability was indicated by faster and stronger depression and enhanced asynchronous release. The paired-pulse ratio was smaller for $Bsn^{\Delta Ex4/5}$ endbulbs at all stimulus rates, while the speed of depression and the estimate of release probability from the EPSC-integration technique differed significantly only at 200 and 333 Hz, respectively. We consider it likely that demonstrating significantly enhanced release probability using the EPSC-integration technique required the stronger synaptic drive of higher stimulus rates because of the lower rate of vesicle replenishment in $Bsn^{\Delta Ex4/5}$ synapses. Impaired replenishment might also explain why the mEPSC frequency rate was unchanged despite the increased release probability."

10.) Throughout, the number of independent repetitions should be stated in the Results text. In all bar graphs which show average value plus/minus SEM; the data points from individual cells could be overlaid (e.g. in Fig. 4B - F).

done

11.) The authors claim that Bsn KO cells "fire more APs for small current injections" (p. 12 top), but the corresponding Figure only shows a set of example traces for a WT cell (Fig. 8A). This example seems to show only a single AP at the onset even with the largest I-injection, whereas the average data for WT suggests about 3-4 APs (Fig. 8C, black symbols). Thus, a more representative example, in which all APs all clearly visible, and example traces for a Bsn KO cell should be shown.

Also, it appears that FigS6A contains the same set of traces as Fig. 8A.

We followed the reviewer's suggestion and now show more representative example traces for both genotypes in Figure 8A, which now also differ from the ones shown in Figure S7 (former Figure S6).

Referee #3

This is an excellent manuscript designed to assess the contribution of the presynaptic active zone protein Bassoon on synaptic function and plasticity within the auditory system. Specifically, the authors describe a set of morphological and electrophysiological experiments assessing functional changes at the endbulb of held forming synapses on bushy cells within the anteroventral cochlear nucleus. Their results show that in the absence of Bassoon, Held synapses are formed normally, in both size and number. While vesicle replenishment and the standing pool of readily releasable pool vesicles is reduced, they noted that postsynaptic densities are larger as these synapses exhibit increases in quantal size and vesicular release probability. Intriguingly, in vivo homoestatic mechanisms appear to compensate for these changes allowing for improved synchronous signaling of the dysfunctional auditory pathway in BsnΔEx4/5 mice.
This is a beautifully designed and executed study. The data are of very high quality and the manuscript is clearly and logically written. I have no concerns.

We would like to thank the reviewer for the appreciation of our work.

2nd Editorial Decision 29 October 2013

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been re-reviewed by referee #2. As you can see below, the referee appreciates the introduced changes, but also has a number of constructive text suggestions that I would like you to take into consideration in a final revision.

REFEREE REPORT

Referee #2

The authors have responded to many of my previous points. However, I think writing of the paper still needs to be improved, especially Abstract, Introduction and start of Results. It needs to be explained at the outset what the aim of this study was, and that the hypothesis of homeostatic plasticity is followed.

In the results, please clearly conclude your main findings for each Figure. For example, for Fig. 1 it should be summarized explicitly that the "truncated" form of the protein was absent in endbulb synapses, even if this might seem trivial to the authors. At the end of the Results for Fig.2, again please summarize the main results; i.e. also specifically mention that Piccolo was upregulated, but that RIM2a and Munc13-1 were unchanged. Please also explain your motivation for chosing RIM2a (as opposed to RIM1?) and Munc13-1 for these investigations, at the start of describing Fig. 2.

In the introduction, the mouse model used here (Bsn-d-Ex4/5 mice) should be introduced more carefully. These improvements are necessary to make the paper accessible to a general readership.

Specific points remaining from the first review:

1) The example image in Fig. 2E shows only few Munc13-1 punctae, and it is was not explained why. Maybe show a more representative example?

New points which I spotted upon the second reading:

Major (relating to the homeostatic effects):

1) p. 15, Discussion:
"Instead, the firing rates of BCs at sound onset were greater than those of ANF."
=> I don't understand this sentence. If the authors refer to the slight difference between the peak rate of ANF in Bsn-d-Ex4/5(Fig. 9C, grey bar) and AVCN neurons in Bsn-d-Ex4/5 (same panel, red bar), then I think this is an over-interpretation. I understand that due to the hair cell deficit, sound-evoked peak ANF frequency is lower, and this effect seems to be passed over onto AVCN neurons (Fig. 9C). But making a point out of such a small difference (Fig. 9C grey vs red), which might easily be caused by sampling biases between the groups of cells, seems not justified.

Similarly, I do not understand statements like (p. 20)
"Here, we demonstrate that the onset firing of BCs is improved over that of ANFs." (... in Bsn-d-Ex4/5 mice, you mean?)
and
"account for the improved onset response."
=> in the Bsn-d-Ex4/5 mice, the absolute onset response was clearly smaller (?)

Further specific points:

2) CR IHC to count the number of endbulb inputs (p. 5).
It is unclear to me how this was done, and I did not find a description in the Method. From the image in Fig. 1A, I assume that every endbulb expresses CR, so I did not understand how CR can be used to distinguish between different "fingers" of the same endbulb. Please explain this in detail in the Methods, and mention it briefly in the Results section. In Fig. 1B, it would be better to change the display to a standard histogram with bars.

3) "what is center of mass distance" => please define at outset in Results and make a link to a suitable explanation in Methods.

4) Mover IHC intensities: How was the "normalized" intensity of IHC punta calculated (please describe in detail in Methods, and mention it briefly in Results). Also, please give the average reduction in the Results text -- this was apparently a small effect (~ 20% reduction?).

5) Fig. S2A:
- indicate length of scalebar
- what is a "presynaptic dense projection" -- please give reference to previous work

6) p.10:
" This functional distinction was confirmed by inspection of cell morphology after filling them with the fluorescent dye Alexa-488 via the pipette in a subset of experiments (Figure S4)"
=> Respectfully, the morphology in Fig. 4SC, cell 2 and 3 are very similar -- both cells have 3 or more "primary dendrites". Please further explain.

7) Fig. 8G:
- please label which symbols correspond to which situation (with/without TTX).
- The fig. legends says " ...but did not enable clamping the voltage at the AIS". -- how was voltage-clamp assessed?

8) p. 17:
"We consider it likely that demonstrating significantly enhanced release probability using the EPSC-integration technique required the stronger synaptic drive of higher stimulus rates because of the lower rate of vesicle replenishment inBsnΔEx4/5 synapses"
=> I did not understand this sentence; please re-write

2nd Revision - authors' response 05 November 2013

Referee #2

1) The authors have responded to many of my previous points. However, I think writing of the paper

still needs to be improved, especially Abstract, Introduction and start of Results. It needs to be explained at the outset what the aim of this study was, and that the hypothesis of homeostatic plasticity is followed.

We have revised the MS accordingly.
In the **abstract**, we now state: "**Here, we studied the structure and function of endbulb synapses in mice that lack the presynaptic scaffold bassoon and exhibit reduced ANF input into the AVCN. "** to set the stage for introducing homeostatic plasticity. Then we emphasize homeostatic plasticity "**We propose that ANF activity deprivation drives homeostatic plasticity in the AVCN involving synaptic upscaling and increased intrinsic BC excitability."**

In the **introduction, page 4, line 15**, we now state: "However, the subsequent auditory brainstem responses were better maintained, suggesting some compensatory mechanism in the AVCN. Whether such improved synchronicity of auditory signaling results from the convergent ANF input to BCs or the AVCN undergoes homeostatic plasticity in response to the reduced ANF remained to be investigated."
And, page 5, line 4:
"We combined quantitative light and electron microscopy, *in vitro* electrophysiology in acute brainstem slices and *in vivo* extracellular recordings of sound driven activity to study the role of bassoon in synaptic transmission from ANFs to BCs and potential homeostatic plasticity in hearing impaired $Bsn^{\Delta Ex4/5}$ mice."

In the **results** we now state:
Beginning of first section:
"We used immunohistochemistry in order to elucidate effects of bassoon disruption and potential homeostatic plasticity on the convergence of ANFs, the number of AZs and their molecular composition."

In the results, please clearly conclude your main findings for each Figure.

2) For example, for Fig. 1 it should be summarized explicitly that the "truncated" form of the protein was absent in endbulb synapses, even if this might seem trivial to the authors.

We had already clearly stated the specific finding, undoubtedly mentioning the remaining presence of low amounts of the truncated bassoon fragment. We now clarify further (page 7, line 4):
"Consistent with previous observations at other synapses (Dresbach et al., 2003; Frank et al., 2010) the remaining fragment was not or less efficiently integrated in to the AZ, as is evident from the diffuse and weak labeling for the truncated bassoon fragment that less accurately co-localized with piccolo. The center of mass distance of the nearest neighboring spots of piccolo and bassoon (Bsn-c-term. antibody) was significantly larger and more variable (F-test: $p<0.001$) in $Bsn^{\Delta Ex4/5}$ terminals than in Bsn^{wt} (Fig. 1G). Therefore, we assume that AZs $Bsn^{\Delta Ex4/5}$ endbulbs lack functional bassoon."

3) At the end of the Results for Fig.2, again please summarize the main results; i.e. also specifically mention that Piccolo was upregulated, but that RIM2a and Munc13-1 were unchanged.

"While the abundance of RIM2a and Munc13-1 was unchanged, piccolo was up-regulated whereas the putative bassoon-effector Mover was down-regulated in endbulbs."

4) Please also explain your motivation for chosing RIM2a (as opposed to RIM1?) and Munc13-1 for these investigations, at the start of describing Fig. 2.

The goal was to analyze exemplary CAZ constituents for whether bassoon disruption would change their abundance. Among the several antibodies tested the ones selected for the immunohistochemical analysis shown provided the most reliable staining in our hands, which was required for a robust comparison of both genotypes.

We now state, page 8, line 3:
"In addition, we studied RIM2a- and Munc13-1, which served as **exemplary** CAZ constituents. The number and fluorescence intensity of RIM2a- and Munc13-1-immunoreactive spots was largely unaltered in $Bsn^{\Delta Ex4/5}$ endbulbs (Figure 2C, D, E, F)."

5) In the introduction, the mouse model used here (Bsn-d-Ex4/5 mice) should be introduced more carefully. These improvements are necessary to make the paper accessible to a general readership.
Done:
"Disruption of bassoon function by deletion of exons 4 and 5 of the *Bsn* gene that code for a large central part of bassoon in mice ($Bsn^{\Delta Ex4/5}$) led to a higher number of presynaptically silent synapses in cultured hippocampal neurons (Altrock et al., 2003), while the remaining synaptic transmission of the functional synapses was unaltered (Altrock et al., 2003; Mukherjee et al., 2010). Working on a cerebellar synapse with high transmission rate an impairment of vesicle replenishment was found in $Bsn^{\Delta Ex4/5}$ and a bassoon gene trap mouse mutant (Hallermann et al., 2010). However, potential accompanying ultrastructural and molecular changes remain to be investigated. Robust morphological and functional phenotypes were observed at the synapses of retinal photoreceptors (Dick et al., 2003) and cochlear inner hair cells (Buran et al., 2010; Frank et al., 2010; Jing et al., 2013; Khimich et al., 2005) of $Bsn^{\Delta Ex4/5}$ mice including detached or missing ribbons and a reduced rate of transmitter release."

Specific points remaining from the first review:

6) The example image in Fig. 2E shows only few Munc13-1 punctae, and it is was not explained why. Maybe show a more representative example?

Done, we now show a more representative example in panel 2E.

New points which I spotted upon the second reading:

7) Major (relating to the homeostatic effects):

1) p. 15, Discussion:
"Instead, the firing rates of BCs at sound onset were greater than those of ANF."
=> I don't understand this sentence. If the authors refer to the slight difference between the peak rate of ANF in Bsn-d-Ex4/5(Fig. 9C, grey bar) and AVCN neurons in Bsn-d-Ex4/5 (same panel, red bar), then I think this is an over-interpretation. I understand that due to the hair cell deficit, sound-evoked peak ANF frequency is lower, and this effect seems to be passed over onto AVCN neurons (Fig. 9C). But making a point out of such a small difference (Fig. 9C grey vs red), which might easily be caused by sampling biases between the groups of cells, seems not justified.

Small, but significant (P = 0.038, Wilcoxon Mann Whitney test), reasonable sample size (n = 32 ANF and n = 12 putative BCs) and relevant:

The relatively well maintained wave II of the ABR in $Bsn^{\Delta Ex4/5}$ mice has been puzzling us and the field. We now provide evidence for an improved onset response of putative BCs compared to ANF and partly attribute it to homeostatic plasticity.

In response to the reviewers request we have toned down our statement in the abstract and discussion.
Abstract:
"*In vivo* recordings from individual mutant BCs demonstrated a slightly improved response at sound onset compared to ANF, likely reflecting the combined effects of ANF convergence and homeostatic plasticity."

Results, page 14, line 6 bottom-up:
"As reported earlier, we found a reduction of evoked spike rates in $Bsn^{\Delta Ex4/5}$ ANFs (Buran et al., 2010; Frank et al., 2010). However, the spike rate at sound onset of putative mutant BCs was slightly less reduced than in ANFs, indicating that brainstem mechanisms partially counteract the cochlear deficit to recover spike rates (Figure 9C; $Bsn^{\Delta Ex4/5}$ ANF: n = 32, $Bsn^{\Delta Ex4/5}$ AVCN: n = 12)."

Discussion, page 16, top: "Instead, the firing rates of BCs at sound onset were slightly higher than those of ANF."

8) Similarly, I do not understand statements like (p. 20)
"Here, we demonstrate that the onset firing of BCs is improved over that of ANFs." (... in Bsn-d-Ex4/5 mice, you mean?)
and
"account for the improved onset response."
=> in the Bsn-d-Ex4/5 mice, the absolute onset response was clearly smaller (?)

changed accordingly:
"Here, we demonstrate that in $Bsn^{\Delta Ex4/5}$ mice the onset firing of BCs was slightly improved over that of ANFs. We postulate that homeostatic plasticity (Turrigiano, 2011) in the AVCN, likely induced by the partial auditory deprivation due to the synaptic defect in hair cells, together with the convergence of ANF in the AVCN (Cao and Oertel, 2010) account for the partially restored sound onset response."

Further specific points:

9) CR IHC to count the number of endbulb inputs (p. 5).
It is unclear to me how this was done, and I did not find a description in the Method. From the image in Fig. 1A, I assume that every endbulb expresses CR, so I did not understand how CR can be used to distinguish between different "fingers" of the same endbulb. Please explain this in detail in the Methods, and mention it briefly in the Results section. In Fig. 1B, it would be better to change the display to a standard histogram with bars.

done as requested:
Results:
was quantified by reconstructing calretinin-stained endbulbs (Caicedo et al., 1997; Chanda and Xu-Friedman, 2010a; Lohmann and Friauf, 1996) as exemplified in Figure 1A and explained in the methods section.

Methods:
Reconstruction of endbulb terminals from calretinin-stained confocal image stacks was done by visually tracing, labeling and computed volume rendering with the Reconstruct software (Fiala, 2005).

Please note that we did not intend to quantify fingers of individual endbulbs.

10) "what is center of mass distance" => please define at outset in Results and make a link to a suitable explanation in Methods.

We consider center of mass distance a standard term. However, on request of the reviewer we specify in results: "center of immunofluorescence mass distance" and provide an explanation in the methods section:
(center of immunofluorescence mass coordinates were calculated by the average pixel position weighted by pixel intensity)

11) Mover IHC intensities: How was the "normalized" intensity of IHC punta calculated (please describe in detail in Methods, and mention it briefly in Results). Also, please give the average reduction in the Results text -- this was apparently a small effect (~ 20% reduction?).

done

12) Fig. S2A:
- indicate length of scalebar
done

- what is a "presynaptic dense projection" -- please give reference to previous work
We added to the MS:

"Given that bassoon is part of the CAZ we also counted presynaptic dense projections (DP), electron-dense appearing specializations that extend from the cytomatrix into the cytoplasm and tether synaptic vesicles (Zhai and Bellen, 2004)…"

13) p.10:
" This functional distinction was confirmed by inspection of cell morphology after filling them with the fluorescent dye Alexa-488 via the pipette in a subset of experiments (Figure S4)"
=> Respectfully, the morphology in Fig. 4SC, cell 2 and 3 are very similar -- both cells have 3 or more "primary dendrites". Please further explain.

In response to the reviewers request we have enlarged the images and enhanced the contrast allowing for better visual inspection and now explain more thoroughly in the figure legend:

Cell 2 exhibits bushy cell-typical mEPSCs and morphology (two primary dendrites that branch extensively in close proximity to the soma and oval shaped soma) as well, but unusually strong depression. In contrast, **cell 3** represents a typical example of a stellate cell with facilitating EPSC amplitudes at the beginning of high frequency stimulation, slower kinetics of both, evoked and spontaneous currents, more numerous (~5) primary dendrites (*) and a sharp cornered, polygon-like soma.

14) Fig. 8G:
- please label which symbols correspond to which situation (with/without TTX).
done
- The fig. legends says " ...but did not enable clamping the voltage at the AIS". -- how was voltage-clamp assessed?

To explain this the figure legend now states:
[...] but did not enable clamping the voltage at the AIS as indicated by the all-or-none-like current and the relatively constant peak current amplitudes, exemplified in G.

15) p. 17:
"We consider it likely that demonstrating significantly enhanced release probability using the EPSC-integration technique required the stronger synaptic drive of higher stimulus rates because of the lower rate of vesicle replenishment inBsnΔEx4/5 synapses"
=> I did not understand this sentence; please re-write

done: "The enhancement of release probability might have been revealed by the EPSC-integration technique only with most rapid RRP depletion protocol (at 333 Hz), because at lower stimulation rates the method tends to overestimate the RRP, and consequently, to underestimate release probability in $Bsn^{\Delta Ex4/5}$ synapses due to their lower rate of vesicle replenishment."

