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Differential regulation of glycinergic and GABAergic nanocolumns at mixed inhibitory synapses

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1st Editorial Decision 15th Jan 2021

Dear Dr. Specht,

Thank you for your patience while your manuscript was peer-reviewed at EMBO reports. We have now received the full set of referee reports that is pasted below.

As you will see, all referees acknowledge that the data are interesting. However, they also all have several suggestions for how the study could be further improved and strengthened. I think that all concerns make sense and should be addressed. Please let me know in case you disagree, so that we can discuss the revisions further.

I would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of major 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.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further.

Regarding data quantification, please specify the number "n" for how many independent experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information must be provided in the figure legends. Please also include scale bars in all microscopy images.

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 providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.
2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

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.

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).
See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

3) 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: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- 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.

4) 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.

5) a complete author checklist, which you can download from our author guidelines <https://www.embopress.org/page/journal/14693178/authorguide>. Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

6) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines <https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>

7) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please remember to provide a reviewer password if the datasets are not yet public. The accession numbers and database should be listed in a formal "Data Availability" section placed after Materials & Method (see also https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please note that the Data Availability Section is restricted to new primary data that are part of this study. * Note - All links should resolve to a page where the data can be accessed. *
If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

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 at <https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

9) Our journal also 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 https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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

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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.

Kind regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

The manuscript "Differential homeostatic regulation of glycinergic and GABAergic nanocolumns at mixed inhibitory synapses" by Yang et al., investigates the nanoscale organization of mixed Glycinergic and GABAAergic inhibitory synapses. By combining a single molecule localization microscopy approach (dSTORM) with conventional epifluorescence imaging, the Authors analyze the relative distribution of 4 different molecular complexes: i) RIM1/2 (at the presynaptic side), ii) Gephyrin, iii) Glycine receptors (GlyRs) and iv) GABAA receptors (GABAARs) (at the postsynaptic side). They found that these proteins are largely organized in sub-synaptic domains (SSDs) and that such SSDs at the postsynaptic side clusters are preferentially aligned with the presynaptic ones thus leading to a nano-columnar modular structure. In addition, in spite of this organization in which the presynaptic elements matches the postsynaptic ones, this study shows that GlyRs and GABAARs SSDs poorly overlap, thus revealing a further level of complexity of inhibitory synaptic organization. Finally, they studied the activity-dependent changes of the aforementioned molecular complexes by manipulating voltage gated ion channels in vitro and found that GABAARs are more susceptible to rearrangements by increased network activity than GlyRs.
This is a solid a well-conceived study that advances our understanding of the molecular arrangement at the nanoscale level in particular during activity-dependent modifications. The study of mixed GlyR/GABAAR synapses offers an excellent opportunity to understand the arrangement of different molecular players coexisting at individual inhibitory synapses. In addition, this study employs high methodological standards in the use of dSTORM technique.

Major Points:
1) I understand that normalization of the plasticity changes observed in 4-AP to that in TTX is intended to maximize the differences observed. However I think that it would be more formally correct to study the differences in both conditions (TTX and 4-AP) from basal conditions. Is there any difference between basal conditions and TTX? For instance in Bannai et al., (2009), the application of TTX and 4-AP significantly reduces and increase the mobility of GABAA receptors, respectively, with respect to basal conditions. I think that the Authors should show whether TTX and 4-AP induces opposite changes. Related to this, what do the Authors expect when, instead of depression, a protocol inducing potentiation would be applied?

2) The Authors propose a mechanism for the differential plasticity of both GABAARs and GlyRs which involves the phosphorylation of S270 in gephyrin. However this conclusion is totally based on the different properties of the rbGPHN and mAb7a antibodies. While I do not question the plausibility of this hypothesis I think that an effort should be done to better demonstrate it. Did the author independently verified that mAb7a antibodies preferentially recognize phospho S270? There are several independent studies from different labs that by analyzing, for instance, the potentiation of GABAergic synapses found similar increase of gephyrin effect by using the mAb7a antibodies and antibody-free experiments. While these don't necessarily argue against that still mAb7a may show preference for phospho gephyrin, they do not support the concept that during inhibitory plasticity scaffold gephyrin is rather fixed while only the level of gephyrin phosphorylation will vary. So, I think that in order to strengthen their conclusions about the mechanisms of differential plasticity at mixed inhibitory synapses the Authors should also show the results with an antibody-free experiment.
3) In a related point, The Authors propose a very intriguing hypothesis that gephyrin in SSDs could be phosphorylated in S270 while outside it might be dephosphorylated. They also propose that the same mechanism should be implicated in the gephyrin interaction with GABAA receptors. However gephyrin molecule shows many other phosphorylation sites that modulate the stability of scaffold and receptors. Do the Authors think that the phosphorylation of S270 is selectively playing a pivotal role in the plasticity proposed here? Owing the fact that gephyrin undergoes heavy post-translational modifications I find difficult that phospho S270 would be the only determinant for such mechanism. Maybe the authors should better frame the role of phospho S270.

4) The Authors through the manuscript mention "homeostatic regulation/plasticity". The term "homeostatic" can be used to describe slightly different processes. What do the Authors precisely mean here? Could the lowering of GABAergic inhibition in response to increased network activity be considered an anti-homeostatic process? Please clarify.

5) I think that a final graphical scheme summarizing the major findings of this study could help the reader to better visualize the spatial organization of mixed inhibitory synapses in basal conditions and after regulation by activity. In particularly I would be interested in visualizing gehyrin distributed inside and outside SSDs.

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1) The sentence: "The identification of gephyrin SSDs and their alignment with pre-synaptic release sites in vivo points to a possible role for synaptic function" maybe needs some rephrasing. It seems that in this study the authors mainly worked ex-vivo. In addition I think that in order to find "a possible role for synaptic function" one doesn't strictly need an approach ex vivo, since many

studies (including this one) have inferred many possible role of nanoscale organization in synaptic function even neuronal cultures in vitro. I suggest to soften it.

2) The sentence: "The low level of overlap between GlyR....." reads SSDS instead of SSDs.

3) In the discussion the Authors state: "Modeling predicts that the positioning of receptors in front of vesicle release sites can increase the transmission efficacy at excitatory synapses (Haas et al., 2018, MacGillavry et a., 2013). Actually some modeling studies tackling the issue of the dependence of the synaptic current from the relative distance of releasing and postsynaptic sites (and receptors distribution) have also been performed at GABAergic synapses in, e.g. Pugh and Raman (2005), and Petrini et al., (2011) (although in these studies the releasing site-receptor distance analysis has not been related to SSDs).

The same comment could be pertinent in the sentence: "The relative vesicle load and binding affinity of the neurotransmitters therefore add to the complexity related to the nanoscale organization that controls the distance of GlyRs and GABAARs to the release site".

Referee #2:

Differential homeostatic regulation of glycinergic and GABAergic nanocolumns at mixed inhibitory synapses
Yang et al. characterized the nanoscale co-organization of glycinergic and GABAergic post-synaptic clusters at the spinal cord synapses. The main conclusions are that (i) both GABAR and GlyR subdomains are trans-synaptically aligned with pre-synaptic release site identified through RIM1/2 labelling. (ii) GlyR and GABAR subdomains barely overlap inside the PSD. (iii) Increase of the neuronal activity regulates only GABAR subdomains and (iv) activity regulation affect the phosphorylation level of gephyrin.
The overall paper is well done and based on solid and high level experiments and techniques. The scientific question is relevant for the community and the amount of experiments is well adapted to answer to the scientific questions. However, some conclusions would need some additional experiments to be validated.
Comments
1) The assessment that the 26 nm difference between the RIM to GlyR and the RIM to GABAR alignment comes from the labelling at the external part or at the internal part of the receptors need to be demonstrated or to be transferred to the discussion part. This 25 % difference between the trans-synaptic alignment could have other artifactual or physiological explanations.
2) When authors compared the GlyR-GABAR SSD overlap and conclude that they almost do not co-localize, they should show what is the 100% co-localization they can reach with the technique, by realizing, for example, dual color super-resolution on the same clustered protein. This will provide both the percentage of co-localization and the centroid-centroid distance.
3) The explanation that GlyR and GABAR SSDs do not co-localize but are both aligned at 80% with pre-synaptic RIM1/2 is difficult to understand. This result is important in terms of synaptic physiology, either the two types of SSD are separated and the authors should observe almost 50% of RIM1/2 clusters aligned with GlyR SSDs and the 50% with GABAR SSDs or there is some issue in the quantification. The organization of this specific synapse is one of the main key points of the paper, the authors could not only explain with words these surprising results but should find a way to demonstrate it.

4) The second paragraph of the discussion corresponds to new and quite important results with numbers which are just noticed without corresponding figure. This has to be documented in the result part.
5) The labelling with m7a antibody is quite strong and comparable to the total antibody, is it possible that this antibody recognized both phosphorylated and non-phosphorylated form of gephyrin? Is it possible to estimate the percentage of phosphorylated gephyrin at the PSD?

Referee #3:

In this work from Specht and colleagues the authors use dual color STORM based super resolution approaches to study the subsynaptic localisation of inhibitory receptors and their scaffold gephyrin. Importantly some of the work is done in native tissue. Interestingly and importantly (but perhaps not surprisingly given what is know for glutamate receptors), the authors show that receptors in subsynaptic domains (SSDs) form nanocolumns that align with Rim positive release sites. Of particular note in this study and which I think makes it particularly interesting, is that they use a spinal cord preparation where inhibitory synapses contain both GABAARs and glycine receptors. While both receptors use gephyrin as a scaffold, their affinity for gephyrin differs. This is therefore very nice preparation to look at nanoscopic distribution of two receptor types in the same synapses. Interestingly they find that GABAARs and glycineRs occupy different subsynaptic domains with only partial overlap. The authors also go on to show that (in agreement with previous work of receptor diffusion dynamics in the same preparation), GABAAR SSDs and glycineR SSDs behave differently upon neural activity changes, which may in part be depedent on gephyin phospho-regulation.

Overall, it's a very nice paper with lots of important and interesting results. I have only relatively minor comments as the work is the done to a high standard throughout.

Mostly my issues are around the activity dependence of SSDs and whether the study could have more dimensions added to it to add depth and impact. It would have been nice to know more about the underlying mechanisms. Given the robust read outs, could the authors not explore a bit more on the underlying signalling.

If gephyrin dephosphorylation is important, then what is the underlying phosphatase driving this? And does blocking that phosphatase block the loss of GABAAR SSDs?

Neural activity increases appear important but what about receptor activity? What happens to SSDs in neurons treated with strychnine or GABA? Does agonist treatment selectively only impact on the target receptor or is there cross talk?

Point-by-point response to the reviews

Referee #1:

The manuscript "Differential homeostatic regulation of glycinergic and GABAergic nanocolumns at mixed inhibitory synapses" by Yang et al., investigates the nanoscale organization of mixed Glycinergic and GABAAergic inhibitory synapses. By combining a single molecule localization microscopy approach (dSTORM) with conventional epifluorescence imaging, the Authors analyze the relative distribution of 4 different molecular complexes: i) RIM1/2 (at the presynaptic side), ii) Gephyrin, iii) Glycine receptors (GlyRs) and iv) GABAA receptors (GABAARs) (at the postsynaptic side). They found that these proteins are largely organized in sub-synaptic domains (SSDs) and that such SSDs at the postsynaptic side clusters are preferentially aligned with the presynaptic ones thus leading to a nano-columnar modular structure. In addition, in spite of this organization in which the presynaptic elements matches the postsynaptic ones, this study shows that GlyRs and GABAARs SSDs poorly overlap, thus revealing a further level of complexity of inhibitory synaptic organization. Finally, they studied the activity-dependent changes of the aforementioned molecular complexes by manipulating voltage gated ion channels in vitro and found that GABAARs are more susceptible to rearrangements by increased network activity than GlyRs.
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1) I understand that normalization of the plasticity changes observed in 4-AP to that in TTX is intended to maximize the differences observed. However I think that it would be more formally correct to study the differences in both conditions (TTX and 4-AP) from basal conditions. Is there any difference between basal conditions and TTX? For instance in Bannai et al., (2009), the application of TTX and 4-AP significantly reduces and increase the mobility of GABAA receptors, respectively, with respect to basal conditions. I think that the Authors should show whether TTX and 4-AP induces opposite changes. Related to this, what do the Authors expect when, instead of depression, a protocol inducing potentiation would be applied?

We have in fact compared the TTX and 4-AP treatments to the basal condition ('CTRL') in naive cultures. The CTRL condition in the pooled experiments was typically close to the TTX condition, indicating that the basal network activity is generally low (revised Fig. EV3 E-J). However, when we compare the results of individual experiments, the control condition can be quite variable, meaning that it is less well defined than the pharmacologically controlled TTX and 4-AP treatments (new Fig. EV5 C-E). Since we had to restrict the number of variables due to the time-

consuming nature of dual colour dSTORM imaging, we did not include the CTRL as a third experimental condition. The new control data are described in the results section (page 7). Together, the available data show that mixed inhibitory synapses undergo dynamic re-modelling in response to bi-directional changes in network activity, represented in our experiments by the TTX and 4-AP activity states (see comment 5).

2) The Authors propose a mechanism for the differential plasticity of both GABAARs and GlyRs which involves the phosphorylation of S270 in gephyrin. However this conclusion is totally based on the different properties of the rbGPHN and mAb7a antibodies. While I do not question the plausibility of this hypothesis I think that an effort should be done to better demonstrate it. Did the author independently verified that mAb7a antibodies preferentially recognize phospho S270? There are several independent studies from different labs that by analyzing, for instance, the potentiation of GABAergic synapses found similar increase of gephyrin effect by using the mAb7a antibodies and antibody-free experiments. While these don't necessarily argue against that still mAb7a may show preference for phospho gephyrin, they do not support the concept that during inhibitory plasticity scaffold gephyrin is rather fixed while only the level of gephyrin phosphorylation will vary. So, I think that in order to strengthen their conclusions about the mechanisms of differential plasticity at mixed inhibitory synapses the Authors should also show the results with an antibody-free experiment.

The specificity of the mAb7a antibody for an epitope that includes the phosphorylated serine residue S270 has been demonstrated beyond doubt (Kuhse et al. 2012 JBC; Hans Maric, personal communication; Khayenko et al. in preparation). Despite this unusual property, it is correct that mAb7a immunoreactivity is a reliable marker that in many cases accurately reflects synaptic gephyrin levels, suggesting that a large proportion of gephyrin at synapses is in fact phosphorylated at this (and possibly other) sites. Only recently, a number of reports have directly compared pS270 and total gephyrin immunoreactivities (e.g. Niwa et al. 2019 iScience; Lorenz-Guertin et al. 2019 Front Cell Neurosci; this study). In our previous work (Niwa et al. 2019) we used an mRFP-gephyrin knock-in mouse strain to visualise total synaptic gephyrin free from antibody labelling. The consensus that seems to emerge is that the two states of gephyrin can be independently regulated, and that pS270 phosphorylation correlates with $GABA_AR$ binding to the synaptic scaffold, in agreement with our observations. It should be kept in mind, however, that the effects (or their magnitude) may be different at mixed inhibitory synapses versus purely GABAergic synapses in the brain. We have discussed this issue more clearly in the revised version of the manuscript (page 12).

3) In a related point, The Authors propose a very intriguing hypothesis that gephyrin in SSDs could be phosphorylated in S270 while outside it might be dephosphorylated. They also propose that the same mechanism should be implicated in the gephyrin interaction with GABAA receptors. However gephyrin molecule shows many other phosphorylation sites that modulate the stability of scaffold and receptors. Do the Authors think that the phosphorylation of S270 is selectively playing a pivotal role in the plasticity proposed here? Owing the fact that gephyrin undergoes heavy post-translational modifications I find difficult that phospho S270 would be the only determinant for such mechanism.

Maybe the authors should better frame the role of phospho S270.

We agree with the reviewer that gephyrin phosphorylation and in fact post-translational modifications of gephyrin more generally provide multiple and complex mechanisms to regulate the organisation, dynamics, and plasticity of inhibitory synapses (e.g. Ghosh et al. 2019 Nat Commun). As mentioned above, these signalling pathways could also vary substantially at mixed versus GABAergic synapses (comment 2). As such, the pS270 site is only one proxy that can inform us about changes in gephyrin phosphorylation and/or conformation (see also Niwa et al. 2019). We have clarified this issue in the discussion (page 12, see also new Fig. 8).

4) The Authors through the manuscript mention "homeostatic regulation/plasticity". The term "homeostatic" can be used to describe slightly different processes. What do the Authors precisely mean here? Could the lowering of GABAergic inhibition in response to increased network activity be considered an anti-homeostatic process? Please clarify.

The term homeostatic is indeed not clear in this context, because it suggests that the induced changes are partially compensated for by the observed downstream effects. For clarity, we have removed the term homeostatic throughout the manuscript.

5) I think that a final graphical scheme summarizing the major findings of this study could help the reader to better visualize the spatial organization of mixed inhibitory synapses in basal conditions and after regulation by activity. In particularly I would be interested in visualizing gehyrin distributed inside and outside SSDs.

We have now added a graphical scheme as a new Figure 8. According to our model, the modulation of network activity induces plastic changes at mixed inhibitory synapses, whereby higher levels of activity are associated with the loss of $GABA_A$Rs from SSDs and the reduction of gephyrin phosphorylation. We believe that this simplified graphical model reflects the major findings of our work.

Minor points:

1) The sentence: "The identification of gephyrin SSDs and their alignment with pre-synaptic release sites in vivo points to a possible role for synaptic function" maybe needs some rephrasing. It seems that in this study the authors mainly worked ex-vivo. In addition I think that in order to find "a possible role for synaptic function" one doesn't strictly need an approach ex vivo, since many studies (including this one) have inferred many possible role of nanoscale organization in synaptic function even neuronal cultures in vitro. I suggest to soften it.

Done.

2) The sentence: "The low level of overlap between GlyR....." reads SSDS instead of SSDs.

Done.

3) In the discussion the Authors state: "Modeling predicts that the positioning of receptors in front of vesicle release sites can increase the transmission efficacy at excitatory synapses (Haas et al., 2018, MacGillavry et a., 2013). Actually some modeling studies tackling the issue of the dependence of the synaptic current from the relative distance of releasing and postsynaptic sites (and receptors distribution) have also been performed at GABAergic synapses in, e.g. Pugh and Raman (2005), and Petrini et al., (2011) (although in these studies the releasing site-receptor distance analysis has not been related to SSDs).

The two articles have been referenced accordingly.

The same comment could be pertinent in the sentence: "The relative vesicle load and binding affinity of the neurotransmitters therefore add to the complexity related to the nanoscale organization that controls the distance of GlyRs and GABAARs to the release site".

We have cited the two articles again in an earlier sentence in the same passage, where the impact of receptor localisation on signal transmission at inhibitory synapses is raised (page 11).

Referee #2:

Differential homeostatic regulation of glycinergic and GABAergic nanocolumns at mixed inhibitory synapses

Yang et al. characterized the nanoscale co-organization of glycinergic and GABAergic post-synaptic clusters at the spinal cord synapses. The main conclusions are that (i) both GABAR and GlyR subdomains are trans-synaptically aligned with pre-synaptic release site identified through RIM1/2 labelling. (ii) GlyR and GABAR subdomains barely overlap inside the PSD. (iii) Increase of the neuronal activity regulates only GABAR subdomains and (iv) activity regulation affect the phosphorylation level of gephyrin. The overall paper is well done and based on solid and high level experiments and techniques. The scientific question is relevant for the community and the amount of experiments is well adapted to answer to the scientific questions. However, some conclusions would need some additional experiments to be validated.

Comments

1) The assessment that the 26 nm difference between the RIM to GlyR and the RIM to GABAR alignment comes from the labelling at the external part or at the internal part of the receptors need to be demonstrated or to be transferred to the discussion part. This 25 % difference between the trans-synaptic alignment could have other artifactual or physiological explanations.

This section has been moved to the discussion as suggested by the reviewer (page 10). However, similar distance measurements using STORM imaging have been performed at excitatory synapses, in which the authors were able to determine subtle differences between N-terminal and C-terminal epitopes of pre- and post-synaptic proteins, illustrating the strength of super-resolution imaging to resolve molecular distances in the range of tens of nanometres (Dani et al. 2010 Neuron).

2) When authors compared the GlyR-GABAR SSD overlap and conclude that they almost do not co-localize, they should show what is the 100% co-localization they can reach with the technique, by realizing, for example, dual color super-resolution on the same clustered protein. This will provide both the percentage of co-localization and the centroid-centroid distance.

This is an important comment that addresses a critical point in our argument. Clearly, the low apparent overlap between the SSDs of GlyRs and $GABA_A$Rs (9%) is an underestimation, since it does not consider the synaptic receptors outside of the SSDs, nor the inherent stochasticity of antibody labelling and single fluorophore detection. We have therefore conducted a control experiment in which the same primary antibody (GlyR) was labelled with two secondary antibodies, one conjugated with Alexa 647, the other with Cy3B, as suggested by the reviewer (new Fig. 3E). Under these conditions, the SSD overlap area was 14%, which represents the maximal value for perfectly co-localised epitopes. In other words, the real co-localisation between GlyRs and $GABA_A$Rs is actually rather good at the synapse level, in line with image correlation analysis (ICA) that we have also performed on the rendered dual-colour dSTORM images (new Fig. 3C).

At the same time, our data demonstrate that the overlap between GlyRs and $GABA_A$Rs is not complete, meaning that the two receptors occupy partially overlapping but distinct domains (Fig. 3C,E). This is also confirmed by the fact that the centroid-centroid distance between GlyR and $GABA_A$R SSDs is larger than between dual-colour GlyR SSD (new Fig. 3F). The relevant section in the results has been thoroughly revised (page 5-6), as well Figure 3 and parts of the discussion (page 10-11).

3) The explanation that GlyR and GABAR SSDs do not co-localize but are both aligned at 80% with pre-synaptic RIM1/2 is difficult to understand. This result is important in terms of synaptic physiology, either the two types of SSD are separated and the authors should observe almost 50% of RIM1/2 clusters aligned with GlyR SSDs and the 50% with GABAR SSDs or there is some issue in the quantification. The organization of this specific synapse is one of the main key points of the paper, the authors could not only explain with words these surprising results but should find a way to demonstrate it.

New control experiments were performed and the data re-analysed in order to measure the true overlap between GlyRs and $GABA_A$Rs more accurately (new Fig. 3C-F). See our response to comment 2 above.

4) The second paragraph of the discussion corresponds to new and quite important results with numbers which are just noticed without corresponding figure. This has to be documented in the result part.

The molecule counting data have been moved to the results section (page 8) and the paragraph in the discussion has been shortened and rewritten accordingly.

5) The labelling with m7a antibody is quite strong and comparable to the total antibody, is it possible that this antibody recognized both phosphorylated and non-phosphorylated form of gephyrin? Is it possible to estimate the percentage of phosphorylated gephyrin at the PSD?

This issue has also been raised by reviewer 1 (see our response to point 2 of reviewer 1). Briefly, the antibody mAb7a is highly specific for phosphorylated gephyrin (Kuhse et al. 2012 JBC). However, it appears that a large proportion of gephyrin at synapses is phosphorylated, making the mAb7a antibody a reliable marker to probe synaptic gephyrin levels. As we and others have shown, relative changes of S270 phosphorylation versus total gephyrin can thus be estimated (e.g. Niwa et al. 2019 iScience; Lorenz-Guertin et al. 2019 Front Cell Neurosci; this study). An absolute quantification of the percentage of gephyrin phosphorylation at synapses, however, has not yet been achieved.

Referee #3:

In this work from Specht and colleagues the authors use dual color STORM based super resolution approaches to study the subsynaptic localisation of inhibitory receptors and their scaffold gephyrin. Importantly some of the work is done in native tissue. Interestingly and importantly (but perhaps not surprisingly given what is know for glutamate receptors), the authors show that receptors in subsynaptic domains (SSDs) form nanocolumns that align with Rim positive release sites. Of particular note in this study and which I think makes it particularly interesting, is that they use a spinal cord preparation where inhibitory synapses contain both GABAARs and glycine receptors. While both receptors use gephyrin as a scaffold, their affinity for gephyrin differs. This is therefore very nice preparation to look at nanoscopic distribution of two receptor types in the same synapses. Interestingly they find that GABAARs and glycineRs occupy different subsynaptic domains with only partial overlap. The authors also go on to show that (in agreement with previous work of receptor diffusion dynamics in the same preparation), GABAAR SSDs and glycineR SSDs behave differently upon neural activity changes, which may in part be depedent on gephyin phospho-regulation.

Overall, it's a very nice paper with lots of important and interesting results. I have only relatively minor comments as the work is the done to a high standard throughout.

Mostly my issues are around the activity dependence of SSDs and whether the study could have more dimensions added to it to add depth and impact. It would have been nice

to know more about the underlying mechanisms. Given the robust read outs, could the authors not explore a bit more on the underlying signalling.

If gephyrin dephosphorylation is important, then what is the underlying phosphatase driving this? And does blocking that phosphatase block the loss of GABAAR SSDs?

We have recently characterised gephyrin S270 phosphorylation downstream of cAMP signalling (Niwa et al. 2019 iScience). Interestingly, this pathway was PKA-independent and involved EPAC and the phosphatase PP1, which is in agreement with an earlier study (Kalbouneh et al. 2014 PLoS One). However, S270 phosphorylation may not necessarily be regulated in the same way in response to changes network activity (this study), since gephyrin can be the target of a wide variety of signalling processes that may involve other post-translational modifications (e.g. Ghosh et al. 2019 Nat Commun), and that are beyond the scope of this study. We have therefore discussed the implications and complexity of gephyrin regulation in more detail (page 12, see our response to reviewer 1, points 2 and 3). We have also supplied a graphical model to illustrate the way in which gephyrin phosphorylation could be regulated at SSDs (new Fig. 8).

Neural activity increases appear important but what about receptor activity? What happens to SSDs in neurons treated with strychnine or GABA? Does agonist treatment selectively only impact on the target receptor or is there cross talk?

This is an interesting question. It was indeed shown that receptor activity can directly affect the mobility of $GABA_A$Rs in a subunit-dependent manner (e.g. Gouzer et al. 2014 Mol Cell Neurosci), and it would be exciting to know if at mixed inhibitory synapses different receptors can be selectively mobilised by neurotransmitter binding. However, the aim of the present study was to investigate the indirect effect of excitatory network activity on the nanoscale organisation at inhibitory synapses. In order to distinguish activity-dependent remodelling from receptor activation itself, we focussed on the application of 4-aminopyridine to increase action potentials. We also carried out experiments in which we applied the receptor blockers strychnine and gabazine (page 7 of the revised manuscript). This treatment induces massive and synchronised synaptic activity in the culture, and leads to a loss of gephyrin S270 phosphorylation and a reduction of $GABA_A$R levels (see revised Fig. EV3), similar to what was observed with 4-AP. However, since activity-dependent and receptor-autonomous effects cannot be separated with strychnine/gabazine application, we did not use this paradigm subsequently in our dual-colour dSTORM experiments.

1st Revision - Editorial Decision 22nd Apr 2021

Dear Christian,

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from the referees that were asked to assess it. As you will see, referee 2 still has concerns that need to be addressed and incorporated before we can proceed with the official acceptance of your manuscript.

Please incorporate your response to referee 2's comments as discussed into the manuscript and change all relevant text passages accordingly. Here are referee 2's comments:
"The answer is fine to me. The authors probably have to add this new analysis into the paper and to clearly explain the technical limitation which can affect
the conclusion inside the discussion."

A few other editorial changes will also be required:

- please reduce the number of keywords to 5

- please add all author contributions, this is mandatory

- the conflict of interest statement needs a heading "Conflict of Interest"

- the reference format should be Harvard style and is currently not correct, only up to 10 authors should be listed and the journal names italicised

- Fig EV1 panels are not called out. Fig EV4C+D panels are not called out.
Fig 5 C-E panels are not called out. Please correct.

- please upload the source data as 1 file or folder per figure

- the methods section is called "Materials and Methods" and should follow the Discussion

I attach to this email a related ms file with comments by our data editors. Please address all comments in the final manuscript.

The synopsis image you sent is good, however, for our website we also need a short (1-2 sentence) summary of your findings and their significance and 2-3 bullet points highlighting key results.

The abstract needs to be written in present tense. Please let me know whether you agree with the following:

Super-resolution imaging has revealed that key synaptic proteins are dynamically organized within sub-synaptic domains (SSDs). To examine how different inhibitory receptors are regulated, we carried out dual-color direct stochastic optical reconstruction microscopy (dSTORM) of GlyRs and GABAARs at mixed inhibitory synapses in spinal cord neurons. We show that endogenous GlyRs and GABAARs as well as their common scaffold protein gephyrin form SSDs that align with pre-synaptic RIM1/2, thus forming trans-synaptic nanocolumns. Strikingly, GlyRs and GABAARs occupy different sub-synaptic spaces, exhibiting only a partial overlap at mixed inhibitory synapses. When

network activity is increased by 4-aminopyridine treatment, the GABAAR copy numbers and the number of GABAAR SSDs are reduced, while GlyRs remain largely unchanged. This differential regulation is likely the result of changes in gephyrin phosphorylation that preferentially occurrs outside of SSDs. The activity-dependent regulation of GABAARs versus GlyRs suggests that different signaling pathways control the receptors' sub-synaptic clustering. Taken together, our data reinforce the notion that the precise sub-synaptic organization of GlyRs, GABAARs and gephyrin has functional consequences for the plasticity of mixed inhibitory synapses.

I look forward to seeing a final version of your manuscript as soon as possible.
Please let me know if you have any questions.

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

The Authors, overall, answered my questions

Referee #2:

The authors answered to the vast majority of my requests, and realized supplemental experiments leading to modifications of a part of their conclusions on the GABAR/ GlyR co-localization. The impact of this paper could be quite high in the field of inhibitory synapses by revealing the pre-post co-organization and the non-overlap of GlyR/ GABAR SSD.
It is difficult with the level of co-localization observed with the dual labelled GlyR (14%) to interpret the GABAR/GlyR co-labeling and so the RIM/ post synaptic SSD too.
With the presented data, we cannot conclude if GlyR SSD co-localizes partially, not at all or almost fully (9% is close to 14%) with GABAR SSD.
Molecularly it means that (i) either they exclude each other, (ii) if they overlap it means that the density inside the SSD is not so high, letting some space for both channel types, (iii) if they co-localize almost entirely, this means they should use the same trapping mechanisms.
With such possibilities, we have to add the effect on pre-post localization, with different adhesion proteins in one case to co-organize RIM/GlyR and separately RIM/GABAR, or the same, both connected to various calcium channel type to have a differential release probability or not.
The general idea of such papers based on super-resolution is to make it possible the visualization of co-organization and to conclude on their activation or regulation as recently done for example by the Choquet's group (Goncalves et al. 2020 PNAS).
Here the results does not allow such interpretation. More experiments, analysis, representations and images has to be done to be able to conclude for one or the other type of co-organization.

Response to reviews

Please incorporate your response to referee 2's comments as discussed into the manuscript and change all relevant text passages accordingly. Here are referee 2's comments:
"The answer is fine to me. The authors probably have to add this new analysis into the paper and to clearly explain the technical limitation which can affect the conclusion inside the discussion."
The manuscript has been revised in line with the comments of reviewer 2. This includes the addition of pointillist images in Fig. 3A, the new analysis of the co-localization data in Fig. 3E, as well as the revision of the corresponding sections of the results, discussion, methods, and figure legends (highlighted in the submitted manuscript).

A few other editorial changes will also be required:

- please reduce the number of keywords to 5
DONE

- please add all author contributions, this is mandatory
DONE

- the conflict of interest statement needs a heading "Conflict of Interest"
DONE

- the reference format should be Harvard style and is currently not correct, only up to 10 authors should be listed and the journal names italicised
DONE

- Fig EV1 panels are not called out. Fig EV4C+D panels are not called out.
Fig 5 C-E panels are not called out. Please correct.
DONE

- please upload the source data as 1 file or folder per figure
Individual zip files for each figure (including EV figures) were uploaded.

- the methods section is called "Materials and Methods" and should follow the Discussion
DONE

I attach to this email a related ms file with comments by our data editors. Please address all comments in the final manuscript.
All comments have been addressed in an earlier response to the data editors.

The synopsis image you sent is good, however, for our website we also need a short (1-2

sentence) summary of your findings and their significance and 2-3 bullet points highlighting key results.
A summary statement (blurb) and bullet points are given on page 1 of the manuscript.

The abstract needs to be written in present tense. Please let me know whether you agree with the following:
Super-resolution imaging has revealed that key synaptic proteins are dynamically organized within sub-synaptic domains (SSDs). To examine how different inhibitory receptors are regulated, we carried out dual-color direct stochastic optical reconstruction microscopy (dSTORM) of GlyRs and GABAARs at mixed inhibitory synapses in spinal cord neurons. We show that endogenous GlyRs and GABAARs as well as their common scaffold protein gephyrin form SSDs that align with pre-synaptic RIM1/2, thus forming trans-synaptic nanocolumns. Strikingly, GlyRs and GABAARs occupy different sub-synaptic spaces, exhibiting only a partial overlap at mixed inhibitory synapses. When network activity is increased by 4-aminopyridine treatment, the GABAAR copy numbers and the number of GABAAR SSDs are reduced, while GlyRs remain largely unchanged. This differential regulation is likely the result of changes in gephyrin phosphorylation that preferentially occurs outside of SSDs. The activity-dependent regulation of GABAARs versus GlyRs suggests that different signaling pathways control the receptors' sub-synaptic clustering. Taken together, our data reinforce the notion that the precise sub-synaptic organization of GlyRs, GABAARs and gephyrin has functional consequences for the plasticity of mixed inhibitory synapses.
The abstract has been updated accordingly.

2nd Revision - Editorial Decision 30th Apr 2021

Dr. Christian Specht
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Dear Dr. Specht,

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

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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Best wishes,
Esther

Esther Schnapp, PhD
Senior Editor
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Corresponding Author Name: Christian G Specht
Journal Submitted to: EMBO Reports
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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- ➔ the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ➔ figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- ➔ graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- ➔ if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be iustified
- ➔ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

- ➔ a specification of the experimental system investigated (eg cell line, species name).
- ➔ the assay(s) and method(s) used to carry out the reported observations and measurements
- ➔ an explicit mention of the biological and chemical entity(ies) that are being measured.
- ➔ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- ➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- ➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- ➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
- ➔ definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Based on our previous experience with imaging-based measurements of pharmacological treatments, we consider that n=20-30 cells per experimental condition provides a good read-out of biologically relevant effects. This typically represents hundreds or thousands of synapses per condition. In the case of two-colour dSTORM, the number of recordings was more restricted due to the complexity of the technique, which is why the number of experimental conditions was kept to a minimum. In this case, the number of synapses was considered as the relevant sample size (approx. n=50-100 per condition).
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Not applicable
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Most experiments were repeated three times or at least twice. If the results of the independent experiments were qualitatively the same, the experiments were pooled, to provide a more representative result and enhance the statistical weight of the data. No experiments were excluded from the analysis based on this criterion.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Not applicable
For animal studies, include a statement about randomization even if no randomization was used.	Not applicable
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No such steps were taken.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Not applicable
5. For every figure, are statistical tests justified as appropriate?	No statistical tests were applied in Figure 1 (qualitative interpretation of images) and Figure 8 (schematic drawing). A range of test was used in all the other figures (Fig. 2-7, EV1-5), depending on the type of data that are compared (pairwise or multiple comparison tests, parametric or non-parametric).
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Data were tested for normality with the D'Agostino & Pearson normality test. Where the data passed the normality test, the analysis was done using Student's t-test or one-way ANOVA followed by Tukey's post-hoc test. Where the data were not normally distributed, statistical analysis was done using non-parametric Mann-Whitney U-test (MW), Kolmogorov-Smirnov test (KS), multi-comparison Kruskal-Wallis test (KW) for unpaired data and Friedman test for paired data, followed by a Dunn's post-hoc multiple comparison test.

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Is there an estimate of variation within each group of data?	Variation was not formally compared, however, the majority of data are shown as cumulative distributions that reflect the range and variability of the dataset.
Is the variance similar between the groups that are being statistically compared?	Not tested.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All antibodies used in this study are listed in the methods section, including their supplier and catalogue number.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	No cell lines were used in this study other than dissociated primary rat spinal cord neuron cultures.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	All procedures using animals follow the regulations of the French Ministry of Agriculture and the Direction départementale des services vétérinaires de Paris (Ecole Normale Supérieure, animalerie des rongeurs, license B 75-05-20). Primary spinal cord neurons were prepared from embryonic Sprague Dawley rats on embryonic day E14. For the data shown in Figure 1, adult male mice (C57BL/6J, 10 weeks old) were deeply anesthetized with pentobarbital, and intracardially perfused with 4% PFA and 0.1% glutaraldehyde in PBS. Animals were purchased from Janvier (France) and housed at the animal facility of the Institut de Biologie de l'Ecole Normale Supérieure (IBENS).
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Not applicable
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	The guidelines were consulted and followed where applicable.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not applicable
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not applicable
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not applicable
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not applicable
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not applicable
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not applicable
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not applicable

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The raw imaging data of this publication have been deposited in the Zenodo database (https://zenodo.org) and assigned the unique identifier DOI: 10.5281/zenodo.4545025.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	In addition to the raw imaging data (repository submission, see above), the source data for all Figures are submitted to EMBO Reports.
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21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	Not applicable

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No