THE EMBO JOURNAL

Manuscript EMBO-2014-88143

A Conformational Switch in Collybistin Determines the Differentiation of Inhibitory Postsynapses

Tolga Soykan, Daniela Schneeberger, Giancarlo Tria, Claudia Buechner, Nicole Bader, Dmitri Svergun, Ingrid Tessmer, Alexandros Poulopoulos, Theofilos Papadopoulos, Frederique Varoqueaux, Hermann Schindelin and Nils Brose

Corresponding author: Nils Brose, Max Planck Institute of Experimental Medicine -Member

Review timeline:

Submission date:	06 February 2014
Editorial Decision:	19 March 2014
Additional Correspondence:	31 March 2014
Revision received:	25 June 2014
Accepted:	09 July 2014

Editor: Karin Dumstrei

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 March 2014

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees. I am still waiting for the third report, but given the comments I can take a preliminary decision at this stage.

As you can see below, both referees find the analysis interesting and suitable for publication here. They raise a number of concerns primarily with the structural work that would have to be resolved before publication here. I am still waiting to hear back from referee # 3 and would like to give the referee until mid next week to return his/her report. I should also add that this decision is still subject to change should referee #3 raise convincing arguments.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

Thank you for the opportunity to consider your work for publication.

REFEREE REPORTS

Referee #1:

© European Molecular Biology Organization

Brose and colleagues perform a thorough set of experiments to test a previously formulated model for protein-protein interactions that direct the organization of postsynaptic protein complexes. They combine structural, biophysical, biochemical and cell biological assays to probe the mechanism underlying collybistin activation and gephyrin recruitment to inhibitory synapses.

The structural work is only of modest resolution and it is not entirely clear whether the most definitive predictions emerged from the comparison with the Asef structure or the new structure presented here. However, for the validity of the conclusions that are presented here this is irrelevant.

I have only two minor points:
1) The discussion could be shortened to contain less repetition of the result section.
2) For the lipid binding experiments in Figure 3D it is unclear what the statistical evaluation and n for the analysis was. This should be noted in the figure legend.

Referee #2:

The adaptor protein Collybistin (CB) regulates the localization of gephyrin - the main scaffolding protein of inhibitory glycinergic and GABAergic postsynapses. The authors of this manuscript investigated the molecular mechanism of CB autoinhibiton through its SH3 domain, as well as of its activation through neuroligin-2 (NL2) binding. They determined a low resolution crystal structure of an SH3-containing CB, which reveals a "closed" form that closely resembles the architecture of Asef - the closest homologue of CB. Based on the structural information, the authors identified determinants (W24 and E262) of interaction between SH3 and the rest of CB (DH/PH domains). Using their biochemical and cell biological data, as well as an "open" form structure of CB solved by another group, the authors conclude that binding of SH3 to DH/PH keeps CB in a closed form and reduces the affinity of PH to membrane phosphoinositides - this is the basis of SH3-mediated autoinhibition. The authors further identified the NL2 binding site (W52 and W63) within the SH3 domain and provided evidence that binding of NL2 to SH3 stabilizes CB in an open conformation and thus relieves the autoinhibition, resulting in increased gephyrin-clustering activity of CB.

The present work was well designed and provides valuable information on the CB-based regulatory mechanism of inhibitory postsynapses. The manuscript should be of sufficient interest to the readers of EMBO J if the following points could be addressed:

Major:
- The authors mentioned that the SH3 domain of CB1_SH3+ is missing from the electron density map and they hypothesize that it is been proteolytic cleaved. Please do show the SDS-PAGE of the CB1_SH3+ crystals. This is a very important piece of information to prove that the missing SH3 domain in density map is really due to proteolysis.

- A correlation coefficient of 0.59 between the SH3 model and map is low. The electron density of the loop connecting the two beta-sheets is largely invisible, so I suggest that these residues should be removed in the final model. In addition, please provide the correlation coefficient for the rest of the structure (DH/PH).

- On page 7, please indicate if the root mean square deviation is for all atoms or main chain atoms only. Please also calculate the rmsd of individual domains (DH, PH) between open and closed forms to demonstrate if there is any intra-domain conformational change. Furthermore, the authors should analyze domain movements between open and closed states in more detail. Are there any differences other than different conformations of the kink helix that connects the DH and PH domains?

- In discussion, the authors conclude that the affinity between the SH3 domain and the DH/PH domain is lower in CB than in Asef, because CB1_SH3+ lost its SH3 domain during crystallization and pulldown assay showed weak binding of CB SH3 domain to CB2_SH3-. This alone, however, does not provide quantitative information about the affinity. Please provide references regarding the affinity in Asef, as well as explanation how you compare the affinities.

-The authors proposed that W24, E262 and R70 are the interface forming residues between SH3 and DH/PH domains. However, they only showed data about W24 and E262. Given that the resolution

© European Molecular Biology Organization

of the structure is low and the exact positions of side chains are uncertain, please provide the data of R70 as well.

Minor:
- Because the "open" form CB (CB2_SH3-) was used for comparison with "closed" form in the manuscript, please include a brief introduction about the "open" form.

- On page 8, first paragraph, there is a citation format error about interface forming residues in Asef.

- On page 15, the author mentioned that NL2 and NL4 contain proline-rich stretches in their C-terminal tails, however, according to sequence alignment shown in Fig. 5D, the proline-rich stretch is only present in NL2. Please check.

Additional Correspondence 31 March 2014

I have just received the third referee report. See below. The referee raises good points that should be fairly straight forward to resolve. It would be good if you could try to incorporate these changes too in the revised version. I am happy to discuss the specific points further if needed.

REFEREE REPORT

Referee #3:

In this paper, Soykan and colleagues have studied the conformational changes of collybistin (CB) and its consequences for the interaction of CB with and the clustering of inhibitory synaptic proteins neuroligin (NL) and gephryin. Using a combination of structural biology, biochemistry and imaging approaches, the authors propose a model whereby CB is 'activated' by NL2, which relieves the inhibitory effect of the SH3 domain and leads to an open conformation of CB. This in turn frees the PH domain of CB for interactions with PIP3 in the plasma membrane, and the recruitment and clustering of additional NL and gephyrin molecules. The proposed model may be helpful to dissect the sequence of events that take place during the formation of inhibitory synaptic assemblies and the regulation of their molecular composition. However, in broad terms this model has been put forward in a previous study including an overlapping group of scientists (Poulopoulos et al, Neuron 2009) and was also discussed in detail in a recent review (Papadopoulos and Soykan, Frontiers in Cellular Neuroscience 2011). Saying that, the strength of the present study lies in the detailed conformational analysis of CB and the characterization of residues that can affect its folding and interaction profile. Also, the paper is very well structured and clearly written, and the topic is most certainly of interest for the readership of the EMBO Journal.

Major concerns:
1. Mutagenesis: The W24A and E262A mutations appear to shift the dynamic equilibrium of CB from a closed to an open conformation. However, this is only a gradual effect, as best seen in Fig3C. In this context it is not clear why the mutagenesis was done exclusively by replacing individual residues with alanine. Clearly, the introduction of a positively charged lysine or arginine at position E262 may have a more pronounced effect, with potentially more obvious downstream consequences for the clustering behavior of NL and gephyrin. Also, it is not obvious whether the chosen residues play a specific role for the autoinhibition of CB by the SH3 domain, or whether any residue in the DH/PH fold could have a similar effect.
2. COS cell experiments (data shown in Fig4, Fig5E-H): The quantitative analysis of triple transfected cells in this assay is far from trivial, since the clustering of CB, NL and gephyrin will be influenced by the relative expression levels and many other factors such as the cell cycle. A more effective conformational change through mutagenesis (e.g. the double mutant W24A/E262A used in the neuron experiments or other amino acid substitutions) may produce more pronounced effects. According to the legends of Fig4,5 only 10-20 cells were used per construct. This seems to be far too few in view of the experimental variability (unless the number of cells is given per experiment and the total number of cells is in fact three times higher). In order to better judge the strength of the observed phenotypes it would be useful to display non-normalized and non-thresholded data in Fig4B and Fig5H. The quantification of the data in Fig5G,H is missing. Also, high-resolution magnifications should be shown in Fig5H for consistency with the previous panels.

© European Molecular Biology Organization

3. Imaging experiments in neurons (data shown in Fig6,7): Soykan et al have rescued the NL2-/- phenotype by overexpression of various CB variants. However, in order to better judge the effect of the NL2 deletion, it would be helpful to include a wild type (NL+/-) control in these experiments (i.e. using littermates from NL2-/- x NL2+/- matings). Again, thus n = 11-41 refer to the total number of neurons or to the number of neurons for each of three experiments? For this type of analysis a total number of more than 50 neurons per condition would be preferable.
4. Cluster number and cluster size: The authors have quantified the clustering effect of CB by looking at the synaptic clusters of VIATT and gephyrin in neurons expression CB variants. However, what is given as the 'size' of gephyrin clusters is in fact the number of clustered gephyrin molecules, in view of the low spatial resolution of fluorescence microscopy. Rather than the apparent size (in um2) it would be better to quantify the total cluster fluorescence (in fluorescence units). Also, it would be interesting to quantify the fluorescence intensity of the VIATT clusters, in order to see whether the effect by CB on gephyrin clustering is paralleled by changes at the presynapse. As to the quantification of the number of gephyrin clusters, the shown data are not conclusive: While CB expression appears to change the gephyrin cluster number in neurons quite dramatically, it has no significant effect on the number of VIATT clusters nor on the co-localization of gephyrin and VIATT, which is not easily reconciled. The proportion of co-localization of gephyrin and VIATT in the absence of CB should be given in Fig6F,7F.

Minor concerns:
1. Data shown in Fig1E: The lower panel with a triple overlay is not very clear.
2. Data shown in Fig1G/H: clearly, there is a DH/PH band in the GST control lanes, suggesting unspecific binding of GST with CB. This makes the quantitative analysis of these data questionable, given the weak specific interaction between SH3 and DH/PH.
3. Fig2D: The color scheme is unclear in the table (especially yellow and orange), which makes the attribution to a specific conformation difficult.
4. The discussion mostly reiterates the results, which makes it rather long and not very captivating to read.
5. Some concluding statements are made that are inaccurate or that do not result from the previous discussion, e.g.: '*This truncated NL2 mutation failed to activate CB2SH3+ and to induce gephyrin microcluster formation, (...) indicating that the proline-rich stretch of NL2 is essential for CB activation*' (page16) This statement is not strictly correct, since the deletion also covers the PDZ binding domain of NL2. '*These results show that CB2SH- only exists in trace amounts...*' (page20) This is not correct, the data only suggest that there is no CB2SH- expression. '*These results show that the ability of CB to mediate gephyrin clustering is directly related to its ability to bind to membrane phosphoinositides*' (page24). This conclusion does not follow from the previous statements.
6. Page8, line4: reference [25] is missing
7. The structural data of the Cdc42-CB2SH3- complex where taken from the paper by Xiang et al, J Mol Biol 2006. This should be made explicit wherever these data are referred to (e.g. page22 line4).
8. Methods: More detail should be given on the way in which gephyrin and VIATT clusters were detected (thresholding, particle detection, measurement of particle size, etc.).

1st Revision - authors' response 25 June 2014

Point-by-Point Response to Reviewers' Comments

(Reviewers' Comments in Bold)

Referee 1

Brose and colleagues perform a thorough set of experiments to test a previously formulated model for protein-protein interactions that direct the organization of postsynaptic protein complexes. They combine structural, biophysical, biochemical and cell biological assays to probe the mechanism underlying collybistin activation and gephyrin recruitment to inhibitory synapses.

© European Molecular Biology Organization

The structural work is only of modest resolution and it is not entirely clear whether the most definitive predictions emerged from the comparison with the Asef structure or the new structure presented here. However, for the validity of the conclusions that are presented here this is irrelevant.

I have only two minor points:

1) The discussion could be shortened to contain less repetition of the result section.
We thank the reviewer for this suggestion. We have shortened the discussion to contain fewer repetitions of the results.

2) For the lipid binding experiments in Figure 3D it is unclear what the statistical evaluation and n for the analysis was. This should be noted in the figure legend.
For this analysis we used the paired Student's t-test for statistical analysis from a total of three independent experiments. This is now clearly stated in the legend for Fig 3D, as well as in the statistics section of the Materials and Methods (page 37).

Referee 2

The adaptor protein Collybistin (CB) regulates the localization of gephyrin - the main scaffolding protein of inhibitory glycinergic and GABAergic postsynapses. The authors of this manuscript investigated the molecular mechanism of CB autoinhibiton through its SH3 domain, as well as of its activation through neuroligin-2 (NL2) binding. They determined a low resolution crystal structure of an SH3-containing CB, which reveals a "closed" form that closely resembles the architecture of Asef - the closest homologue of CB. Based on the structural information, the authors identified determinants (W24 and E262) of interaction between SH3 and the rest of CB (DH/PH domains). Using their biochemical and cell biological data, as well as an "open" form structure of CB solved by another group, the authors conclude that binding of SH3 to DH/PH keeps CB in a closed form and reduces the affinity of PH to membrane phosphoinositides - this is the basis of SH3-mediated autoinhibition. The authors further identified the NL2 binding site (W52 and W63) within the SH3 domain and provided evidence that binding of NL2 to SH3 stabilizes CB in an open conformation and thus relieves the autoinhibition, resulting in increased gephyrin-clustering activity of CB.

The present work was well designed and provides valuable information on the CB-based regulatory mechanism of inhibitory postsynapses. The manuscript should be of sufficient interest to the readers of EMBO J if the following points could be addressed:

Major:

- The authors mentioned that the SH3 domain of CB1_SH3+ is missing from the electron density map and they hypothesize that it is been proteolytic cleaved. Please do show the SDS-PAGE of the CB1_SH3+ crystals. This is a very important piece of information to prove that the missing SH3 domain in density map is really due to proteolysis.
The SDS-PAGE of the CB1SH3+ crystals has now been included as supplementary Fig S2D. For comparison we also included an SDS-PAGE of CBSH3+ to show that this protein was not degraded.

- A correlation coefficient of 0.59 between the SH3 model and map is low. The electron density of the loop connecting the two beta-sheets is largely invisible, so I suggest that these residues should be removed in the final model. In addition, please provide the correlation coefficient for the rest of the structure (DH/PH).
After additional DEN-refinement in CNS, the maps were improved substantially, resulting in correlation coefficients of 0.74 for the SH3 domain and of 0.87 for the DH/PH tandem (page 7, line 21 and supplementary Fig S2). The electron density for the loop is clearly visible and we hence decided to include the corresponding residues in the model.

- On page 7, please indicate if the root mean square deviation is for all atoms or main chain atoms only. Please also calculate the rmsd of individual domains (DH, PH) between open and closed forms to demonstrate if there is any intra-domain conformational change.

© European Molecular Biology Organization

Furthermore, the authors should analyze domain movements between open and closed states in more detail. Are there any differences other than different conformations of the kink helix that connects the DH and PH domains?
The root mean square deviation (rmsd) on page 7 was calculated for main chain atoms only. We followed the suggestion of this reviewer and analyzed the individual domains and their movements in more detail. There are no major intra-domain conformational changes, however, the PH domain rotates substantially with respect to the DH domain. The detailed results of this analysis are summarized in Table S2 and mentioned in the text (page 9, lines 1-4 and 13-14).

- In discussion, the authors conclude that the affinity between the SH3 domain and the DH/PH domain is lower in CB than in Asef, because CB1_SH3+ lost its SH3 domain during crystallization and pulldown assay showed weak binding of CB SH3 domain to CB2_SH3-. This alone, however, does not provide quantitative information about the affinity. Please provide references regarding the affinity in Asef, as well as explanation how you compare the affinities.
In additional microscale thermophoresis (MST) measurements, we quantified the SH3-DH/PH interaction strength with a KD of 273 µM. This is in (surprisingly) good agreement with a KD of 56 µM calculated with PISA and the van 't Hoff equation from the SH3-DH/PH structural interface. In contrast, the calculated KD of the Asef SH3 DH/PH interface is 11 µM. While the exact value will be different (see our results for CB), we believe that the analysis allows us to conclude that the interdomain interaction in Asef is stronger than in CB. These data are now included in the text (pages 9, 10 and 21) and in Fig 1G.

-The authors proposed that W24, E262 and R70 are the interface forming residues between SH3 and DH/PH domains. However, they only showed data about W24 and E262. Given that the resolution of the structure is low and the exact positions of side chains are uncertain, please provide the data of R70 as well.
To analyze the interaction of the SH3 domain with the DH/PH tandem we performed MST measurements, which, in the new version of our manuscript, now replace the pulldown data of the previous version (Fig 1G, now supplementary Fig S1B). We measured wild type proteins and the W24A, R70A (SH3), and E202A (DH/PH) mutants and found that each single point mutation impairs the interaction significantly. As can be seen from supplementary Fig S1 the R70A mutant weakens the interaction to such a degree that it can no longer be measured by MST.
In addition to the MST measurements, we also included the R70A mutant in the COS cell-based CB autoactivation assay (Fig 4A,B). As compared to WT CB2SH3+, the R70A mutant leads to a more frequent occurrence of submembraneous gephyrin microclusters. Since the effect of the R70A mutation in this assay was not as strong as that of the W24A and E262A mutations, we decided to use the W24A-E262A double point mutants for the subsequent neuron based assays.

Minor:

- Because the "open" form CB (CB2_SH3-) was used for comparison with "closed" form in the manuscript, please include a brief introduction about the "open" form.
This variant is now introduced in the introduction (page 4, end of second paragraph, and page 5, line 2-3).

- On page 8, first paragraph, there is a citation format error about interface forming residues in Asef.
This error has been corrected.

- On page 15, the author mentioned that NL2 and NL4 contain proline-rich stretches in their C-terminal tails, however, according to sequence alignment shown in Fig. 5D, the proline-rich stretch is only present in NL2. Please check.
The alignment has been modified to include all potential proline-rich SH3 domain binding sites in NLs, as determined by the SH3-Hunter software (http://cbm.bio.uniroma2.it/SH3-Hunter/home.html) (Fig 5D and corresponding legend). The software detected these motifs only in NL2 (at three independent sites) and NL4 (at two independent sites). We provide evidence in this manuscript that NL2 binds to the SH3 domain via the proline-rich motif starting from P798 (Fig 5G,H). Despite the presence of several prolines in the cytoplasmic regions of NL1 and NL3, no specific motif for SH3 domain binding was detected in these NL variants, which is in accordance

© European Molecular Biology Organization

with our previous data, according to which NL1 and NL3 do not bind to or activate CB2SH3+ (Poulopoulos et al., 2009; Hoon et al., 2011).

Referee 3

In this paper, Soykan and colleagues have studied the conformational changes of collybistin (CB) and its consequences for the interaction of CB with and the clustering of inhibitory synaptic proteins neuroligin (NL) and gephryin. Using a combination of structural biology, biochemistry and imaging approaches, the authors propose a model whereby CB is 'activated' by NL2, which relieves the inhibitory effect of the SH3 domain and leads to an open conformation of CB. This in turn frees the PH domain of CB for interactions with PIP3 in the plasma membrane, and the recruitment and clustering of additional NL and gephyrin molecules. The proposed model may be helpful to dissect the sequence of events that take place during the formation of inhibitory synaptic assemblies and the regulation of their molecular composition. However, in broad terms this model has been put forward in a previous study including an overlapping group of scientists (Poulopoulos et al, Neuron 2009) and was also discussed in detail in a recent review (Papadopoulos and Soykan, Frontiers in Cellular Neuroscience 2011). Saying that, the strength of the present study lies in the detailed conformational analysis of CB and the characterization of residues that can affect its folding and interaction profile. Also, the paper is very well structured and clearly written, and the topic is most certainly of interest for the readership of the EMBO Journal.

Major concerns:

1. Mutagenesis: The W24A and E262A mutations appear to shift the dynamic equilibrium of CB from a closed to an open conformation. However, this is only a gradual effect, as best seen in Fig3C. In this context it is not clear why the mutagenesis was done exclusively by replacing individual residues with alanine. Clearly, the introduction of a positively charged lysine or arginine at position E262 may have a more pronounced effect, with potentially more obvious downstream consequences for the clustering behavior of NL and gephyrin. Also, it is not obvious whether the chosen residues play a specific role for the autoinhibition of CB by the SH3 domain, or whether any residue in the DH/PH fold could have a similar effect.

We agree with this reviewer in that the effect of W24A and E262A mutations are gradual and we do not achieve a total disinhibition of the protein like the one seen with the ΔSH3 mutant. Also, the combination of two alanine mutations (W24A-E262A) did not have a substantial effect on CB autoactivation beyond the effects of the respective single mutants, as seen in Figure 4A,B. Therefore, it is prudent to assume that replacement of E262 with oppositely charged Lys or Arg residues will have more pronounced effects, as suggested by this reviewer. However, we decided to perform the mutagenesis by replacing individual residues by Ala to minimize the risk of introducing unpredictable side effects on the protein structure, such as misfolding, which could have led to misinterpretations of our data. Given that all three alanine mutants we chose to generate in this study (W24A, R70A and E262A) led to partial disinhibition of the gephyrin clustering activity of CB (as shown in COS7 cell assays) and to a weakening of interdomain interactions between the SH3 and DH/PH domains (as shown by MST measurements), and in view of the shifted equilibrium of the E262A mutant structure towards a less compact state (as shown by SAXS and AFM), we are confident that these are critical residues for maintaining CB in an inactive state in the absence of the corresponding activating ligands. Furthermore, since alanine mutations of other residues in the SH3 domain (e.g. W52A and W63A, as shown in Fig 5E,F) or in the DH domain (N292A-E293A, as shown in Mayer *et al*, 2013) do not lead to CB autoactivation, we believe that the selected residues (W24, R70, E262) do play a specific role for autoinhibition. We sincerely hope that this reviewer will concur with our assessment.

2. COS cell experiments (data shown in Fig4, Fig5E-H): The quantitative analysis of triple transfected cells in this assay is far from trivial, since the clustering of CB, NL and gephyrin will be influenced by the relative expression levels and many other factors such as the cell cycle. A more effective conformational change through mutagenesis (e.g. the double mutant W24A/E262A used in the neuron experiments or other amino acid substitutions) may produce more pronounced effects. According to the legends of Fig4,5 only 10-20 cells were used per

© European Molecular Biology Organization

construct. This seems to be far too few in view of the experimental variability (unless the number of cells is given per experiment and the total number of cells is in fact three times higher).

We agree with this reviewer's criticism of sample size, and have now increased sample sizes to a minimum of 33 cells per construct in the CB-autoactivation assay (Figure 4A,B) and a minimum of 38 cells in the NL2-activation assay (Figure5E,F). In our hands, this sample size is sufficient, and we hope that this reviewer will concur. Further, we also included the single R70A mutant and the double W24A-E262A mutant in the COS cell-based autoactivation assay. However, we failed to detect any pronounced additional effect of the W24A-E262A double mutation beyond the effects of the corresponding single mutations.

In order to better judge the strength of the observed phenotypes it would be useful to display non-normalized and non-thresholded data in Fig4B and Fig5H.

We present the non-normalized data for all COS cell based assays performed in this study (as seen in Fig 4B, 5F, 5H, 5J).

The quantification of the data in Fig5G,H is missing. Also, high-resolution magnifications should be shown in Fig5H for consistency with the previous panels.

We now included the quantifications for the data in Fig 5G,H (now 5I), as well as high-magnification panels for all experiments.

3. Imaging experiments in neurons (data shown in Fig6,7): Soykan et al have rescued the NL2-/- phenotype by overexpression of various CB variants. However, in order to better judge the effect of the NL2 deletion, it would be helpful to include a wild type (NL+/-) control in these experiments (i.e. using littermates from NL2-/- x NL2+/- matings).

The effect of the loss of NL2 was carefully demonstrated in one of our previous studies (Poulopoulos et al. 2009). In the present study, our focus was on the activation of CB and its interplay with NL2. Therefore, we concentrated our efforts on studying CB function (i) in CB-/- neurons, in order to assess the basic function of the corresponding constructs, and (ii) in NL2-/- neurons, in order to assess the dependence of the corresponding CB variants on the presence of NL2. In this context, we feel that our data are comprehensive and very informative. In essence, our data obtained with CB-/- cells (Fig 6) show the dependence of CB function on the reversal of autoinhibition, and our data obtained with NL2-/- neurons (Fig 7) show that this reversal of autoinhibition is - at least in part - mediated by NL2. We truly feel that additional – and very time consuming – experiments on WT neurons would not add any further relevant information, and therefore ask this reviewer sincerely to waive the requirement for such experiments.

Again, thus n = 11-41 refer to the total number of neurons or to the number of neurons for each of three experiments? For this type of analysis a total number of more than 50 neurons per condition would be preferable.

We have now expanded our sample size to a total of around 45 neurons per construct in the CB-/- background (Fig 6 and the corresponding legend) and to around 30 neurons per construct in NL2-/- background (Fig 7 and the corresponding legend). In our hands, this sample size is sufficient, and we hope that this reviewer will concur.

4. Cluster number and cluster size: The authors have quantified the clustering effect of CB by looking at the synaptic clusters of VIATT and gephyrin in neurons expression CB variants. However, what is given as the 'size' of gephyrin clusters is in fact the number of clustered gephyrin molecules, in view of the low spatial resolution of fluorescence microscopy. Rather than the apparent size (in um2) it would be better to quantify the total cluster fluorescence (in fluorescence units).

In addition to the gephyrin cluster size, we now also present the mean fluorescence intensity of gephyrin clusters (Fig 6C and Fig 7C). As expected, the differences in mean fluorescence values between one condition and another (e.g. clusters induced by CB2SH3+ vs. CB2/ΔSH3) nicely

© European Molecular Biology Organization

reflect the corresponding differences in the average size of the clusters (Fig 6D and Fig 7D). We additionally present the total cluster fluorescence of gephyrin clusters (the product of the cluster density, cluster size and mean fluorescence intensity), which can be seen in supplementary Fig 6A,B.

Also, it would be interesting to quantify the fluorescence intensity of the VIATT clusters, in order to see whether the effect by CB on gephyrin clustering is paralleled by changes at the presynapse.

To date, there is no strong evidence for a transsynaptic signaling effect caused by postsynaptic CB and mediated via its interaction with NLs or other synaptic components that would be able to signal transsynaptically. In our study, the different CB variants we tested did not have a strong effect on presynaptic innervation as assessed by VIAAT cluster density (Fig 6E, 7E). Nevertheless, we followed the advice of this reviewer and looked into this issue more carefully. Indeed, we detected very minor increases (~10%) in the mean fluorescence intensity and average size of VIAAT clusters in CB-/- and NL2-/- neurons expressing GFP alone, as compared to neurons expressing CB2SH3+. These changes are likely of homeostatic nature and can be explained by a compensatory increase in the presynaptic input strength due to the corresponding postsynaptic perturbations. Overexpression of constitutively active CB2/ΔSH3 in CB-/- neurons also led to increased VIAAT cluster size and intensity, which is best compatible with the opposite scenario, where significantly larger gephyrin clusters may induce the expansion of the corresponding presynaptic input. These data are now shown in supplementary Figs S6C-F and discussed in the Discussion section, page 25, end of second paragraph.

As to the quantification of the number of gephyrin clusters, the shown data are not conclusive: While CB expression appears to change the gephyrin cluster number in neurons quite dramatically, it has no significant effect on the number of VIATT clusters nor on the co-localization of gephyrin and VIATT, which is not easily reconciled. The proportion of co-localization of gephyrin and VIATT in the absence of CB should be given in Fig6F,7F.

This point of the reviewer is important but can be explained. In CB-/- and NL2-/- neurons, VIAAT clusters are generally more dense (0.13-0.17 clusters per mm2) and larger (0.20-0.27 mm2) than gephyrin clusters (0.06-0.17 clusters per mm2, 0.13-0.27 mm2 in size), indicating presynaptic inputs with little or no postsynaptic gephyrin, so that VIAAT clusters outnumber gephyrin clusters. This is typically observed in the culture system we are using, and may be due to some 'orphan' presynapses. Correspondingly, new gephyrin clusters induced by CB2/ΔSH3 or CB2SH3+/W24A-E262A were formed at these orphan presynapses and were thus still synaptic. This is reflected by essentially unaltered proportions of synaptic gephyrin clusters, indicating that newly induced gephyrin clusters formed at sites with prior innervation. The corresponding complete dataset on the proportion of co-localization of gephyrin and VIATT in the absence of CB is now presented in Figs 6F and 7F and discussed in the Discussion section, page 25, beginning of second paragraph.

Minor concerns:
1. Data shown in Fig1E: The lower panel with a triple overlay is not very clear.
The indicated panel has been updated. We hope that the improvement of the presentation is adequate.

2. Data shown in Fig1G/H: clearly, there is a DH/PH band in the GST control lanes, suggesting unspecific binding of GST with CB. This makes the quantitative analysis of these data questionable, given the weak specific interaction between SH3 and DH/PH.
We have now replaced the GST-pulldown data with more quantitative microscale thermophoresis (MST) measurements (Fig 1G), which clearly show that W24A, R70A and E262A mutations exhibit highly significant effects on the interdomain interactions between SH3 and DH/PH domains.

3. Fig2D: The color scheme is unclear in the table (especially yellow and orange), which makes the attribution to a specific conformation difficult.
The color scheme has been modified for easier matching of the SAXS-derived values on the table with the surface representations.

4. The discussion mostly reiterates the results, which makes it rather long and not very

captivating to read.
We have now revised the discussion and eliminated reiterations of the results.

5. Some concluding statements are made that are inaccurate or that do not result from the previous discussion, e.g.: 'This truncated NL2 mutation failed to activate CB2SH3+ and to induce gephyrin microcluster formation, (...) indicating that the proline-rich stretch of NL2 is essential for CB activation' (page16) This statement is not strictly correct, since the deletion also covers the PDZ binding domain of NL2. 'These results show that CB2SH- only exists in trace amounts...' (page20) This is not correct, the data only suggest that there is no CB2SH- expression. 'These results show that the ability of CB to mediate gephyrin clustering is directly related to its ability to bind to membrane phosphoinositides' (page24). This conclusion does not follow from the previous statements.
We are grateful for these comments. As regards the functional characteristics of the truncated NL2 mutation and their interpretation, we agree with this reviewer and phrased the corresponding text passage more conservatively (Page 17, end of first paragraph). Regarding the statement on the abundance of CBSH3-, we wanted to be careful because this variant has featured prominently in the literature. It is true that our data indicate that no such variant is expressed in brain, but we cannot exclude that (i) it is expressed in a wide-spread manner but only in trace amounts that have escaped our detection, or that (ii) it is expressed only in very restricted brain regions or certain types of cells so that it escaped our detection. We therefore prefer to maintain the more conservative statement of the original version of our manuscript, with a slight modification ('These results indicate that CB2SH3- might exist only in trace amounts…', Page 20, last sentence) and hope that this reviewer will concur. Concerning the statement on the relationship between phosphoinositide binding and gephyrin clustering by CB, we agree with this reviewer's comment. The sentence immediately preceding the indicated statement in our original manuscript does not really allow for such a strong conclusion. However, we feel strongly that our data in their entirety (i.e. including all points made in the entire preceding paragraph) do confirm that the ability of CB to mediate gephyrin clustering depends on its ability to bind to membrane phosphoinositides, and that they show in addition that the conformational activation of CB facilitates phosphoinositide binding and gephyrin clustering. The corresponding passage in the discussion (Page 24, lines 8-13) has been revised accordingly, and we hope that these changes find the approval of this reviewer.

6. Page8, line4: reference [25] is missing
We thank the reviewer for pointing this out. The mistake has been corrected.

7. The structural data of the Cdc42-CB2SH3- complex where taken from the paper by Xiang et al, J Mol Biol 2006. This should be made explicit wherever these data are referred to (e.g. page22 line4).
We thank the reviewer for pointing this out. The reference has been inserted (Page 22, end of second paragraph).

8. Methods: More detail should be given on the way in which gephyrin and VIATT clusters were detected (thresholding, particle detection, measurement of particle size, etc.).
A better description of the data analysis for neuron-based assays is now provided in the Materials and Methods (Page 37, line 2-11).

Accepted 09 July 2014

Thank you for submitting your revised manuscript to The EMBO Journal. Your revision has now been re-reviewed by referee #2. As you can see below the referee appreciates the introduced changes. I am therefore very pleased to accept the paper for publication here.

REFEREE REPORT

Referee #2

The authors have addressed all of my previous concerns.