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Synaptotagmin-11 inhibits clathrin-mediated and bulk endocytosis

Changhe Wang , Yeshi Wang , Meiqin Hu , Zuying Chai , Qihui Wu , Rong Huang , Weiping Han, Claire Xi Zhang, and Zhuan Zhou

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Transaction Report:

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

1st Editorial Decision 19 May 2019

Thank you for your presubmission enquiry to EMBO reports. Barbara cannot handle it due to conflict of interest and therefore I will be the primary handling editor. I have now read the files you sent and related literature, and discussed them within our editorial team. We agree that the study could be suitable for publication in EMBO reports and would invite its submission, to be sent for peer-review.

Before sending the study to experts, however, I would recommend a change in format. It is our policy to leave formatting decisions up to the authors at this initial stage, as the final outcome of the peer-review process is unknown. However, in this case, I think it would be beneficial to spend some time on this because the current extremely condensed format of the study does not allow for an efficient description of the results, or placing them in the context of the field. In addition, most of the data is presented as supplementary, which we do not allow.

EMBO reports publishes two types of manuscripts, Scientific Reports and Articles. For detailed instructions about their format, please consult our Guide to Authors on the web. Whether the study ultimately falls into one or the other category would be decided further down the process if a revision is invited, so you can now format it as you consider best for your data.

Please contact me if I can be of any help during the preparation of your study for peer-review.

1st Revision - authors' response 28 May 2015

Author made suggested editorial changes.

2nd Editorial Decision 26 June 2015

Thank you for your patience during the peer-review of your study at EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, although all of them find the topic of interest and the study overall well conducted, referees 1 and 2 especially raise a number of technical and other issues that decrease the conclusiveness of your results.

Given that all referees provide constructive suggestions on how to make the work more conclusive, I would like to give you the opportunity to revise your manuscript. If the referee concerns can be adequately addressed, we would be happy to consider your manuscript for publication.

In this case, it will be important to address the issues related to the overexpression and localization of Syt-11, the concerns raised by referees 1 and 2 regarding the EM data, the issue regarding the capacitance data and address referee 2's major point 5.

It would also be good to address the concerns raised with respect to the lack of specificity of the clathrin and dynamin inhibitors used, but this would not be a precondition for acceptance of the study. Alternatively, the text would have to discuss the problems and the claims modified accordingly. Please also rephrase the discussion of CME vs bulk endocytosis, mentioned by referee 2 in his/her point 4, although I would not remove the data.

Please also address the minor points raised by the referees, although I would not combine main figures, as referee 2 suggests in his/her first minor point (number 6), as these are already large. Please do not remove figure 5, and I would also not favor removing figure 6 (although the claims would need to be toned down), but I leave this up to you. However, supplementary figures 5 and 7 can and should be combined.

Please note that it is EMBO reports policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

Revised manuscripts must be submitted within three months of a request for revision unless previously discussed with the editor; they will otherwise be treated as new submissions. Your study will be published in Article format, which can have as many main figures as required to depict the main data, contains all material and methods in the main text and has separate Results and Discussion sections.

In addition, EMBO reports can accommodate the inclusion of extra figures (up to five) in the online version of the manuscript. These are presented in an expandable format inline in the main text so that readers who are interested can access them directly as they read the article. They are also provided for download in a separate typeset PDF to accompany the Article PDF. These should be those of particular value to specialist readers, but which are not required to follow the main thread of the paper (and not additional controls or reagent optimization). These should be labeled expanded view, and the rest of the figures should be combines into one Appendix.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision)
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files in high resolution
- a separate PDF file of any Supplementary information (in its final format)

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, please contact me if I can be of any assistance.

REFEREE REPORTS

Referee #1:

In this article, Changhe Wang and colleagues report for the first time a negative role in endocytosis for Syt11, whose neuronal function was so far unknown. The identification of negative regulators of endocytosis is of great interest to cell biologists. This category of proteins is indeed poorly characterized, yet it is likely to play important physiological roles by fine-tuning all cellular processes that rely on endocytosis, including neurotransmission.

The authors convincingly show that Syt11 selectively inhibits endocytosis, both constitutive and upon stimulation, without affecting exocytosis. Their conclusions are based on capacitance measurements, fluorescent dye uptake and EM in Syt11 KD dorsal root ganglion neurons, providing a complete set of data. The experiments appear to have been conducted carefully and in general the appropriate controls have been included.

1. My main concern relates to the rescue of the Syt11 KD phenotype by expression of different Syt11 constructs. I was a bit surprised that the FL construct leads to a perfect rescue of the FM4-64, Tf and dextran uptake defect. If Syt11 is a negative regulator of these pathways, one would expect that Syt11 overexpression causes a reduction of dye uptake compared to control, no? I therefore encourage the authors to include a western blot showing the expression levels of the different Syt11 constructs compared to the endogenous protein; how much OE are we talking about?

2. Similarly, the authors should show the expression level of Myc-Syt11. As figure 4 indicates that this tagged protein co-localizes with all but one of the markers that were tested, I am concerned that this broad intracellular distribution might be an overexpression artefact. Moreover, an additional panel showing the specificity of the anti-Myc staining should be included.

3. I am a bit puzzled by the EM quantification results shown in figure 8G-H. There appears not to be a difference in the amount of endocytic structures found at the plasma membrane in control and KD neurons after 100 mM K+ stimulation for 2 minutes. Yet under these conditions an increase in FM1-43 uptake was observed in Syt11 KD neurons compared to control (figure 2C). I am thus not convinced by the explanation proposed by the authors that "... Syt11 specifically limits the internalization sites of both CME and bulk endocytosis ..." (line 281, see also line 36). At least upon stimulation, it appears that there are just as many internalization sites, but that the turnover of endocytic events is faster in the absence of Syt11. In my opinion the authors should thus reformulate their conclusion. Along the same idea, the authors also wrote in the discussion that "... Syt11 inhibits CME and bulk endocytosis at the stage of membrane deformation..." (line 273). Yet upon 100 mM K+ stimulation the same proportion of the plasma membrane appears to be invaginated. Here again, this is an overstatement and the sentence should thus be carefully re-written.

4. The inhibitors for clathrin and dynamin the authors used to determine which endocytic pathway is affected by Syt11 KD are indeed used by many, but they have also been shown to be not entirely specific. I agree that the data shown are consistent with the EM data but it would still be nice to confirm these results using another independent methodology or at least state the limitations of the inhibitors they used very clearly in the text.

I also have some minor concerns:

5. Line 103: "These results demonstrated the functional specificity of Sy11 in endocytosis". At this stage of the paper the authors haven't yet shown that the reduced Cm jump observed upon Syt11 KD is due to increased endocytosis during stimulation and not to defective exocytosis. This sentence should thus be reformulated.

6. Figure 7 B and C: indicate "diameter of SVs" and "diameter DCVs" on the graph axis to improve clarity.

7. Figure 8A: I recommend including an inset with a higher magnification picture, since with the current images it is not obvious that there are more HRP-labelled structures in the Syt11 KD neurons compared to control.

8. Figure 9 B-D: the dashed lines used to indicate the control levels make the graphs look messy.

9. Final comment: I would strongly recommend having the manuscript checked by a native English speaker. In its current state it contains several spelling and grammar mistakes, and some sentences are quite unclear. The figures are clear and consistently designed.

Referee #2:

This paper reports on the presumed inhibitory function of Syt-11 in DRG and hippocampal neurons in culture. KD of Syt-11 is shown to reduce capacitative membrane increase due to elevated compensatory membrane uptake via dynamin- and clathrin-mediated mechanisms including bulk endocytosis. Rescue experiments indicate that endocytosis inhibition involves both C2 domains as well as the polybasic stretch within C2B of Syt-1. Based on these data the authors suggest differential regulation of exo-endocytosis at synapses by distinct Syt isoforms.

This Ms suffers from a variety of experimental flaws that dampen my enthusiasm for the potentially interesting role of Syt-11 as a negative regulator of endocytosis in neurons. These caveats preclude publication of the paper, at least in its current format.

1. I wonder how robust the capacitance data really are as the apparent rates seem to differ vastly between experiments (i.e. comparing the traces in Fig. 1B-E or Fig. 3 to those Fig. 6C-D). Surprisingly, no kinetic analysis of endocytic rate constants is presented. This gap needs to be filled to substantiate the central claim of the paper.

2. Similar concerns hold for the EM analysis. First, in Fig. 7 only histograms of WT DRG neurons are shown. Where are the data for Syt-11 KD vs scr-siRNA treated controls? Second, and most important: I fail to see how the expected variability of SV and DCV sizes (overt also from the presented histograms) caused by both biological variation as well as by different planes of sectioning through individual organelles can yield the extremely low SEM values shown in the bar diagrams in panels D and E. This is inconsistent and frankly, I do not believe the low variability can be correct. Moreover, for ALL data mean{plus minus}SD rather than SEM must be shown to get a feeling for the variability between experiments. I further urge the authors to make sure that all statistical testing is done using the number of independent experiments as n (not the number of cells, boutons or alike).

3. MDC rather than inhibiting clathrin is used to perturb and monitor autophagy, hence, the specificity of the effects shown in Fig.5 is unclear. In line with this, the effects of MDC are distinct from those of clathrin inhibition (S4). It would therefrore seem appropriate to remove the MDC data from the paper, while elaborating more on the effects of clathrin inhibition (i.e. concentration, effect of inactive control compound etc.). Moreover, recent work from De Camilli has cast doubt on the specificity of dynasore and dynoles, which have off-target effects on the actin cytoskeleton (Park et al, J Cell Sci). Hence, dynasore/dynole data need to be interpreted with caution. This concern is important as one of the key arguments supporting the claim that Syt-11 negatively regulates endocytosis without affecting release is based on these inhibitors. Hence, aadditional evidence is required to rule out effects of Syt-11 on exocytosis.

4. In Fig. 6 the authors use the size of FM or transferrin puncta as a surrogate measure for CME vs bulk endocytosis. This is an unwarranted leap given the poor temporal and spatial (diffraction-limited optics) resolution of these experiments and the fact that primary endocytic vesicles fuse with each other and with endosomes on a timescale of seconds (see Watanabe et al., Nature 2014). I therefore suggest to eliminate these data from the Ms.

5. I wonder about the contribution of Syt-11 to vesicle replenishment. A prediction from the data is that KD of Syt-11 faciliates recovery of exocytic responses after rundown? Data in this direction would greatly add to the value of the paper.

Additional points:

6. The MS is way too long given the length limits of EMBO Rep. Some shortening can be easily accomplished by moving data to the supplement (i.e. Fig. 4), combining figures (i.e. 1-3, 7+8) and by deleting data that in my view do not support the central claim (i.e. Figs. 5,6).

7. The images shown in Fig. 2G are of poor quality and ought to be replaced by more compelling examples.

8. The legends are extremely sketchy and many times the reader is left at odds with respect to what is actually shown.

9. On p.5 the authors state that "These phenomena were also observed ...with the other two shRNAs (Figures 1E-H)". Looking at the figures quoted I find no evidence that this statement is correct. Where are the data?

Referee #3:

In this study, the authors analyze the function of synaptotagmin 11 in stimulus-evoked and basal endocytosis in cultured superior ganglion neurons. The authors use primarily RNA-knockdown to reduce the expression levels of the targeted protein and then analyze endocytosis both by capacitance patch clamping and by uptake of tracers (FM 1-43, and labeled dextrans). The authors find that both clathrin-mediated and bulk endocytosis is significantly increased in the knockdown cells whereas exocytosis appears to be unaffected. Rescue experiments using expression f synaptotagmin variants revealed interesting differences of certain mutations on bulk vs. clathrin-mediated exocytosis.

In my opinion, this is an excellent and expertly conducted study that sheds new light on the regulation of neuronal endocytosis. The experiments are well controlled, and the conclusions are sound although I am sure that in this busy field some of the conclusions will be contested by other groups.

Obviously there are loose ends, which is hard to avoid in a study of this complexity. Most importantly, it is unfortunate that the intracellular localization of Syt11 could not be determined. The authors have tried to solve this problem by carrying out double labeling of neurons overexpressing tagged syt 11, an approach that has shortcomings but is unavoidable considering that no decent antibodies are available. What is missing is a double labeling with a vesicle marker or with a marker for large dense core vesicles such as synaptophysin, VAMP2 (or perhaps syt 1) or with one of the chromogranins/secretogranins. Note that I do not consider such data as essential for publication but they would help to obtain a better idea as to whether Syt 11 is located to secretory vesicles or not.

2nd Revision - authors' response 15 September 2015

RESPONSE TO EDITOR

Thank you for your patience during the peer-review of your study at EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, although all of them find the topic of interest and the study overall well conducted, referees 1 and 2 especially raise a number of technical and other issues that decrease the conclusiveness of your results.

Given that all referees provide constructive suggestions on how to make the work more conclusive, I would like to give you the opportunity to revise your manuscript. If the referee concerns can be adequately addressed, we would be happy to consider your manuscript for publication.
In this case, it will be important to address the issues related to the overexpression and localization of Syt-11, the concerns raised by referees 1 and 2 regarding the EM data, the issue regarding the capacitance data and address referee 2's major point 5.

Reply: We thank you and all three referees very much for the thoughtful comments and positive assessment of this work. All the concerns of the three reviewers are greatly appreciated and have been carefully addressed (please see below). Based on the new evidence and the overall improvement of the manuscript, we hope that the reviewers are now satisfied and that this paper can be accepted for publication in *EMBO Reports*.

It would also be good to address the concerns raised with respect to the lack of specificity of the clathrin and dynamin inhibitors used, but this would not be a precondition for acceptance of the study. Alternatively, the text would have to discuss the problems and the claims modified accordingly. Please also rephrase the discussion of CME vs bulk endocytosis, mentioned by referee 2 in his/her point 4, although I would not remove the data.

Reply: We have stated the limitations of the inhibitors in the revised manuscript (line 192) and rephrased the related conclusive sentences (lines 160, 192 and 285). We have also removed the puncta size analysis from the revised manuscript following the advice of referee #2.

Please also address the minor points raised by the referees, although I would not combine main figures, as referee 2 suggests in his/her first minor point (number 6), as these are already large. Please do not remove figure 5, and I would also not favor removing figure 6 (although the claims would need to be toned down), but I leave this up to you. However, supplementary figures 5 and 7 can and should be combined.

Reply: We have kept the old figures following your advice. Considering that Supplementary Fig 5 showed little additional information, we have removed it from the revised manuscript.

RESPONSE TO REFEREES

Referee #1:
In this article, Changhe Wang and colleagues report for the first time a negative role in endocytosis for Syt11, whose neuronal function was so far unknown. The identification of negative regulators of endocytosis is of great interest to cell biologists. This category of proteins is indeed poorly characterized, yet it is likely to play important physiological roles by fine-tuning all cellular processes that rely on endocytosis, including neurotransmission.

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Reply: We thank the reviewer very much for the positive assessment of this work.

1. *My main concern relates to the rescue of the Syt11 KD phenotype by expression of different Syt11 constructs. I was a bit surprised that the FL construct leads to a perfect rescue of the FM4-64, Tf and dextran uptake defect. If Syt11 is a negative regulator of these pathways, one would expect that Syt11 overexpression causes a reduction of dye uptake compared to control, no? I therefore encourage the authors to include a western blot showing the expression levels of the different Syt11 constructs compared to the endogenous protein; how much OE are we talking about?*

Reply: We indeed found that Syt11 overexpression decreased Tf uptake in DRG neurons (Fig 5B), as well as in HEK293 cells (Fig R1). In addition, we found decreased FM uptake in Syt11-overexpressing DRG neurons (Fig R2), confirming the inhibitory role of Syt11 in endocytosis.
As overexpression of Syt11 inhibited endocytosis, the expression levels in rescued constructs indeed become critical, as you noted. To investigate the total expression level of Syt11 in rescued neurons under our experimental conditions (electroporation with ~10% transfection efficiency), we collected the transfected cells with a fluorescence-activated cell sorter. Western blot analysis revealed similar expression levels of total Syt11 in control and rescued cells (rescued ~ 1.07-fold that in control cells, Fig R3A and B). Due to the difficulty in collecting enough transfected DRG neurons for Western blots, we did not test the expression levels in the four Syt11 mutants. However, to rule out possible

misfolding of mutants after deletion or point mutation, we checked the expression levels of these mutations in HEK293 cells. We found expression levels similar to full-length Syt11 (Fig R3C and D), except that ΔC2A was hardly detectable due to deletion of part of the antibody epitope. These experiments suggested that most of the rescued constructs expressed at similar levels and the total Syt11 protein levels were close to that of the endogenous protein. This could be due to the high expression level of endogenous Syt11 in DRG neurons (Fig R4).

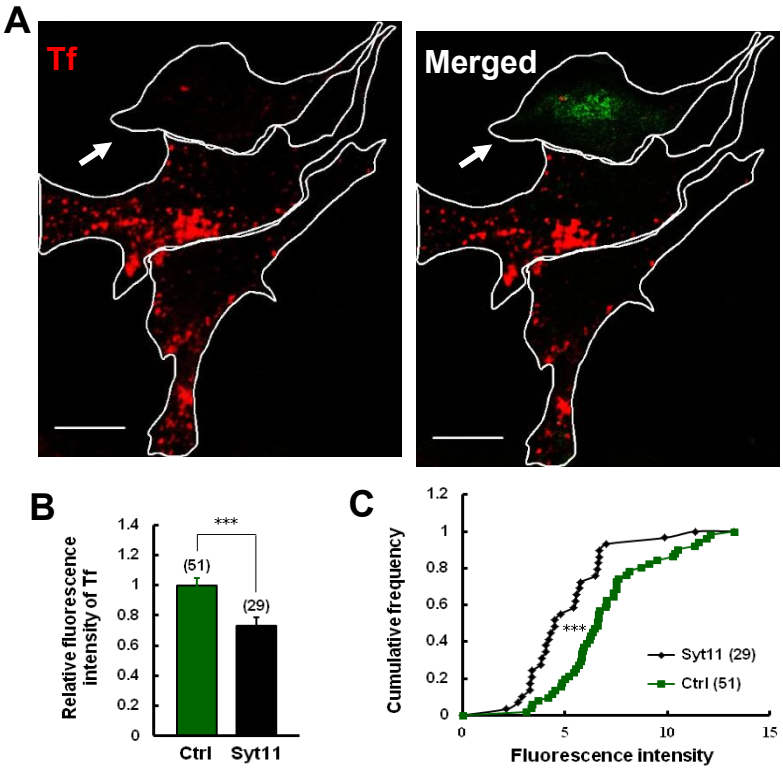

Figure R1. Transferrin uptake by HEK293A cells is inhibited by Syt11 overexpression
A. Constitutive transferrin (Tf, red) uptake by HEK293A cells with (GFP-positive, arrows) or without Syt11 overexpression; scale bars, 10 μm.
B. Quantification of Tf uptake in Syt11-overexpressing and control HEK293A cells. Mean ± s.e.m.; Student's *t*-test, ***$P < 0.001$.
C. Cumulative frequency histograms of Tf fluorescence. Kolmogorov-Smirnov test, ***$P < 0.001$.

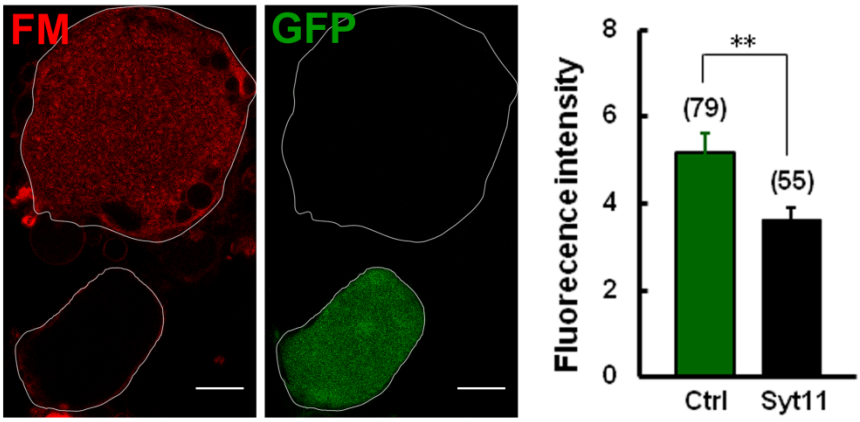

Figure R2. Syt11 overexpression inhibits FM uptake in DRG neurons
Representative micrographs (left and middle panels) and statistics (right panel) showing the decreased FM 4-64 uptake (red) in Syt11-overexpressing DRG neurons (GFP-positive) in response to 100 mM K^+ for 2 min. Student's *t*-test, **$P < 0.01$; scale bars, 20 μm.

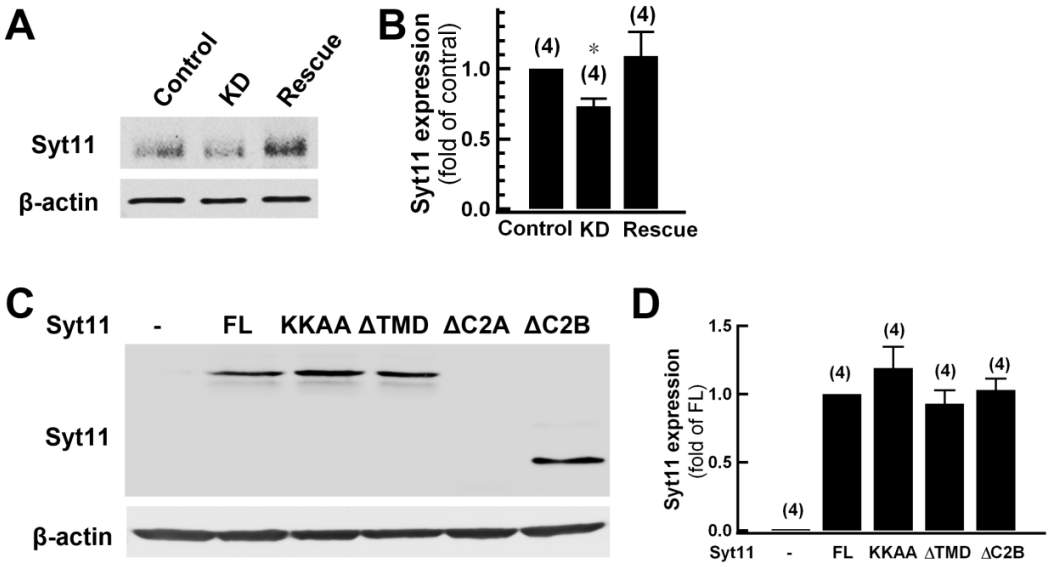

Figure R3. Syt11 expression levels in Syt11-KD and rescued DRG neurons.
A, B. Immunoblotting for Syt11 expression in control (scrambled shRNA), KD (shSyt11-2), and Syt11-rescued DRG neurons. DRG neurons were transfected with plasmids expressing scrambled shRNA, or shSyt11-2 (Syt11 KD, KD) with or without Syt11-rescue and cultured for 5 days. Transfected cells were collected with a fluorescence-activated cell-sorter and the whole-cell lysates were used for immunoblotting analysis.
C, D. Immunoblotting for the expression of the different Syt11 mutations in HEK293 cells. HEK293 cells were transfected with plasmids expressing full-length Syt11 (FL), or Syt11 harboring a deletion of the transmembrane domain (TMD), C2A, or C2B, or the KKAA mutant (replacing the two conserved lysine residues with alanines in the AP-2-binding site) and immunoblotting was performed ~ 24 h after transfection. Please note that we failed to detect expression of the C2A-truncated form of Syt11, probably due to disturbance of the targeting sequence (the linker region before C2A) of this antibody (270003, Synaptic Systems).
One-way ANOVA, $*P < 0.05$.

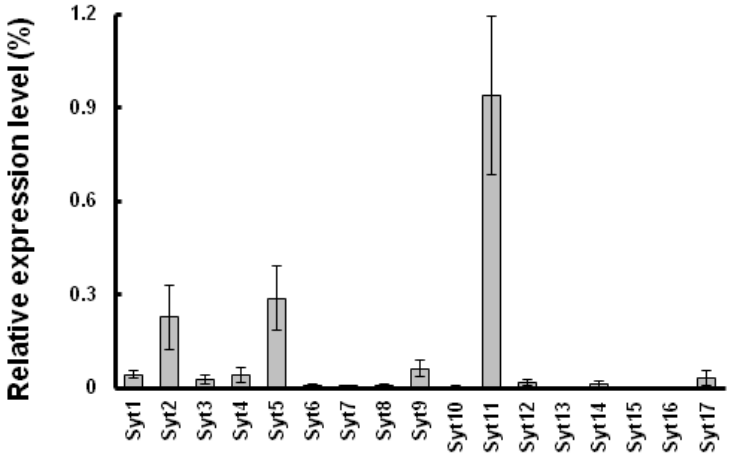

Figure R4. Relative mRNA levels of 17 synaptotagmin isoforms in rat DRG neurons
Expression levels of synaptotagmin 1–17 mRNAs were quantified by real-time PCR and normalized to 18S ribosomal RNA (mean ± s.e.m., 3 independent experiments, each performed in triplicate).

2. *Similarly, the authors should show the expression level of Myc-Syt11. As figure 4 indicates that this tagged protein co-localizes with all but one of the markers that were tested, I am concerned that this broad intracellular distribution might be an overexpression artefact. Moreover, an additional panel showing the specificity of the anti-Myc staining should be included.*

Reply: We also worried about an overexpression artifact and used a low plasmid concentration for electroporation. Furthermore, we imaged cells with low expression levels only. Since we previously showed that the total expression level of Syt11 rescue was similar to that of endogenous protein (~ 1.07-fold that in control cells, Fig R3A and B), we expected that the expression level of Myc-Syt11 would not be higher than the endogenous level. The specificity of the anti-Myc staining was shown in the Tf uptake assay (Fig 5B), in which only Myc-Syt11-expressing neurons showed positive staining.

3. *I am a bit puzzled by the EM quantification results shown in figure 8G-H. There appears not to be a difference in the amount of endocytic structures found at the plasma membrane in control and KD neurons after 100 mM K+ stimulation for 2 minutes. Yet under these conditions an increase in FM1-43 uptake was observed in Syt11 KD neurons compared to control (figure 2C). I am thus not convinced by the explanation proposed by the authors that "... Syt11 specifically limits the internalization sites of both CME and bulk endocytosis ..." (line 281, see also line 36). At least upon stimulation, it appears that there are just as many internalization sites, but that the turnover of endocytic events is faster in the absence of Syt11. In my opinion the authors should thus reformulate their conclusion. Along the same idea, the authors also wrote in the discussion that "... Syt11 inhibits CME and bulk endocytosis at the stage of membrane deformation..." (line 273). Yet upon 100 mM K+ stimulation the same proportion of the plasma membrane appears to be invaginated. Here again, this is an overstatement and the sentence should thus be carefully re-written.*

Reply: For EM quantification we counted the number of clathrin-coated pits immediately after the 2-min stimulation, while the FM uptake reflected the total endocytic events during stimulation. These EM data were consistent with our finding that the increase of FM uptake was more prominent at shorter stimulation times as the dye uptake plateaued within 1 min in Syt11 KD neurons (Fig 2C). Further increases of both structures on the plasma membrane were not found in Syt11 KD compared with control neurons (Fig 8G and H), probably due to the saturation of internalization sites. Following your advice, we have changed "... Syt11 inhibits CME and bulk endocytosis at the stage of membrane deformation..." to "... Syt11 inhibits CME and bulk endocytosis most plausibly at the stage of membrane deformation..." (line 290). Line 37 has also been rephrased accordingly.

4. *The inhibitors for clathrin and dynamin the authors used to determine which endocytic pathway is affected by Syt11 KD are indeed used by many, but they have also been shown to be not entirely specific. I agree that the data shown are consistent with the EM data but it would still be nice to confirm these results using another independent methodology or at least state the limitations of the inhibitors they used very clearly in the text.*

Reply: Following your advice, we have stated the limitations of these inhibitors in the revised manuscript (line 192).

I also have some minor concerns:

5. *Line 103: "These results demonstrated the functional specificity of Sy11 in endocytosis". At this stage of the paper the authors haven't yet shown that the reduced Cm jump observed upon Syt11 KD is due to increased endocytosis during stimulation and not to defective exocytosis. This sentence should thus be reformulated.*

Reply: We have followed this suggestion and changed "These results demonstrated the functional specificity of Sy11 in endocytosis" into "These results demonstrated the isoform-specific role of Syt in endocytosis" in the revised manuscript.

6. *Figure 7 B and C: indicate "diameter of SVs" and "diameter DCVs" on the graph axis to improve clarity.*

Reply: Thank you; we have followed this suggestion in the revised manuscript.

7. *Figure 8A: I recommend including an inset with a higher magnification picture, since with the current images it is not obvious that there are more HRP-labelled structures in the Syt11 KD neurons compared to control.*

Reply: Higher magnification images of HRP-labeled structures are shown in Fig 8B. Following your advice, we have also included an enlarged inset in revised Fig 8A.

8. *Figure 9 B-D: the dashed lines used to indicate the control levels make the graphs look messy.*
Reply: Thank you; we have changed the figure in the revised manuscript.

9. *Final comment: I would strongly recommend having the manuscript checked by a native English speaker. In its current state it contains several spelling and grammar mistakes, and some sentences are quite unclear. The figures are clear and consistently designed.*

Reply: We have followed this suggestion and asked Dr. Iain C. Bruce for a final read of the revised manuscript. Thank you.

Referee #2:

This paper reports on the presumed inhibitory function of Syt-11 in DRG and hippocampal neurons in culture. KD of Syt-11 is shown to reduce capacitative membrane increase due to elevated compensatory membrane uptake via dynamin- and clathrin-mediated mechanisms including bulk endocytosis. Rescue experiments indicate that endocytosis inhibition involves both C2 domains as well as the polybasic stretch within C2B of Syt-1. Based on these data the authors suggest differential regulation of exo-endocytosis at synapses by distinct Syt isoforms.

This Ms suffers from a variety of experimental flaws that dampen my enthusiasm for the potentially interesting role of Syt-11 as a negative regulator of endocytosis in neurons. These caveats preclude publication of the paper, at least in its current format.

Reply: Thank you very much for your critical comments. With the new evidence and the overall improvement of the manuscript, we hope that you are now satisfied with the revised manuscript.

1. *I wonder how robust the capacitance data really are as the apparent rates seem to differ vastly between experiments (i.e. comparing the traces in Fig. 1B-E or Fig. 3 to those Fig. 6C-D). Surprisingly, no kinetic analysis of endocytic rate constants is presented. This gap needs to be filled to substantiate the central claim of the paper.*

Reply: The C_m recordings in Figs 1B-E and 3 were made under different conditions. To investigate the dynamin inhibitors, 0.1% DMSO was included in the peptide solution during the recordings in Fig 3. Thus, the slight C_m difference between the two figures was probably due to the DMSO. Importantly, the inhibitory role of Syt11 in endocytosis was reproduced in the presence of DMSO (Fig 3G and H), which enabled us to investigate the nature of Syt11 KD-accelerated endocytosis with dynamin inhibitors. We followed your advice to analyze the kinetics of C_m decay and found that most of the KD neurons showed typical bi-exponential endocytic C_m decay (Fig 5C and R5B). Thus, we used a double-exponential function to fit the total C_m decay in DRG neurons and found that both the fast and slow components of endocytosis were dramatically accelerated in KD neurons (Fig R5), supporting our findings that both bulk and clathrin-mediated endocytosis were accelerated by Syt11 KD.

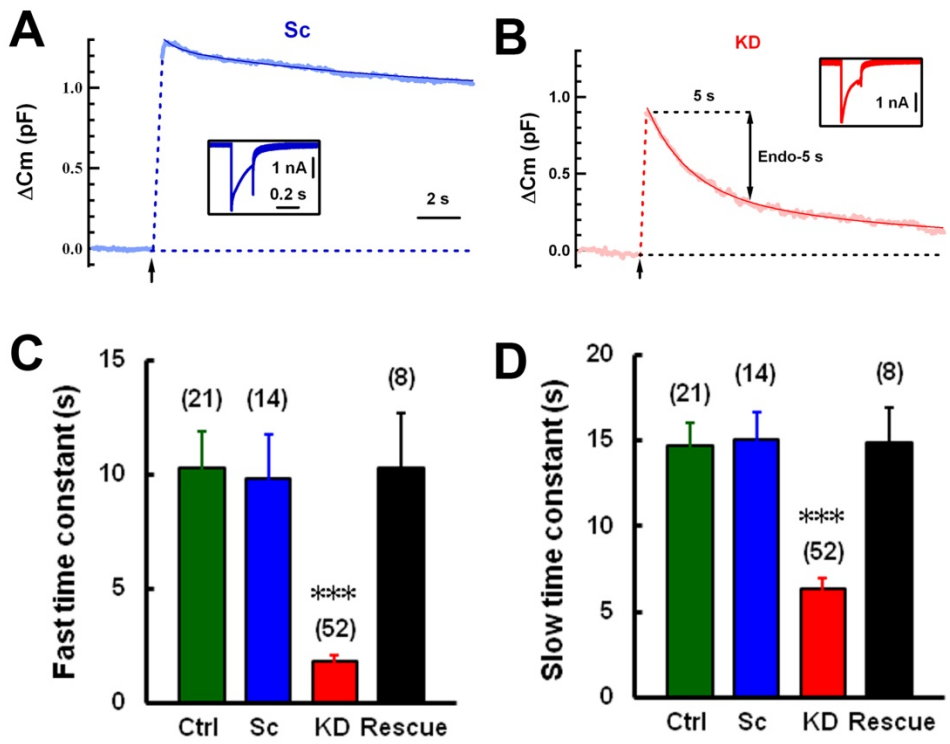

Figure R5 (revised supplementary Fig S2A-D). Syt11 KD accelerates endocytic C_m decay in DRG neurons

A, B. Representative C_m traces recorded from DRG neurons induced by a 200-ms depolarizing pulse (arrows). The endocytic C_m decay was fitted to a double-exponential function (solid blue and red, fitted curves). DRG neurons were transfected with plasmids expressing shSyt11-2 (Syt11 KD, KD) or scrambled shRNA (Sc) and C_m recordings were made 5 days after transfection. Insets show Ca^{2+} currents recorded in the same neurons.

C, D. Time-constants of fast and slow phases of endocytosis in Control (Ctrl), scrambled (Sc), KD, and rescued (with the shSyt11-2-resistant form of Syt11) DRG neurons.

One-way ANOVA, ***$P < 0.001$.

2. *Similar concerns hold for the EM analysis. First, in Fig. 7 only histograms of WT DRG neurons are shown. Where are the data for Syt-11 KD vs scr-siRNA treated controls? Second, and most important: I fail to see how the expected variability of SV and DCV sizes (overt also from the presented histograms) caused by both biological variation as well as by different planes of sectioning through individual organelles can yield the extremely low SEM values shown in the bar diagrams in panels D and E. This is inconsistent and frankly, I do not believe the low variability can be correct. Moreover, for ALL data mean{plus minus}SD rather than SEM must be shown to get a feeling for the variability between experiments. I further urge the authors to make sure that all statistical testing is done using the number of independent experiments as n (not the number of cells, boutons or alike).*

Reply: Following your advice, the diameter distributions of SVs and DCVs in KD neurons are included in revised Fig 7. Regarding your concerns about the variability of SV and DCV sizes, we indeed found variations that fit a Gaussian distribution (Fig 7B and C). We counted a large number of vesicles for the statistics to make sure that we did not miss small differences between control and KD neurons; that is the reason for the smaller SEMs in Fig 7D and E. We used SEM and n values here just following the conventional statistical methods widely used in this field, e.g. [1-8]. For single-cell EM imaging, only one cell was embedded in each sectioning sample. Most of the electrophysiological recordings and uptake assays were performed cell by cell as well. That is why we used the number of cells for most statistical tests, and importantly we found that most of the papers in this field with similar experimental conditions use the number of cells or boutons (for synaptic analysis) for statistical analysis [1-8].

3. *MDC rather than inhibiting clathrin is used to perturb and monitor autophagy, hence, the specificity of the effects shown in Fig.5 is unclear. In line with this, the effects of MDC are distinct from those of clathrin inhibition (S4). It would therefrore seem appropriate to remove the MDC data from the paper, while elaborating more on the effects of clathrin inhibition (i.e. concentration, effect of inactive control compound etc.). Moreover, recent work from De Camilli has cast doubt on the specificity of dynasore and dynoles, which have off-target effects on the actin cytoskeleton (Park et al, J Cell Sci). Hence, dynasore/dynole data need to be interpreted with caution. This concern is important as one of the key arguments supporting the claim that Syt-11 negatively regulates endocytosis without affecting release is based on these inhibitors. Hence, additional evidence is required to rule out effects of Syt-11 on exocytosis.*

Reply: We agree with you that these drugs may have off-target effects (in De Camilli's *Journal of Cell Science* paper, dynasore and dyngo-4a also inhibit fluid-phase endocytosis and peripheral membrane ruffling, but dynole remains untested); that is why we used different drugs/assays to test the inhibitory role of Syt11 in endocytosis. Following your advice, we have stated the limitations of the inhibitors in the revised manuscript (line 192) and rephrased the related conclusive sentences (lines 160, 192 and 285). Considering that it is difficult to completely separate exocytosis from endocytosis, we have removed the statement '*without affecting exocytosis*' throughout the manuscript.

4. *In Fig. 6 the authors use the size of FM or transferrin puncta as a surrogate measure for CME vs bulk endocytosis. This is an unwarranted leap given the poor temporal and spatial (diffraction-limited optics) resolution of these experiments and the fact that primary endocytic vesicles fuse with each other and with endosomes on a timescale of seconds (see Watanabe et al., Nature 2014). I therefore suggest to eliminate these data from the Ms.*

Reply: Thank you; we have removed these data from the revised manuscript.

5. *I wonder about the contribution of Syt-11 to vesicle replenishment. A prediction from the data is that KD of Syt-11 faciliates recovery of exocytic responses after rundown? Data in this direction would greatly add to the value of the paper.*

Reply: We indeed found faster vesicle replenishment with paired-pulse stimulation (Fig 1I and J). Furthermore, we made new C_m recordings from DRG neurons during a 1-Hz train of 10 pulses and found accelerated replenishment of releasable vesicle pools under this sustained stimulation (Fig R6).

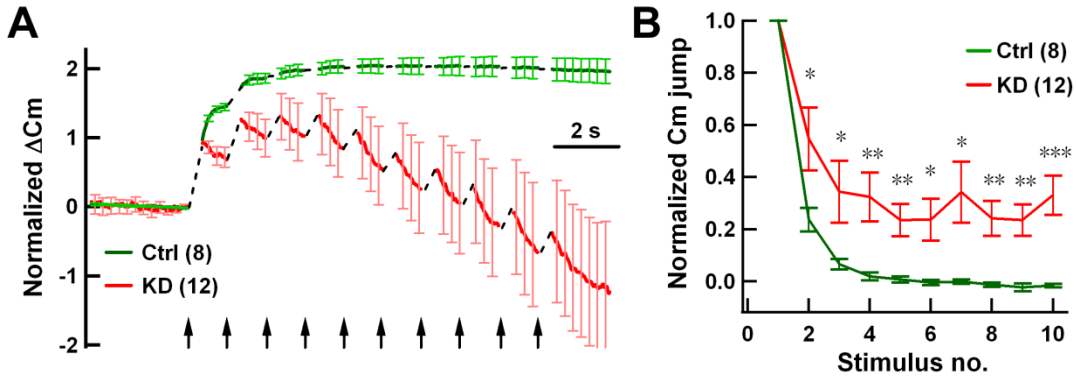

Figure R6 (revised supplementary Fig S3). Syt11 KD accelerates vesicle pool replenishment in DRG neurons
A, B. Normalized ΔC_m (A) and C_m jumps (B) induced by a 1-Hz train of 10 pulses (arrows). The normalized C_m jump was calculated by dividing the ΔC_m value in response to each pulse with that induced by the first one.
Student's *t*-test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Additional points:
6. *The MS is way too long given the length limits of EMBO Rep. Some shortening can be easily accomplished by moving data to the supplement (i.e. Fig. 4), combining figures (i.e. 1-3, 7+8) and by deleting data that in my view do not support the central claim (i.e. Figs. 5,6).*
Reply: We have submitted this manuscript as an article paper.

7. *The images shown in Fig. 2G are of poor quality and ought to be replaced by more compelling examples.*

Reply: We have replaced this figure with a more compelling one.

8. *The legends are extremely sketchy and many times the reader is left at odds with respect to what is actually shown.*

Reply: Following your advice, the figure legends are more detailed in the revised manuscript.

9. *On p.5 the authors state that "These phenomena were also observed ...with the other two shRNAs (Figures 1E-H)". Looking at the figures quoted I find no evidence that this statement is correct. Where are the data?*

Reply: The statistical data of KD neurons was from all three shRNAs. To make this clear, we now show these shRNAs separately (revised Fig 1F-H).

Referee #3:

In this study, the authors analyze the function of synaptotagmin 11 in stimulus-evoked and basal endocytosis in cultured superior ganglion neurons. The authors use primarily RNA-knockdown to reduce the expression levels of the targeted protein and then analyze endocytosis both by capacitance patch clamping and by uptake of tracers (FM 1-43, and labeled dextrans). The authors find that both clathrin-mediated and bulk endocytosis is significantly increased in the knockdown cells whereas exocytosis appears to be unaffected. Rescue experiments using expression f synaptotagmin variants revealed interesting differences of certain mutations on bulk vs. clathrin-mediated exocytosis.

In my opinion, this is an excellent and expertly conducted study that sheds new light on the regulation of neuronal endocytosis. The experiments are well controlled, and the conclusions are sound although I am sure that in this busy field some of the conclusions will be contested by other groups.

Obviously there are loose ends, which is hard to avoid in a study of this complexity. Most importantly, it is unfortunate that the intracellular localization of Syt11 could not be determined. The authors have tried to solve this problem by carrying out double labeling of neurons overexpressing tagged syt 11, an approach that has shortcomings but is unavoidable considering that no decent antibodies are available. What is missing is a double labeling with a vesicle marker or with a marker for large dense core vesicles such as synaptophysin, VAMP2 (or perhaps syt 1) or with one of the chromogranins/secretogranins. Note that I do not consider such data as essential for publication but they would help to obtain a better idea as to whether Syt 11 is located to secretory vesicles or not.

Reply: We thank the reviewer for the thoughtful comments and positive assessment of this work. We agree that Syt11 may partly localize to secretory vesicles and performed double-labeling of Myc-Syt11 and VAMP2 (Syb2) in DRG neurons (**Fig 4**). Following your advice, we performed further double-labeling and found partial co-localization of Myc-Syt11 and calcitonin gene-related peptide (**Fig R7**), indicating the partial localization of Syt11 in dense-core vesicles as well in DRG neurons.

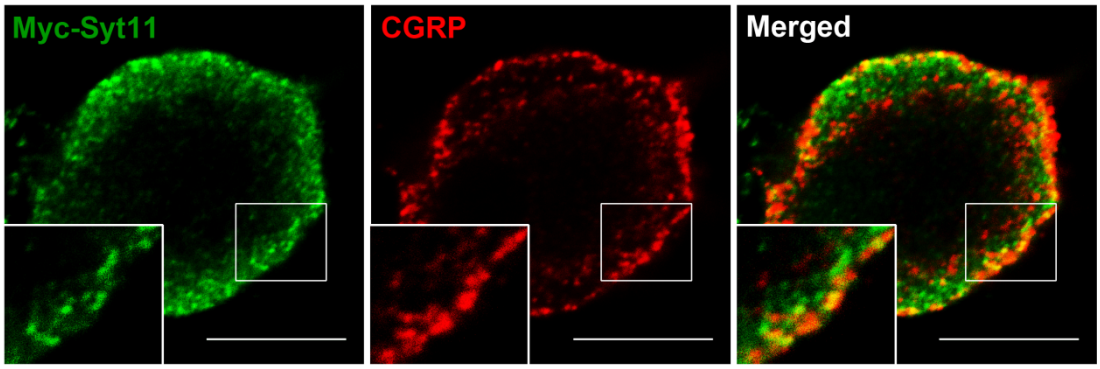

Figure R7. Syt11 partly localizes to dense-core vesicles in DRG neurons
DRG neurons expressing Myc-Syt11 were immunostained for Myc-Syt11 and the endogenous neuropeptide calcitonin gene-related peptide (CGRP). Scale bars, 10 μm.

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3rd Editorial Decision 08 October 2015

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

As you will see, all three referees are very positive about the study. However, referee 1 requests some changes to the text and figures that have to be integrated before we can proceed with the official acceptance of your study. The figure showing the expression levels of Syt11 in control and rescued cells can be integrated either in the main manuscript or in the Appendix/Expanded View.

From the editorial side, there are also a few things that we need.

- Regarding data quantification, can you please specify the number "n" for how many experiments were performed and the bars and error bars (e.g. SEM, SD) in the respective figure legends? This information is currently incomplete and must be provided in all figure legends, also in the Supplementary information.

- Please provide a completed authors checklist, which you can download from our author guidelines

(http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where the requested information can be found.

- You have the option to include up to 5 figures in the Expanded View format. For figures that are not promoted to the Expanded View, please label the file Appendix instead of Supplementary information. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure S1 throughout the text and also relabel the figures according to this nomenclature.

- Every EMBO reports paper now includes a 'Synopsis' to further enhance its discoverability. Synopses are displayed on the html version and they are freely accessible to all readers. The synopsis includes a short standfirst text (205 characters) as well as 2-4 one sentence bullet points that summarize the paper. These should be complementary to the abstract - i.e. not repeat the same text. This is a good place to be more informative and include, as appropriate, key acronyms and organism (yeast, mammalian cells, etc) information. This will be accompanied by a Synopsis image (500 x 400 pixel) of your choice. Could you please provide the standfirst text, bullet points and a synopsis image?

- 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."

We look forward to seeing a final version of your manuscript as soon as possible.

REFEREE REPORTS

Referee #1:

I am overall positive over the revised manuscript, yet I don't feel that the authors have addressed all of my comments adequately. I would thus recommend additional changes that in my view are needed to make the manuscript suitable for publication in EMBO reports:

1) The authors have performed additional experiments to address my main concern related to Syt11 expression levels. However, the data are only shown as a reviewer figure. In my opinion this is a critical point and these new data should be included in the main manuscript, e.g. as an additional figure (panel).

2) The higher magnification EM images now shown in Fig 8B are satisfying. Yet my point was specifically about the images shown in Fig 8A. The enlarged inset now shown in this figure are barely enlarged, and as such are of limited interest; larger images/zooms are needed.

3) I had requested the authors to rephrase the sentence "These results demonstrated the functional specificity of Syt11 in endocytosis" because - as far as I understood - at that point in the manuscript reduced Cm jump in SytKD neurons could still be explained, at least in part, by a role for Syt11 in exocytosis (which is only excluded based on experiments that are shown later in the manuscript). I am thus not happy with the new sentence proposed by the authors and the conclusion should be stated differently, because as it is now, the conclusion at this stage of the manuscript is overstated.

4) Importantly, I am still not convinced that the effect of Syt11 on endocytosis occurs at the level of membrane deformation, as suggested by the authors (line 290). Based on their comment in the rebuttal I would argue that the EM quantification of the number of endocytic structures should have been performed at an earlier time point after the start of stimulation. With the currently available data, I don't think there is evidence that the membrane deformation step is altered by Syt11 KD.

Moreover "membrane deformation" is quite vague and could refer to initial plasma membrane invagination (which, again does not seem to be affected at the time point that was imaged by the authors), or to subsequent vesicle neck formation and/or fission of the endocytic vesicle. I thus would like to ask the authors to rephrase their sentence again to clarify their point and not overstate their conclusions.

Referee #2:

All my previous questions and concerns have been adequately addressed. I would like to congratulate the authors on their findings and strongly support publication of this Ms in EMBO Rep.

I still think the Ms may be somewhat too long but this can be discussed with the editor.

Referee #3:

During revision, the authors have very carefully addressed the points raised by myself (which were minor anyway) and, at least in my opinion, also those of the other referees. As stated before, this paper will certainly result in (perhaps controversial) discussions but this is what science is about. The MS is of high quality and well written, and thus I recommend acceptance after revision.

3rd Revision - authors' response 10 October 2015

RESPONSE TO EDITOR

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Reply: Following your advice, we have included the expression levels of Syt11 in control and rescued cells in the revised manuscript (new Fig S5). Thank you very much.

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Reply: Following your advice, we have included this information in all figures.

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Reply: Following your advice, the inset in Fig 8A have been enlarged.

3) I had requested the authors to rephrase the sentence "These results demonstrated the functional specificity of Syt11 in endocytosis" because - as far as I understood - at that point in the manuscript reduced Cm jump in SytKD neurons could still be explained, at least in part, by a role for Syt11 in exocytosis (which is only excluded based on experiments that are shown later in the manuscript). I am thus not happy with the new sentence proposed by the authors and the conclusion should be stated differently, because as it is now, the conclusion at this stage of the manuscript is overstated.

Reply: Following your advice, we have removed this sentence.

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Reply: Following your advice, we have rephrased the sentence to "We surprisingly discovered that Syt11 inhibited both CME and bulk endocytosis (Figs 5-9), which contributed to the accelerated endocytosis in Syt11 KD neurons." to avoid the overstatement of our conclusions (line 301 in the revised manuscript).

Referee #2:
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Reply: Thank you very much for your great help and also your positive evaluation of this work.

4th Editorial Decision 21 October 2015

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.

