EMBO reports

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SNARE and regulatory proteins induce local membrane protrusions to prime docked vesicles for fast calcium-triggered fusion.

Tanmay A.M. Bharat, Jorg Malsam, Wim J.H. Hagen, Andrea Scheutzow, Thomas H. Sollner and John A.G. Briggs

Corresponding authors: John A.G. Briggs, European Molecular Biology Laboratory, Heidelberg, Thomas H. Sollner, Heidelberg University

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Editorial Decision:	15 August 2013
Correspondence:	30 August 2013
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Revision received:	18 December 2013
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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Esther Schnapp

1st Editorial Decision 15 August 2013

Thank you for the submission of your manuscript to our journal. It has been sent to three referees, and so far we have received reports from two of them. As both referees feel that the manuscript is interesting and recommend that you should be given a chance to revise it, I would like to ask you to begin revising your manuscript according to the referees' comments. Please note that this is a preliminary decision made in the interest of time, and that it is subject to change should the third referee offer very strong and convincing reasons for this. I will be in touch as soon as we receive the final report on your manuscript.

As you will see, while both referees acknowledge that the findings are potentially interesting, they also raise several important concerns that would need to be successfully addressed for publication of the study here. Both referees point out that the discrepancy with earlier findings need to be addressed experimentally, and referee 3 further remarks that the functional significance of the membrane protrusions for vesicle fusion needs to be strengthened. Both referees also ask for different control experiments.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as mentioned above and in their reports) must be fully addressed and their suggestions taken on board. 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.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Also, the revised manuscript may not exceed 30,000 characters (including spaces and references) and 5 main plus 5 supplementary figures, which should directly relate to their corresponding main figure. Parts of the materials and methods can be moved to the supplementary information, but please note that the materials and methods essential for the understanding of the experiments described in the main manuscript file must remain in the main methods section.

Regarding data quantification, can you please specify the number "n" for how many 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.

We recently decided to offer authors the possibility to submit "source data" with their revised manuscript that will be published in a separate supplemental file online along with the accepted manuscript. If you would like to use this opportunity, please submit the source data (for example entire gels or blots, data points of graphs, additional images, etc.) for all or your key experiments together with the revised manuscript.

When submitting your revised manuscript, please include:

A Microsoft Word file of the manuscript text, editable high resolution TIFF or EPS-formatted figure files, a separate PDF file of any Supplementary information (in its final format), a letter detailing your responses to all the referee comments, and a two sentence summary of your findings and their significance.

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

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.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

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

REFEREE REPORTS:

Referee #1:

This is an interesting paper that describes the cryo-EM observation of the membrane protrusion induced by neuronal membrane fusion machinery. Authors reconstituted v-SNARE into SUV and t-SNARE into GUV. They also included regulatory proteins synaptotagmin 1 in SUV and complexin and Munc18-1 in solution. The authors observed the protrusion on t-SNARE-carrying GUV with

cryo EM, which is new and interesting in view of the recent lipid-dimple model by Kozlov and coworkers. It is also interesting that such protrusions were not observed in previous two EM studies by the Brunger group and the Jahn group. If the observation turns out to be true the results are certainly a step forward in the field. Here are my concerns and suggestions:
(1) It appears that when there is a protrusion the inter-membrane gap is still in the range of 20-30 nanometers for some of them. I am wondering if such a large inter-membrane gap is possible when SNAREs are partially assembled.
(2) The authors should repeat the experiments of Brunger and Jahn groups to explain why they observed the protrusion while the other groups did not.
(3) The authors must carry out the control experiment in the absence of synaptotagmin 1.
(4) I am wondering if they are observing simply the mechanical pulling of SUV that are diffusing away from GUV surface. I would recommend repeating the experiment under slow cooling conditions.

Referee #3:

The mechanisms by which proteins fuse membranes in diverse membrane remodeling processes remain to be clarified. Some of the existing hypotheses have suggested that fusion-inducing proteins generate membrane protrusions of high positive curvature. Release of this membrane stress upon fusion drives fusion process. In this study, Bharat et al. provide an important experimental evidence for the existence and potential functional importance of the membrane protrusions in an in vitro reconstituted system mimicking synaptic vesicle fusion. The authors apply cryo-electron tomography imaging to examine the morphology of the contact between proteoliposomes with SNAREs and regulatory proteins that are set to undergo a robust fusion (lipid mixing) upon application of Ca2+. The pre-fusion protrusions of the proteoliposome mimicking the plasma membrane observed in many contact sites depend on the formation of a trans-SNARE complex and are hypothesized to represent a high-energy structure primed for fast fusion upon Ca2+-induced release of the complexin clamp. While the paper presents an exciting and potentially important finding that will be of interest for the EMBO J. readership, the study has important limitations.

Specific comments.
1) I believe the work needs to strengthen the evidence that membrane protrusions are important for fusion. Higher frequency of the protrusions for the liposomes with all components present is the only and indirect evidence for this conclusion. I think the work needs additional correlations between fusion and protrusions. For instance, all analysis is carried out at a single time point, 1 min after mixing the liposomes. Are there any time points where changes in the frequency of protrusions prior to calcium application correlate with changes in the extent of a fast increase in lipid mixing after Ca2+ application? If protrusions represent a primed state in which the membranes wait Ca2+, should we expect an increase in the frequency of protrusions with time after liposome mixing?
2) Protrusions are formed only by GUV mimicking plasma membrane. Asymmetrical deformation can reflect either asymmetrical distribution of protein machinery between the membranes or different deformability of lipid bilayer in SUV and GUV. This question can be addressed by changing the distribution of the proteins between SUV and GUV. Can the use of GUV in this work explain why protrusions were not observed in earlier studies that utilized only SUV (25,26)?
3) The authors conclude that the proteins "stabilize the membrane in a high energy state". This would suggest that protrusions are long-living structures. However, lifetime of the protrusions is not explored here. Can the authors exclude a possibility that protrusions represent short-living deformations caught by freezing? If not, this should not be stated in the paper.
4) The authors may want to cite the first (I think) paper suggesting that exocytotic fusion starts with the functionally important dimpling of plasma membrane towards the vesicle (Monck, J. R., and J. M. Fernandez. 1992. The exocytotic fusion pore. J. Cell Biol. 119:1395-1404.).
5) In the abstract I suggest to replace "target membrane" with "plasma membrane". The meaning of the term "target" is unclear in the case of fusion machinery distributed between two membranes.

Correspondence - editor 30 August 2013

We have finally received the missing referee report for your manuscript, which I paste below. It seems that the referee only asks for minor revisions, and I therefore think that all his/her concerns should be addressed.

REFEREE REPORT:

Referee #2:

This is an exciting paper reporting membrane protrusions on relatively flat membranes towards docked vesicles as an intermediate in the membrane fusion process. There is great interest in sorting out what the intermediates are in the fusion process and here the cryoEM approach gives a beautiful picture of what is going on.
We and others have hypothesised a 'high curvature' or rather an intermediate that would be under curvature stress. Initially we hypothesis this for the insertion of synaptotagmin C2 domains and then we extended this to include the SNARE proteins which will likely participate in the same intermediate. The present study shows that SNARE proteins and synaptotagmin (in the absence of calcium) can generate this. One would hypothesis that the curvature would be more extreme in the presence of synaptotagmin+Calcium where the C2 domains are inserted in membranes. It would just be a small step potentially for the authors to get further intermediates in fusion with the setup the author has, as they can almost certainly spray calcium onto the samples as they plunge into the freezer.
Perhaps a more feasible control that should be done would be to try the same reactions in the absence of synaptotagmin in the SUVs, as we sometimes found that some of the C2 domains would partially insert even in the absence of calcium, in other words they were already poised for full insertion.
I am not sure I completely understand the authors proposal at the end of their discussion where the role of calcium is simply to release the complexin block. They ought to take into account that an establioshed role of calcium is to fully insert the C2 domains.
Finally I wonder what the authors would say about the 1 SNARE hypothesis and whether protrusion could form under these conditions.

1st Revision - authors' response 18 November 2013

Referee #1:

This is an interesting paper that describes the cryo-EM observation of the membrane protrusion induced by neuronal membrane fusion machinery. Authors reconstituted v-SNARE into SUV and t-SNARE into GUV. They also included regulatory proteins synaptotagmin 1 in SUV and complexin and Munc18-1 in solution. The authors observed the protrusion on t-SNARE-carrying GUV with cryo EM, which is new and interesting in view of the recent lipid-dimple model by Kozlov and coworkers. It is also interesting that such protrusions were not observed in previous two EM studies by the Brunger group and the Jahn group. If the observation turns out to be true the results are certainly a step forward in the field. Here are my concerns and suggestions:
(1) It appears that when there is a protrusion the inter-membrane gap is still in the range of 20-30 nanometers for some of them. I am wondering if such a large inter-membrane gap is possible when SNAREs are partially assembled.

The distance between membranes can appear larger than it is for some docked SUVs when the slice through the tomographic reconstruction that is presented in a figure does not section that SUV at the point of closest approach. Nevertheless, the reviewer is correct that some of the docked SUVs are up to 20 nm from the GUV membrane. SUVs at distances larger than approximately 20 nm from the GUV were not scored as "docked" in the visual analysis.

Based on theoretical calculations and biophysical data, we can estimate the maximum distance between membranes for a partially assembled SNARE complex in a number of ways:

1. A partially zippered SNARE has an assembly breakpoint at the ionic layer of the SNARE motif, which corresponds to R56 in VAMP2 and Q226 in syntaxin 1 (Gao et al. 2012, Science; Min et al., 2013, Nature Communications). This leaves aa 57-94 preceding the transmembrane domain of VAMP2 and aa 227-265 preceding the transmembrane domain of syntaxin 1 to bridge the two membranes. Assuming that a single aa extends an unstructured peptide by 0.37 nm, these regions of VAMP2 and syntaxin1 cover a stretch of 13.5 and 13.9 nm, respectively, giving a maximum distance of approximately 27 nm.

2. If instead we assume that syntaxin is assembled together with SNAP-25 into a t-SNARE complex, adopting an alpha helical conformation, syntaxin 1 aa (227-265) would only cover a distance of 5.7 nm (one helical turn = 0.54 nm). In this case the half zippered SNAREpin (e.g. further stabilized by complexin) would cover a maximum distance of 19.2 nm.

3. Experimental measurements probing single SNARE complex unzipping reveal that the unzipped C-terminal half of the SNARE motif covers around 10 nm (Min et al., 2013, Nature Communications). Adding the unstructured linker regions of VAMP2 (aa 81-94) and of syntaxin 1 (aa 251- 265) would increase the distance to approximately 20 nm. We consider it unlikely that syntaxin beyond layer +8 is structured in a prefusion state, but if it adopted an alpha helical conformation a distance of 18 nm could be covered.

These estimates suggest that distances of up to 20 nm could be bridged by partially assembled SNARE complexes.

We have added this clarification in the Supplementary Materials and Methods:

> "Docking sites in large-scale cryoET data were identified by visual inspection of tomograms. SUVs that were closer than ~ 20 nm from the GUV membrane were considered as docked."

(2) The authors should repeat the experiments of Brunger and Jahn groups to explain why they observed the protrusion while the other groups did not.

The experiments of the Brünger and Jahn groups mixed SUVs carrying t-SNAREs with SUVs carrying v-SNAREs. Our experiments in contrast, made use of GUVs containing t-SNAREs. As requested by the reviewer we have therefore carried out experiments using SUV-SUV mixtures. Consistent with the results reported by the Brünger and Jahn groups, we did not observe protrusions. These results suggest to us that the protrusions are not observed in the SUV-SUV systems due to the high tension in SUV membranes. The GUV membrane, like the plasma membrane that is the real target membrane, is more flexible. The results of this experiment are shown in Figure S5A-B, and are described in the text as follows:

> "This protruding target membrane intermediate was not observed in the recent cryoEM studies of SNARE mediated fusion in SUV mixtures in the absence of complexin (Diao et al, 2012; Hernandez et al, 2012). In these studies either close contact between SUVs, or an extended contact region, was suggested to represent the pre-fusion intermediate. We hypothesize that protrusions are less easily visualized in SUVs than in GUVs due to the higher membrane curvature and membrane tension in the SUVs. We carried out an additional experiment where v- and t-SNARE proteins were each reconstituted into separate populations of SUVs that were then mixed in the presence of complexin and Munc18-1 and imaged by cryoEM and cryoET (Figure S5A). Consistent with the recent studies, we did not observe the formation of protrusions at contact sites between SUVs (Figure S5A). We found that in this reaction, the similar sizes of the two SUV populations prevented discrimination of contacts between v- and t-SNARE containing SUVs from contacts between SUVs that both contain t-SNAREs or both contain v-SNAREs."

We also would like to refer the reviewer to our response to Reviewer 3 point 2.

(3) The authors must carry out the control experiment in the absence of synaptotagmin 1.

The same control experiment was suggested by reviewer 2. As requested, we have carried out this experiment and described the results in the manuscript as follows:

> "In a control experiment in which synaptotagmin 1 was omitted, only a background level of docking was observed (~8%, Table S4). This is consistent with previous studies indicating that synaptotagmin 1 is required to mediate vesicle docking (de Wit et al, 2009; Malsam et al, 2012; Parisotto et al, 2012)."

(4) I am wondering if they are observing simply the mechanical pulling of SUV that are diffusing away from GUV surface. I would recommend repeating the experiment under slow cooling conditions.

It is not possible to carry out cryo-electron microscopy under slow cooling conditions, since rapid cooling is essential to avoid the formation of crystalline ice that prevents cryo-electron microscopy imaging. The reviewer suggests that docking, followed by freezing, would lead to a mechanical pulling force and induce the protrusion. This is a reasonable hypothesis. However, our data rule out this hypothesis. We have clarified this point in the text.

> "These experiments indicate that docking of vesicles to the target membrane, though mediated by synaptotagmin 1, it is not sufficient to induce protrusions on the target membrane. (This observation rules out the possibility that protrusions are formed mechanically by docked SUVs being pulled away from the GUV membrane during sample preparation: in this case one would expect protrusion formation to correlate with docking.) Formation of the protruding membrane intermediate is dependent on formation of a trans-SNARE complex."

In addition, we note that in new experiments we inverted the system, reconstituting VAMP2 and synaptotagmin 1 into GUVs and the t-SNARE proteins into SUVs: in this case we did not observe membrane protrusions (please also see comment to point 2 of reviewer #3). This observation further supports that protrusions do not result from mechanical pulling.

Referee #2:

This is an exciting paper reporting membrane protrusions on relatively flat membranes towards docked vesicles as an intermediate in the membrane fusion process. There is great interest in sorting out what the intermediates are in the fusion process and here the cryoEM approach gives a beautiful picture of what is going on.

We and others have hypothesised a 'high curvature' or rather an intermediate that would be under curvature stress. Initially we hypothesis this for the insertion of synaptotagmin C2 domains and then we extended this to include the SNARE proteins which will likely participate in the same intermediate. The present study shows that SNARE proteins and synaptotagmin (in the absence of calcium) can generate this. One would hypothesis that the curvature would be more extreme in the presence of synaptotagmin+Calcium where the C2 domains are inserted in membranes. It would just be a small step potentially for the authors to get further intermediates in fusion with the setup the author has, as they can almost certainly spray calcium onto the samples as they plunge into the freezer. Perhaps a more feasible control that should be done would be to try the same reactions in the absence of synaptotagmin in the SUVs, as we sometimes found that some of the C2 domains would partially insert even in the absence of calcium, in other words they were already poised for full insertion.

Spraying calcium on the reaction is an excellent suggestion, but will require a careful experimental setup to catch in a reproducible and convincing manner transient fusion intermediates, which is beyond the scope and timeline of this revision. We agree with the reviewer that carrying out the reaction in the absence of synaptotagmin is the more feasible control. Reviewer 1 also requested that we carry out the control reaction in the absence of synaptotagmin. We have done this experiment

and included the results in the manuscript, please see our response to Reviewer 1 point 3.

I am not sure I completely understand the authors proposal at the end of their
discussion where the role of calcium is simply to release the complexin block. They ought to take into account that an establioshed role of calcium is to fully insert the C2 domains.

We apologize for this confusion, and have modified the sentence in the discussion to reflect the correct role of Ca^{2+} activation on the system.

> "In this primed state, activation with Ca^{2+} induces the insertion of the C2 domains of synaptotagmin 1 into the target membrane. This releases the complexin block, allowing the system to go energetically downhill towards full membrane fusion."

Finally I wonder what the authors would say about the 1 SNARE hypothesis and whether protrusion could form under these conditions.

Unfortunately, we are not able to identify individual SNARE complexes by cryo-EM and without these experimental data, we do not feel comfortable to speculate on this issue.

Referee #3:

The mechanisms by which proteins fuse membranes in diverse membrane remodeling processes remain to be clarified. Some of the existing hypotheses have suggested that fusion-inducing proteins generate membrane protrusions of high positive curvature. Release of this membrane stress upon fusion drives fusion process. In this study, Bharat et al. provide an important experimental evidence for the existence and potential functional importance of the membrane protrusions in an in vitro reconstituted system mimicking synaptic vesicle fusion. The authors apply cryo-electron tomography imaging to examine the morphology of the contact between proteoliposomes with SNAREs and regulatory proteins that are set to undergo a robust fusion (lipid mixing) upon application of Ca2+. The pre-fusion protrusions of the proteoliposome mimicking the plasma membrane observed in many contact sites depend on the formation of a trans-SNARE complex and are hypothesized to represent a high-energy structure primed for fast fusion upon Ca2+-induced release of the complexin clamp. While the paper presents an exciting and potentially important finding that will be of interest for the EMBO J. readership, the study has important limitations.

Specific comments.
1) I believe the work needs to strengthen the evidence that membrane protrusions are important for fusion. Higher frequency of the protrusions for the liposomes with all components present is the only and indirect evidence for this conclusion. I think the work needs additional correlations between fusion and protrusions. For instance, all analysis is carried out at a single time point, 1 min after mixing the liposomes. Are there any time points where changes in the frequency of protrusions prior to calcium application correlate with changes in the extent of a fast increase in lipid mixing after Ca2+ application? If protrusions represent a primed state in which the membranes wait Ca2+, should we expect an increase in the frequency of protrusions with time after liposome mixing?

As requested by the reviewer, we have carried out the fusion reaction at different time points, finding that under conditions where lower fusion is observed, fewer protrusions are observed, and vice versa. The results of these experiments support our conclusions and are shown in Figure S4. They are described in the text as follows:

> "We next asked whether at time points where priming was incomplete there was a corresponding lower number of protrusions. We therefore carried out an experiment in which a mixture of v-SNARE SUVs and t-SNARE GUVs was incubated on ice for different time periods in the presence of complexin. Subsequently we measured the extent of docking using a previously described liposome sedimentation assay (Parisotto et al, 2012) (Figure S4A, Table S4), and measured the degree of fusion upon Ca^{2+} addition (Figure S4B-C). Further, we collected cryoET data (Figure S4D) at the different time points and "blindly" quantified the degree of protrusion formation. Combining these data, we found that between 1 minute, 5 minute, and 60 minute time points, the amount of docking and of protrusion formation increased (Figure S4E). Simultaneously, the number

> of vesicles primed for fusion, as indicated by the extent of fusion upon Ca^{2+} addition, also increased (Figure S4C). Therefore, where priming was incomplete, a lower number of protrusions was observed."

Our data show that in the primed fusion state, the target membrane displays a protrusion. We show that the formation of protrusions correlates (in terms of protein components and now in terms of timing), with priming. These data provide strong evidence that when the fusion machinery assembles a primed state, it induces a protrusion in the target membrane. Strictly, however, this kind of study cannot address whether the protrusions are "important for fusion", because we cannot design a primed state with no protrusion to see how it behaves.

2) Protrusions are formed only by GUV mimicking plasma membrane. Asymmetrical deformation can reflect either asymmetrical distribution of protein machinery between the membranes or different deformability of lipid bilayer in SUV and GUV. This question can be addressed by changing the distribution of the proteins between SUV and GUV. Can the use of GUV in this work explain why protrusions were not observed in earlier studies that utilized only SUV (25,26)?

We have carried out two experiments to address this. Firstly, please see our response to Reviewer 1 point 2, where we carried out new experiments using only SUVs and included these in the manuscript. We did not observe protrusions, suggesting that the use of GUVs (which better mimic the plasma membrane) is a critical factor. Secondly, as suggested by the reviewer we have carried out the experiment changing the distribution of the proteins between SUVs and GUVs. These results are shown in the new Figure S5C-E and are described in the text as follows:

> "We therefore repeated the reaction with all the components included (as in Figure 1C), but reconstituted the t-SNARE proteins into the SUVs and the v-SNARE proteins into the GUVs (Figure S5C). Although fusion priming was efficient in this reaction (Figure S4E), the formation of protrusions was inefficient (Figure S5D, Table S4). These two observations together suggest that protrusion formation is specific to the t-SNARE containing membrane, and is hindered by the high tension of SUVs."

3) The authors conclude that the proteins "stabilize the membrane in a high energy state". This would suggest that protrusions are long-living structures. However, lifetime of the protrusions is not explored here. Can the authors exclude a possibility that protrusions represent short-living deformations caught by freezing? If not, this should not be stated in the paper.

The data we have added in response to this reviewer's point 1 shows that the number of protrusions increases between 1, 5 and 60 minutes pre-incubation on ice. This excludes that they are short-lived intermediates caught by the rapid freezing. We note that the fact that large numbers of protrusions were observed in our original data already argued against them existing on a timescale shorter than seconds.

4) The authors may want to cite the first (I think) paper suggesting that exocytotic fusion starts with the functionally important dimpling of plasma membrane towards the vesicle (Monck, J. R., and J. M. Fernandez. 1992. The exocytotic fusion pore. J. Cell Biol. 119:1395-1404.).

A citation to this paper has been added as requested.

> "These data suggest that formation of a primed pre-fusion state is associated with a protruding membrane intermediate. Such protrusions have been hypothesized to be important for SNARE-mediated membrane fusion (Monck & Fernandez, 1992)."

5) In the abstract I suggest to replace "target membrane" with "plasma membrane". The meaning of the term "target" is unclear in the case of fusion machinery distributed between two membranes.

Done as suggested.

2nd Editorial Decision 09 December 2013

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed reports from the referees that were asked to assess it. As you will see, both referees are happy with the revised version and support its publication. Only referee 3 has one very minor suggestion that I would like you to incorporate before we can proceed with the official acceptance of your manuscript.

I noticed that the manuscript uses the wrong reference style. I may have forgotten to mention this in my last email, I apologize. Can you please change it to the numbered EMBO reports style? It also is a little strange that the main manuscript file only contains 2 main figures and the supplementary file 5 supplementary figures. The supplementary figures should be directly related and linked to their corresponding main figures at EMBO reports. Would it be possible to move some SF to the main manuscript? The character count is currently 25.000 and can go up to 30.000 for revised manuscripts, so you still have space. The main Materials and Methods section could also be expanded.

Can you please specify the error bars and number of experiments for SF 4A,B and SF 5B in the figure legends? Thank you.

From January onwards, we will include synopses with all our scientific reports online. Can you please send us a 1-2 sentences that summarize the main findings and their significance, and also 2-3 bullet points that highlight the key findings and are complementary to the abstract for the synopsis? We also include a synopsis image that should represent the key findings. We could either use the image in figure 2D, or, if you prefer, you can send us an illustration or new image for the synopsis image. The size of the synopsis image is 211 pixels wide x 157 pixels high. Please let me know if you have questions.

I look forward to seeing a new revised version of your manuscript as soon as possible.

REFEREE REPORTS:

Referee #1:

The authors addressed my concerns extensively with the new experimental data. I am happy with the revision. The paper can now be published in EMBO Reports.

Referee #3:

The revised paper is an excellent contribution to a very active field of research. Added data have fully covered my concerns/comments and I recommend publication of this exciting work. I have only a minor suggestion. I suggest to modify the added citation to the Monck & Fernandez paper to replace "... to be important for SNARE-mediated membrane fusion..." with "... to be important for exocytosis..." or "... to be important for intracellular membrane fusion..." . It looks like SNAREs have not been mentioned in this Monck & Fernandez paper.

2nd Revision - authors' response 18 December 2013

Thanks very much for consideration of our manuscript. We have dealt with all the points raised in your email. A point-by-point response is below (our responses in blue).

With thanks and best wishes

John

Dear John,

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed reports from the referees that were asked to assess it. As you will see, both referees are happy with the revised version and support its publication. Only referee 3 has one very minor suggestion that I would like you to incorporate before we can proceed with the official acceptance of your manuscript.

I noticed that the manuscript uses the wrong reference style. I may have forgotten to mention this in my last email, I apologize. Can you please change it to the numbered EMBO reports style?

Sorry about this, we have now used the endnote style provided on the EMBO Reports website to reformat all the references.

It also is a little strange that the main manuscript file only contains 2 main figures and the supplementary file 5 supplementary figures. The supplementary figures should be directly related and linked to their corresponding main figures at EMBO reports. Would it be possible to move some SF to the main manuscript?

We had originally preferred to keep the old figures S4 and S5 in the supplement to keep readers attention focused on the key experiments, which are in figures 1 and 2. We have now moved old Figure S4 and Table S1 into the main text as the new Figure 3 and Table 1 respectively. We refer to Figures S1-3 in the legends of the main figures. While there is no direct reference to the old Figure S5 (new S4) in a main text figure legend, it is referenced from the main text and we prefer if possible to keep it in the supplement.

The character count is currently 25.000 and can go up to 30.000 for revised manuscripts, so you still have space. The main Materials and Methods section could also be expanded.

We have expanded the main Materials and Methods by a few sentences to make them more self-explanatory. However, many of the experimental procedures employed have been previously reported and in these cases we have referenced the original publications and included further details in the supplement.

Can you please specify the error bars and number of experiments for SF 4A,B and SF 5B in the figure legends? Thank you.

We have added this information as requested.

From January onwards, we will include synopses with all our scientific reports online. Can you please send us a 1-2 sentences that summarize the main findings and their significance, and also 2-3 bullet points that highlight the key findings and are complementary to the abstract for the synopsis?

Main Findings

Cryo-electron tomography of regulated SNARE-mediated membrane fusion, reconstituted *in vitro*, suggests that synaptic vesicles may be primed for fusion by inducing a protrusion in the target membrane.

Bullet point highlights

- We present cryo-electron tomography of regulated, SNARE-mediated membrane fusion, reconstituted *in vitro*.
- Vesicles that are docked and primed for fusion form an intermediate state characterized by a protrusion in the target membrane.
- High local membrane curvature of the protrusion may represent a high-energy state from which fast, coordinated fusion can occur upon Ca^{2+} activation.

We also include a synopsis image that should represent the key findings. We could either use the image in figure 2D, or, if you prefer, you can send us an illustration or new image for the synopsis image. The size of the synopsis image is 211 pixels wide x 157 pixels high. Please let me know if you have questions.

We suggest that you use Figure 1C as the synopsis image.

I look forward to seeing a new revised version of your manuscript as soon as possible.

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The authors addressed my concerns extensively with the new experimental data. I am happy with the revision. The paper can now be published in EMBO Reports.

Referee #3:

The revised paper is an excellent contribution to a very active field of research. Added data have fully covered my concerns/comments and I recommend publication of this exciting work. I have only a minor suggestion. I suggest to modify the added citation to the Monck & Fernandez paper to replace "... to be important for SNARE-mediated membrane fusion..." with "... to be important for exocytosis..." or "... to be important for intracellular membrane fusion..." . It looks like SNAREs have not been mentioned in this Monck & Fernandez paper.

We have modified this sentence and replaced “SNARE-mediated membrane fusion” with “exocytosis”.

3rd Editorial Decision 18 December 2013

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.

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