Bassoon controls synaptic vesicle release via regulation of presynaptic phosphorylation and cAMP

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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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)
Dear Dr. Fejtova

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, while both referees acknowledge that the findings are potentially interesting they also point out several technical concerns and have a number of suggestions for how the study should be strengthened, and I think that all of them should be addressed. Please also address point 5 from referee 1 and display the exact p-values in the figure legends and text.

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

We invite you to submit your manuscript within three months of a request for revision. This would be December 1st in your case. However, we are aware of the fact that many laboratories are not fully functional due to COVID-19 related shutdowns and we have therefore extended the revision time for all research manuscripts under our scooping protection to allow for the extra time required to address essential experimental issues. Please contact us to discuss the time needed and the revisions further.

***IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section is missing.
2) Your manuscript contains error bars based on n=2. Please use scatter blots showing the individual datapoints in these cases. The use of statistical tests needs to be justified.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.***

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure). Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) a .docx formatted letter INCLUDING the reviewers’ reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines.

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as "Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called "Appendix", which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:
- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Please note that a Data Availability section at the end of Materials and Methods is now mandatory. In case you have no data that requires deposition in a public database, please state so instead of refereeing to the database. See also <https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available.

9) Regarding data quantification
The following points must be specified in each figure legend:
- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
Discussion of statistical methodology can be reported in the materials and methods section, but figure legends must contain a basic description of n, P and the test applied.
- Please also include scale bars in all microscopy images.

10) Our journal encourages inclusion of "data citations in the reference list" to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at.

11) As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely

Martina Rembold, PhD
Senior Editor
EMBO reports

Referee #1:

Bassoon is an active-zone protein which has been implicated in regulating the recruitment of vesicles to the readily releasable pool. In this manuscript, the authors present evidence for a role for Bassoon in controlling cell signaling in presynaptic terminals and its relevance to the regulation of the division of synaptic vesicles into functional pools. Specifically, they explore Cdk5, calcineurin and PKA signaling in neurons devoid of bassoon, and the role that PDE4 plays in this cascade. The authors also explore the effect of this signaling cascade on key proteins that play a role in regulating vesicle pools and vesicle exocytosis like synapsin and SNAP25, respectively. The authors find that in the absence of Bassoon (from BsnGT mice) the fraction of vesicles in the recycling pool is lower, meaning that the fraction of vesicles which are resistant to release is larger. In agreement, the
refractory pool is relatively smaller as well. Interestingly, the authors find that the effects differ for excitatory and inhibitory synapses. When exploring the signaling cascades, the authors find that Cdk5 is active to a higher extent in BsnGT neurons, as is phosphorylation of synapsin I at S551, in agreement with their functional findings. Furthermore, inhibiting Cdk5 pharmacologically (with roscovitine) equalized BsnGT and WT neurons. Likewise, inhibition of calcineurin (with FK506) resulted in opposite effects (as it opposes Cdk5 activity). Activation of PKA with forskolin (which activates the enzyme adenyl cyclase) or directly with 6-Bzn-cAMPs (a cAMP analog), or inhibition of PDE4 with rolipram (which slows the breakdown of cAMP) resulted in increases in the recycling pool and in the RRP, as well as phosphorylation of synapsin I and S9. All these treatments had a stronger effect on BsnGT neurons, reaching similar final levels as WT neurons (because they start at an initially lower fraction).

I find this manuscript well written, interesting, and that the information included within is important for the advancement of the field. The consequences of the involvement of signaling cascades in the control of the division of synaptic vesicles into functional pools has not been explored in enough detail, although this topic was introduced quite a few years ago.

I find that there are several issues which the authors need to address in order to tighten up the manuscript:

1. Although the authors present data that the effect of manipulating Bsn is different in inhibitory and excitatory neurons (Fig. 1, no effect on inhibitory synapses), all the data starting at figure 2 does not refer to this issue - i.e., the authors do not differentiate between excitatory and inhibitory neurons. My feeling is that this omission may mask some of their findings. Perhaps the authors should address this issue experimentally, for one or more key findings (retrospective immunolabeling?) or explain why this is not a significant issue. While I am aware that inhibitory synapses comprise only a minority of the synapses in a neuronal culture, this in itself does not void my argument.

2. The effect on the total vesicle pool needs to be documented better. While the authors present (in the text) data supporting their claim that the total pool is not different in WT and BsnGT neurons, based on alkalization of syphy, a more direct approach is to quantify vGlut1 and VGAT - data which they already have, and thus just need to analyze.

3. Fig. 1C: I am troubled by the images showing SytI staining following spontaneous network activity in BsnGT neurons. The staining pattern does not appear to be synaptic, or at least does not correlate well with either VGlut1 or VGAT (more the former). The authors need to rule out unspecific staining (for example, by staining fixed but unpermeabilized neurons). Furthermore, is it possible that vesicle clustering is deficient in BsnGT neurons, at least in the time scale of these measurements? Could this contribute to the results show in the rest of figure 1?

4. The authors used biochemical assays to measure PKA, calcineurin and PDE4 activity in tissue lysates. The results obtained in this manner are interesting and agree well with other results presented in the manuscript. However, all these activities are dependent on the specific environment in which the enzymes are situated, which are most certainly not preserved when cells are lysed. Could the authors explain why these assays can provide information which is specifically relevant to their claims in the manuscript? Specifically - these enzymes are themselves controlled by signaling cascades that are probably disrupted in the assays.

5. The authors don't report specific p values for the statistical tests. Is this allowed by the Journal's policy? Did the authors test for the normality of the distribution of the data? The authors use one-way ANOVA in situations in which two-way ANOVA may be more appropriate (such as when different treatments are given to two different genotypes).

Minor issues:

1. There are a few papers which the authors should consider discussing in this manuscript: Two papers by Chi Greengard and Ryan discuss the effect of synapsin phosphorylation and synaptic activity on synaptic function (including both S551 and S9 which are discussed in the current manuscript). Orenbuch et al., J Neurosci 2012 - specifically addresses the effect of Cdk5 on synaptic vesicle mobility. Finally, 10.1016/j.neuroscience.2006.04.061 discusses the effect of Cdk5 on calcium channels. Considering Bassoon has been implicated in controlling calcium channels in the synapse, this appears to be of relevance.

2. In Fig. 3D, there must be an error. While the authors claim that S551 is phosphorylated to a higher degree in BsnGT neurons, the image shows the opposite.

3. The duration of TTX and Bic treatments used in the experiments shown in Fig. 3 is sufficient to induce homeostatic plasticity. In my opinion a shorter and more acute period would have been more appropriate to support the argument of the authors concerning the activity-dependence of the phosphorylation of synapsin at S551.

4. In Fig. 1, the authors explain that co-localization with synapsin was used in order to define SytI spots as being synapses. However, the synapsin images are not shown at all.

5. In P. 8 the authors report tendency of the data which is not statistically significant. If there is no significance, tendencies are not useful. The same can be said about the following statement in P. 9: "The same treatment had a minor effect on RRP and TRP in BsnGT".

6. As the authors state in the discussion, Forskolin has a significant effect on release probability. This is true also for spontaneous release of miniature events. The authors should consider whether this impacts their interpretation of the results in Fig. 5.

7. Did the authors consider direct imaging of cAMP using fluorescent indicators of cAMP such as Flamindo or R-FlincA?

8. P. 17 - odder should be order
Montenegro-Venegas et al. present the manuscript entitled "Bassoon controls synaptic vesicle release via regulation of presynaptic phosphorylation and cAMP".

The study deals with the investigation of the consequences for neurotransmission upon deletion of the presynaptic scaffold protein bassoon using the gene-trap mutant. Bassoon is known as a key player in neurotransmission and for example regulates the SVs mobilization from the three functional pools (reserve, recycling and readily-releasable vesicle pool). The authors assessed the vesicle pool size as well as the release competence of vesicles and importantly the underlying molecular mechanism(s). They use a combination of imaging experiments of cultured hippocampal neurons together with biochemical approaches and pharmacological interference of relevant signaling pathways. The investigation of the SV release competence in neurons lacking bassoon in comparison to wild type controls revealed a clear decrease in the mutant. Moreover, a reduced RRP and recycling pool size was found in bassoon mutants and at the same time, the reserve pool was increased. The authors hypothesize that the reduction might be associated with the dysregulation of CDK5/calcineurin balance and cAMP/PKA presynaptic signaling. The authors thoroughly worked out that the CDK5/calcineurin and cAMP/PKA presynaptic signaling pathways appeared disturbed. This resulted in an aberrant phosphorylation of their downstream effectors synapsin 1 and SNAP25.

Overall, the authors present a plethora a new insight into the function of bassoon and how it is involved in SV pool regulation via regulating the presynaptic phosphorylation and cAMP homeostasis. I believe that this is a very important contribution to our understanding of synaptic transmission and will be of interest for a broad community. The study is thoroughly done and after revision, I strongly recommend publication in EMBO Reports.

There is one specific point, which should be considered in the manuscript:

It is not discussed, whether the observed effects might be partially also the result of an altered abundance of other presynaptic proteins. The lack of bassoon was shown to influence the expression levels of different proteins, such as piccolo or munc13. For example, in the exon4-5 mutant for bassoon the intensity of piccolo puncta was increased at endbulb of Held active zones, moreover, munc13-1 expression was altered and Mover expression was downregulated (Mendoza Schulz et al., 2014). Are the levels of piccolo (or other proteins like munc13) in the gene-trap bassoon mutant changed on the level of individual synapses? In this context, it will be important to thoroughly discuss this issue and even better, provide data that show an unchanged or changed expression of other presynaptic proteins like piccolo or munc13.

Minor

-Please define, what exactly is meant by release competence of individual SVs
-Please revise carefully for typos etc.
-Typos/abbreviations
  -Page 3, please use the abbreviation for synaptic vesicles (SVs) consistently
  -In Fig. 1I shift of the bars for ns, in general the figures could be revised for alignment of the panels
  -Page 6, I guess button should be bouton?
  -Please refer to Fig. 3A in the main text
  -Page 7: "....we detected a significantly higher pSer551Syn1 labelling in synapses..." What is meant, more puncta or higher intensity? Larger puncta?
  -Page 11: Bassoon should be abbreviated to bsn
  -Page 21: "PDE4 immunoprecipitation and icitivity assay", should be activity
  -Please refer in the main text the Lentiviral particles production
We thank reviewers for their positive evaluation of the original manuscript. In this revised version we addressed all questions raised by reviewers. The main focus was given to experiments in support of synapse-type specific effects of Bsn loss on release competence of synaptic vesicles (SVs). We performed quantitative immunostainings for phosphorylated Ser9 and Ser551 of synapsin 1, the main phosphoprotein dysregulated in BsnGT neurons, in excitatory and inhibitory synapses. The analyses confirmed changes in phosphostatus of both sites in BsnGT in excitatory but not in inhibitory synapses. This further supports our claim that Bsn controls release competence of SVs via regulation of presynaptic phosphorylation specifically at excitatory synapses. We performed analogous immunostainings for Pclo and Munc13-1, which were both previously shown to be regulated in BsnGT synapses. We confirmed the regulation of these proteins as shown previously and importantly, we could show now that Pclo and Munc13 are regulated at both excitatory and inhibitory synapses, indicating 1) importance of Bsn in synapse assembly independently of synapse-type and 2) that the overall role of Bsn in synapse assembly is functionally distinct form regulation of presynaptic phosphorylation evident in excitatory synapses.

We hope that the revised manuscripts will be suitable for publication in EMBO reports.

In following we answer all specific questions of reviewers:

Referee #1:
Bassoon is an active-zone protein which has been implicated in regulating the recruitment of vesicles to the readily releasable pool. In this manuscript, the authors present evidence for a role for Bassoon in controlling cell signaling in presynaptic terminals and its relevance to the regulation of the division of synaptic vesicles into functional pools. Specifically, they explore Cdk5, calcineurin and PKA signaling in neurons devoid of bassoon, and the role that PDE4 plays in this cascade. The authors also explore the effect of this signaling cascade on key proteins that play a role in regulating vesicle pools and vesicle exocytosis like synapsin and SNAP25, respectively. The authors find that in the absence of Bassoon (from BsnGT mice) the fraction of vesicles in the recycling pool is lower, meaning that the fraction of vesicles which are resistant to release is larger. In agreement, the readily releasable pool is relatively smaller as well. Interestingly, the authors find that the effects differ for excitatory and inhibitory synapses. When exploring the signaling cascades, the author find that Cdk5 is active to a higher extent in BsnGT neurons, as is phosphorylation of synapsin I at S551, in agreement with their functional findings. Furthermore, inhibiting Cdk5 pharmacologically (with roscovitine) equalized BsnGT and WT neurons. Likewise, inhibition of calcineurin (with FK506) resulted in opposite effects (as it opposes Cdk5 activity). Activation of PKA with forskolin (which activates the enzyme adenylyl cyclase) or directly with 6-Bzn-cAMPs (a cAMP analog), or inhibition of PDE4 with rolipram (which slows the breakdown of cAMP) resulted in increases in the recycling pool and in the RRP, as well as phosphorylation of synapsin I and S9. All these treatments had a stronger effect on Bsn GT neurons, reaching similar final levels as WT neurons (because they start at an initially lower fraction).

I find this manuscript well written, interesting, and that the information included within is important for the advancement of the field. The consequences of the involvement of signaling cascades in the control of the division of synaptic vesicles into functional pools has not been explored in enough detail, although this topic was introduced quite a few years ago.

I find that there are several issues which the authors need to address in order to tighten up the manuscript:

1. Although the authors present data that the effect of manipulating Bsn is different in inhibitory and excitatory neurons (Fig. 1, no effect on inhibitory synapses), all the data starting at figure 2 does not refer to this issue - i.e., the authors do not differentiate between excitatory and inhibitory neurons. My feeling is that this omission may mask some of their findings. Perhaps the authors should address this issue experimentally, for one or more key findings (retrospective immunolabeling?) or explain
why this is not a significant issue. While I am aware that inhibitory synapses comprise only a minority of the synapses in a neuronal culture, this in itself does not void my argument.

We completely see the point of the reviewer. To strengthen our claim about synapse type-specific effect of Bsn deletion, we did additional experiments. We analysed abundance of pSer95Syn1 and pSer551Syn1 in excitatory and inhibitory synapses in WT and BsnGT neurons (Fig EV2, Fig EV3). In line with our reasoning that aberrant phospho-balance underlies the changes in SV release competence specifically in excitatory synapses, we demonstrated that the phosphorylation status of both analysed residues was unchanged in inhibitory synapses, while their levels in excitatory synapses were elevated. Interestingly, the changes in abundance of Munc13-1 and Pclo were similar in both synapse types, confirming the importance of Bsn expression for proper synapse organization in both synapse types, but the excitatory synapse-specific role of Bsn-dependent control of phospho-balance.

2. The effect on the total vesicle pool needs to be documented better. While the authors present (in the text) data supporting their claim that the total pool is not different in WT and BsnGT neurons, based on alkalization of syphy, a more direct approach is to quantify vGlut1 and VGAT - data which they already have, and thus just need to analyze.

We want to stress that within the presented article our goal was not addresses regulation of SV numbers per synapse in the absence of Bsn. Instead, their distribution in to functional pools and mechanisms behind were the primary subject of investigation. In our syphHy live imaging experiments, the fraction of recycling vesicles was always expressed as a proportion of the total synaptic expression of this sensor. The sensor was expressed at similar levels in synapses of both genotypes as quantified in this manuscript. For syt1 uptake experiments, the situation is similar, our previously published data show unchanged synaptic abundance of Syt1 in BsnGT (Hoffmann-Conaway et al, 2020).

The number of SV in Bsn mutants was addressed elsewhere previously, with to some extent conflicting outcomes. While a normal number of SVs in in hippocampal tissue was detected in ultrastructural analyses (Altrock et al, 2003) and normal abundance of synaptic vesicle proteins including synaptophysin were found in cultured neurons from Bsn mutant (Davydova et al, 2014), levels of SV2B and VAMP2 were decreased by about 20 and 25%, respectively, and about 25% lower density of SV in the presynapses of cultured neurons was reported in a recent publication (Hoffmann-Conaway et al, 2020). As mentioned above, the same publication, however, claims unchanged abundance of Syt1, indicating that it is not straightforward to approximate the number of SVs solely from the immunoreactivity of SV-integral or associated proteins. Moreover, we know that expression of VGLUT1 and VGAT are under strong control of neuronal network activity, which is different upon deletion of Bsn as documented by appearance of epileptic seizures in Bsn KO animals (Altrock et al., 2003).

3. Fig. 1C : I am troubled by the images showing Syt1 staining following spontaneous network activity in BsnGT neurons. The staining pattern does not appear to be synaptic, or at least does not correlate well with either VGlut1 or VGAT (more the former). The authors need to rule out unspecific staining (for example, by staining fixed but unpermeabilized neurons). Furthermore, is it possible that vesicle clustering is deficient in BsnGT neurons, at least in the time scale of these measurements? Could this contribute to the results show in the rest of figure 1?

Immunoreactivity of synapsin (FigEV2 and EV3) and synaptophysin (Davydova et al., 2014) are not changed in these cultures, antibodies are KO verified (www.sysy.com). We showed previously using similar protocol that block of endocytosis or spontaneous network activity significantly interfere with the Syt1 uptake documenting the reliability of syt1 antibody uptake approach (Davydova et al., 2014; Ivanova et al, 2020). We changed the images in figure 1G and J for more representative ones.

4. The authors used biochemical assays to measure PKA, calcineurin and PDE4 activity in tissue lysates. The results obtained in this manner are interesting and agree well with other results presented in the manuscript. However, all these activities are dependent on the specific environment
in which the enzymes are situated, which are most certainly not preserved when cells are lysed. Could the authors explain why these assays can provide information which is specifically relevant to their claims in the manuscript? Specifically - these enzymes are themselves controlled by signaling cascades that are probably disrupted in the assays.

>> In the method part, we are describing exactly, how the activity assays were done. The assays are done on P2 (membrane fraction, highly enriched in synaptosomes) not on crude lysates. This protocol is adapted from Gray and Whittaker and used for decades to obtain metabolically active structures capable of evoked neurotransmitter release (Gray & Whittaker, 1962). All homogenization and fractionations steps were done in the presence of phosphatase inhibitors, to preserve the in vivo phospho-status of material. Thus, we argue that both natural environment and phosphostatus of the enzymes are preserved using these protocols. Moreover, the data on changed phosphorylation levels of presynaptic substrates obtained by stainings and by Western blotting provide an additional readout for the kinase/ phosphatase activity in the presynaptic compartment and further support our conclusion about changes in presynaptic phosphorylation in Bsn mutant synapses.

5. The authors don’t report specific p values for the statistical tests. Is this allowed by the Journal's policy? Did the authors test for the normality of the distribution of the data? The authors use one-way ANOVA in situations in which two-way ANOVA may be more appropriate (such as when different treatments are given to two different genotypes).

>> The tests and p values were provided in the figure legend. For experiments, where the effects of treatments on different genotypes are quantified, we changed the statistics to two-way ANOVA as requested. Together with the revised manuscript, we are uploading a table with all details of testing applied in all experiments.

Minor issues:
1. There are a few papers which the authors should consider discussing in this manuscript: Two papers by Chi Greengard and Ryan discuss the effect of synapsin phosphorylation and synaptic activity on synaptic function (including both S551 and S9 which are discussed in the current manuscript). Orenbuch et al., J Neurosci 2012 - specifically addresses the effect of Cdk5 on synaptic vesicle mobility. Finally, 10.1016/j.neuroscience.2006.04.061 discusses the effect of Cdk5 on calcium channels. Considering Bassoon has been implicated in controlling calcium channels in the synapse, this appears to be of relevance.

>> I have to apologize, but I did not find the aspect of cdk5 dependent regulation in the Orenbuch et al., J Neurosci 2012, and despite this paper investigates effects of synapsin triple KO, I do not see direct connection to our work. Similarly, the given citation “10.1016/j.neuroscience.2006.04.061” leads me to a paper with title: Activity-related redistribution of presynaptic proteins at the active zone by J.-H.Tao-Cheng, which does not address the calcium channel/cdk5 relationship. We included the (Chi et al, 2003) in the paper.

2. In Fig. 3D, there must be an error. While the authors claim that S551 is phosphorylated to a higher degree in BsnGT neurons, the image shows the opposite.

>> We are sorry for this mistake. We corrected the wrongly placed images.

3. The duration of TTX and Bic treatments used in the experiments shown in Fig. 3 is sufficient to induce homeostatic plasticity. In my opinion a shorter and more acute period would have been more appropriate to support the argument of the authors concerning the activity-dependence of the phosphorylation of synapsin at S551.

>> In this experiment, we exactly followed a previously published protocol to achieve the best possible comparison (Verstegen et al, 2014). It is correct that lack of inactivity-induced changes in phosphorylation likely also affect the ability of Bsn-deficient neurons to adapt to inactivity.
4. In Fig. 1, the authors explain that co-localization with synapsin was used in order to define SytI spots as being synapses. However, the synapsin images are not shown at all.
>> For space reasons, we omitted the synapsin images in the first submission. In the revised submission we included these images again in Fig 1.

5. In P. 8 the authors report tendency of the data which is not statistically significant. If there is not significance, tendencies are not useful. The same can be said about the following statement in P. 9: "The same treatment had a minor effect on RRP and TRP in BsnGT"
>> We changed the respective wording (now on page 9 of the manuscript).

6. As the authors state in the discussion, Forskolin has a significant effect on release probability. This is true also for spontaneous release of miniature events. The authors should consider whether this impacts their interpretation of the results in Fig. 5.
>> Forskolin increases both the number of release sites and vesicular p. Sampling rate used in our imaging experiments enables us to address the RRP size but not the kinetics of RRP release, so we can’t make direct conclusion form our experiments. Previous electrophysiological studies indicate decreased RRP but unchanged release probability in hippocampal synapses of Bsn mutant mice, which is in good agreement with the effect of reduced PKA activity on RRP in the absence of Bsn (Altrock et al., 2003).

7. Did the authors consider direct imaging of cAMP using fluorescent indicators of cAMP such as Flamindo or R-FlincA?
>> This is an excellent idea and exactly our plan for future work.

8. P. 17 - odder should be order
>> Sorry for this typo. We changed it.

Referee #2:
Montenegro-Venegas et al. present the manuscript entitled "Bassoon controls synaptic vesicle release via regulation of presynaptic phosphorylation and cAMP". The study deals with the investigation of the consequences for neurotransmission upon deletion of the presynaptic scaffold protein bassoon using the gene-trap mutant. Bassoon is known as a key player in neurotransmission and for example regulates the SVs mobilization from the three functional pools (reserve, recycling and readily-releasable vesicle pool). The authors assessed the vesicle pool size as well as the release competence of vesicles and importantly the underlying molecular mechanism(s). They use a combination of imaging experiments of cultured hippocampal neurons together with biochemical approaches and pharmacological interference of relevant signaling pathways. The investigation of the SV release competence in neurons lacking bassoon in comparison to wild type controls revealed a clear decrease in the mutant. Moreover, a reduced RRP and recycling pool size was found in bassoon mutants and at the same time, the reserve pool was increased. The authors hypothesize that the reduction might be associated with the dysregulation of CDK5/calcineurin balance and cAMP/PKA presynaptic signaling. The authors thoroughly worked out that the CDK5/calcineurin and cAMP/PKA presynaptic signaling pathways appeared disturbed. This resulted in an aberrant phosphorylation of their downstream effectors synapsin 1 and SNAP25. Overall, the authors present a plethora a new insight into the function of bassoon and how it is involved in SV pool regulation via regulating the presynaptic phosphorylation and cAMP homeostasis. I believe that this is a very important contribution to our understanding of synaptic transmission and will be of interest for a broad community.

The study is thoroughly done and after revision, I strongly recommend publication in EMBO Reports. There is one specific point, which should be considered in the manuscript: It is not discussed, whether the observed effects might be partially also the result of an altered abundance of other presynaptic proteins. The lack of bassoon was shown to influence the expression levels of different proteins, such as piccolo or munc13. For example, in the exon4-5 mutant for bassoon the intensity of piccolo puncta was increased at endbulb of Held active zones, moreover,
munc13-1 expression was altered and Mover expression was downregulated (Mendoza Schulz et al., 2014). Are the levels of piccolo (or other proteins like munc13) in the gene-trap bassoon mutant changed on the level of individual synapses? In this context, it will be important to thoroughly discuss this issue and even better, provide data that show an unchanged or changed expression of other presynaptic proteins like piccolo or munc13.

>>We performed the requested analyses and added the data in Fig. EV1

Minor
-Please define, what exactly is meant by release competence of individual SVs
  >>We included our definition in section introduction on page 2
- The Western blots presented in Figs 3-6 should show the corresponding loading controls
  >>The blots of total and phosphorylated SNAP25 and PDE4 were performed on the same membrane and the signal for phosphor-antibody was directly normalized to total immunoreactivity at the same membrane. Thus, no loading control was necessary for these experiments. The data on Syn1 phosphorylation provided during the first submission was based on normalization to a loading control. Unfortunately, we lost the original data for blots of loading controls during the lab relocation that happened in 2020/2021. Therefore, we repeated these analyses and show now new analyses based multiplexed WBs, where total and phospho-Syn1 were detected on the same membrane. For analysis of total Syn1 abundance the total protein stain was used to get the most unbiased control of loading, which is now included in Fig 3 and 5. We adjusted the respective descriptions in the method section.
- The findings as well as the figures are very complex, I like the schemes of the pathways in each figure, but a summary of all findings would help to easier digest the key findings of the MS.
  >>We added a graphical summary of the results in Figure 7.
- Please revise carefully for typos etc. Page 3, please use the abbreviation for synaptic vesicles (SVs) consistently
  >>We apologise for all typos. We tried to improve this point and carefully corrected and proof-read the whole article.
- In Fig. 1 shift of the bars for ns, in general the figures could be revised for alignment of the panels
  >>We revised and rearranged all figures.
- Page 6, I guess button should be bouton?
  >>corrected
- Please refer to Fig. 3A in the main text
  >>corrected, page 7
- Page 7: "....we detected a significantly higher pSer551Syn1 labelling in synapses..." What is meant, more puncta or higher intensity? Larger puncta?
  >>We clarified this in the text: We detected a significantly higher pSer551Syn1 IF intensity in synapses
- Page 11: Bassoon should be abbreviated to bsn
  >>We prefer to abbreviate to Bsn as this is also the symbol for the mouse bassoon protein and gene.
- Page 21: "PDE4 immunoprecipitation and activity assay", should be activity
  >>corrected
- Please refer in the main text the Lentiviral particles production
  >>We added this- page 4

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Verstegen AM, Tagliatti E, Lignani G, Marte A, Stolero T, Atlas M, Corradi A, Valtorta F, Gitler D, Onofri F et al (2014) Phosphorylation of synapsin I by cyclin-dependent kinase-5 sets the ratio between the resting and recycling pools of synaptic vesicles at hippocampal synapses. *J Neurosci* 34: 7266-7280
Dear Dr. Fejtova

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the report from the referee who was asked to assess it (copied below).

As you will see, the referee is very positive about the study and supports publication.

Browsing through the manuscript myself, I noticed a few editorial things that we need before we can proceed with the official acceptance of your study.

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- Table EV1: Please add the legend to the file.

- Please add a callout to Figure 7. I also noticed that you used BioRender to generate the figure. Please ensure that you have a BioRender Publication license.

- TTX is an HHS select toxin, as you correctly indicated in the Author checklist. You might want to state that the use of limited amounts of TTX is not subject to special regulations in Germany in the materials and methods section.

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Martina Rembold, PhD
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***************

Referee #1:

In my opinion, the authors have addressed my comments adequately and no additional changes are necessary.
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- A specification of the experimental system investigated (e.g., cell line, species name).
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- An explicit mention of the biological and chemical entity(ies) that are being measured.
- An explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- The exact sample size (n) for each experimental group/condition, given as a number, not a range.
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