



SNAP23 deficiency causes severe brain dysplasia through the loss of radial glial cell polarity

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November 22, 2019

Re: JCB manuscript #201910080

Prof. Akihiro Harada
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Japan

Dear Prof. Harada,

Thank you for submitting your manuscript entitled "SNAP23 deficiency causes severe brain dysplasia through the loss of neural progenitor cell polarity". Your manuscript has been assessed by expert reviewers, whose comments are appended below. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

You will see that, although one of the reviewers is concerned that the current study does not represent a sufficient advance for JCB, the other reviewers are somewhat more enthusiastic. However, all three reviewers raise a number of substantive concerns which will need to be addressed before the paper would be deemed suitable for publication in JCB. These reviewers have also provided fairly clear direction on how you might proceed and so we hope that you will be able to address all of the reviewers' concerns in full.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. Our typical timeframe for revisions is three to four months; if submitted within this timeframe, novelty will not be reassessed. We would be open to resubmission at a later date; however, please note that priority and novelty would be reassessed.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Your manuscript may have up to 10 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, <http://jcb.rupress.org/site/misc/ifora.xhtml>. All figures in accepted manuscripts will be screened prior to publication.

*****IMPORTANT:** It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication.

Please ensure that you have access to all original microscopy and blot data images before submitting your revision.***

Supplemental information: There are strict limits on the allowable amount of supplemental data. Your manuscript may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Yukiko Gotoh, PhD
Monitoring Editor
Journal of Cell Biology

Tim Spencer, PhD
Executive Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

This paper presents a thorough description of the effects of SNAP23 gene disruption or knockdown on brain development, together with a less complete, but still quite convincing, study of effects of VAMP8 and Stx1B deletion (by in utero Crispr). In all cases the apical junctional complexes of the neural progenitors are disrupted and all other defects follow logically from there. The phenotypes strongly resemble those caused by N-cadherin gene disruption and mutations in other polarity genes. So the main finding is that this particular SNARE complex is needed for traffic of N-cadherin, and potentially other junction proteins, to the cell surface. I question whether the paper goes far enough for JCB unless the authors can show that this specific SNARE complex is needed for N-cadherin specifically. It is possible that SNAP23, VAMP8 and Stx1B are the only SNARE proteins involved in docking secretory vesicles to the plasma membrane in these cells? If so, the results are to be expected.

1. The conclusion that "SNARE-mediated localization of N-cadherin is essential for AJC formation, NPC polarization and brain development" as claimed in the abstract, is an over statement, since other proteins besides N-cadherin may be also be trafficked incorrectly. The conclusion is almost certainly true (given the known effect of N-cadherin gene disruption), but to prove that N-cadherin is the essential SNARE cargo in this case would require some kind of rescue experiment.

2. The finding that SNARE complexes regulate fusion of vesicles carrying specific cargoes is not

new. What is missing here is some insight into how the specificity is achieved. Is SNAP23 required for all Golgi to plasma membrane transport in the apical progenitors? (SNAP25 appears not to be expressed in the apical progenitors). Is VAMP8 enriched on secretory vesicles containing N-cadherin and not on secretory vesicles containing other cargo? Do Stx1B and SNAP23 localize to the AJCs or are they distributed along the basal process?

Minor points:

1. The cerebellar defect may be better illustrated by sagittal sections.
2. p12. It is not valid to conclude that the increased neuronal death seems to be the cause of the severe brain malformation. The increased death may increase the severity of the phenotype and small brain size, but the malformations seen are common to mutations that affect progenitor polarity.
3. Figs 7C, 8E, F would benefit from including data for another biotinylated cell surface protein. Is all surface protein transport inhibited in the SNAP23-depleted NPCs?
4. The RUSH experiments are interesting, and show nicely that SBP-EGFP-N-cadherin co-localizes with mCherry-VAMP8. This assay could be taken further - eg, how about mCherry-VAMP2? SBP-EGFP-E-cadherin? Also, the traffic wasn't followed to the surface. With some modifications, this approach could be used to follow the kinetics of SBP-EGFP-N-cadherin transport from ER to surface in NPCs lacking Stx1B or SNAP23.
5. Fig 10 would benefit from more negative controls: eg, guide RNA against SNAP25.

Reviewer #2 (Comments to the Authors (Required)):

In the submitted manuscript, Kunii et al. describe the function of SNAP23 SNARE complex protein in the development of the brain.

SNAP23 is a SNARE protein that is involved in exocytosis in multiple tissues.

The authors, for the first time, investigated its role in the developing brain utilizing a conditional knock-out mouse they generated previously. They could show that deletion of SNAP23 in the brain with Nestin-Cre line causes severe malformation of the dorsal forebrain as well as cerebellum. They show that SNAP23 is important for the localization of N-cadherin to the cell membrane in cortical progenitors, and this complex is necessary for the apico-basal polarization of NPCs and subsequent normal brain development. Furthermore, they identified VAMP8 and Stx1B as binding partners and demonstrated that they induce a similar phenotype in loss-of-function experiments.

The manuscript, for the first time provides a mechanistic insight into the role of exocytosis in the cortical stem cells polarity and differentiation.

This is an impressive body of work that uses variable approaches. The results are convincing and well described.

I have only minor critique points, mostly about writing and presentation of the results. The manuscript is too heavy, contains ten main figures and seven supplementary. I would suggest rearranging the figures, fusing some of them and transferring some phenotype description figures into the supplement. On the other hand, I would suggest taking data on Notch and beta-catenin signaling as well as the final diagram from the supplement into the main figures.

Below are some other minor points:

-I suggest using more conventional terminology, for example "ventral or dorsal cerebral cortex" terms are usually used for primate brain. For the mouse it is more appropriate, to call respective regions lateral or medial cortex.

-It would be also useful for PubMed searchability to also use radial glia cells along with NPC.

"The primordium of the hippocampus was completely absent " (Fig1 for P0 brain) - typically we call primordium of the hippocampus the structure that we see earlier, at E15-16, but not at P0

- Statement that "SNAP23 was localized to the apical side of NPCs (Fig. 2C, arrows) " might be too strong, as it can be understood as it is restricted to the apical side, while it looks like it is on the the entire cell membrane.
- may be it would be more appropriate to describe first the expression of SNAPs and then the phenotype of the mutants
- Fig 3 AB do not contain markers used for IHCh
- cilium seems to be affected in the mutant. Did the authors consider looking at Shh pathway

Reviewer #3 (Comments to the Authors (Required)):

This manuscript reports the effects of CNS ablation of SNAP23 in mice (NcKO mice), showing that this results in severe hypoplasia of the neocortex and no hippocampus or cerebellum. The authors showed that the lack of SNAP23 causes the disruption of apical junctional complexes (AJCs) and loss of polarity in neural progenitor cells in the developing brain as well as a downregulation of beta-catenin and notch signalling. They observed that a variety of AJC markers including N-cadherin were substantially decreased or lost at the ventricular surface in the NcKO cerebral cortex and cerebellum. In wild type mouse embryos SNAP23 was observed to colocalize with N-cadherin in the apical processes of NPCs. The authors therefore investigated whether and how SNAP23 was involved in localizing N-cadherin to the apical plasma membrane. Using cultured, isolated NPCs from the E13.5 cerebral cortex and an siRNA knockdown protocol, they demonstrated that depletion of SNAP23 decreased localization of N-cadherin to the plasma membrane and inhibited cell aggregation. The authors immunoprecipitated SNAP23 and in further knockdown experiments found that other SNAREs required for N-cadherin localization and cell aggregation were VAMP8 and Stx1B. They also carried out RUSH experiments in COS7 cells and showed co-localization of tagged N-cadherin and tagged VAMP8 in some post-Golgi vesicles migrating to the cell surface. The authors concluded that a SNARE complex composed of SNAP23, VAMP8 and Stx1B plays a crucial role in AJC formation, NPC polarization and brain development. There are some major points which the authors need to address with respect to their experiments on the SNAREs required for delivery of N-cadherin to the cell surface, given the prominence of the claim concerning the SNARE complex composed of SNAP23, VAMP8 and Stx1B.

Major points

1. In their siRNA knockdown experiments for various SNAREs (e.g. in Figures 7, 8 and S2), the authors show the effects of only one siRNA and never attempt any rescue experiments by over-expressing siRNA-resistant SNAREs. A minimal requirement should be 2 independent siRNAs and a gold-standard for this type of experiment is to 'rescue' if possible.
2. Other than for SNAP23 in Figure 7, SNARE knockdown is only shown at the level of mRNA by RTPCR (see Figure 2) rather than demonstrating protein depletion by immunoblotting. For VAMP8 and Stx1B, the authors do have antibodies which they have used for immunofluorescence microscopy in Figure 8A and for other SNAREs knocked down in Figure S2B there are commercially available antibodies that are claimed to react with the mouse as well as human VAMP. It is not clear why immunoblotting has not been used to show SNARE protein depletion other than for SNAP23.
3. In Figure S2B, the authors show the effects of siRNAs to several SNAREs on cell aggregation. However, the data are not quantified. This should be done in the same way as for the knockdown of SNAP23 in Figure 7F.
4. In Figure 8, the authors show the effects of knocking down VAMP8 and Stx1B on delivery of N-cadherin to the cell surface. This experiment should have further controls e.g. the knockdown of

VAMP3 and the knockdown of Stx1A. These controls are very important given that only 1 peptide for Stx1B was found in the mass spec of the immunoprecipitates of SNAP23 (Table 1) i.e. fewer than for VAMP3 and the authors speculate on page 24 that Stx1A may compensate for loss of Stx1B. Given the presence of only 1 Stx1B peptide in the mass spec analysis of the SNAP23 immunoprecipitate, it may also be useful to blot the immunoprecipitate with an antibody to Stx1A to see if it is present.

5. The RUSH experiments on COS7 cells shown add very little if anything to the present manuscript. Whilst evidence is presented for co-localization of tagged N-cadherin and tagged VAMP8 in post-Golgi vesicles migrating to the cell surface there is no real evidence that in this cell type the pathway has similar characteristics/requirements to the pathway in cultured NPCs. For example, in COS7 cells can the delivery of N-cadherin to the cell surface be inhibited by depletion of SNAP23, VAMP8 and Stx1B ?

Minor point

1. P8. Bottom paragraph. The second sentence in this paragraph reports disorganization of ventricular walls in the dorsal cortex and cerebellum in the NcKO E13.5 embryos. The third sentence says that SNAP23 was expressed in NPCs and neurons at E13.5. Presumably this refers to Figure 2C. The authors should make it clearer in the text that this expression is in the wild type/control embryos.

Aug.23, 2020

Dear Editor,

Please find enclosed our manuscript titled “SNAP23 deficiency causes severe brain dysplasia through the loss of radial glial cell polarity”. We thank you and all the reviewers for their valuable comments and suggestions. We have added data and rewritten the previous manuscript according to the valuable suggestions from all reviewers. We have included these additional data in the figures and supplemental figures. In addition, we changed the last part of the title (‘radial glial cell polarity’) of the manuscript from the previous one (‘neural progenitor cell polarity’) according to the advice of the second reviewer. If you think it is inappropriate to change during the revision, we are ready to use the previous title.

In response to the reviewer’s comments, the authors revised the manuscript as follows.

Reviewer #1

Major points:

1. The conclusion that "SNARE-mediated localization of N-cadherin is essential for AJC formation, NPC polarization and brain development" as claimed in the abstract, is an over statement, since other proteins besides N-cadherin may be also be trafficked incorrectly. The conclusion is almost certainly true (given the known effect of N-cadherin gene disruption), but to prove that N-cadherin is the essential SNARE cargo in this case would require some kind of rescue experiment.

We appreciate the comment from this reviewer. As mentioned above, the localization of a number of plasma membrane (PM) proteins might decrease in SNAP23-depleted RGCs. To determine the amount of PM proteins in SNAP23-depleted cells, we first performed a comprehensive analysis of the biotinylated PM proteins in the control and SNAP23-depleted cells by mass spectrometry. However, it was difficult to determine the difference in the amount of PM proteins between these two groups. The nonspecific binding of unbiotinylated proteins to streptavidin beads might have influenced the number of peptides detected because of the high sensitivity of the mass spectrometer. Therefore, we compared the amount of several biotinylated proteins by immunoblotting. The results showed that, in addition to N-cadherin, the PM localization

of β 1-integrin and Ephrin-B1 was decreased in the SNAP23-depleted RGCs. In contrast, the PM localization of low-density lipoprotein receptor (LDLR) and Na^+/K^+ -ATPase was relatively unaffected in the SNAP23-depleted RGCs (Fig. 5, C and D), which indicates the selectivity of SNAP23 in the delivery of PM proteins.

To show the involvement of N-cadherin in the phenotype acquisition of SNAP23 KO, we attempted rescue experiments as suggested by this reviewer by *in utero* electroporation (IUE). First, we simply electroporated the N-cadherin-mCherry plasmid and Cas9 with the SNAP23 sgRNA plasmid into the cerebral cortex with the hope that overexpressing N-cadherin might overcome the defects caused by the reduction in SNAP23. However, the formation of AJCs and the shape of the ventricular surface were not rescued (Fig. 5F), suggesting that even when N-cadherin is overexpressed, N-cadherin might not be delivered to the PM without SNAP23. Then, we modified a part of the N-cadherin structure to try to localize N-cadherin to the PM. As described previously, the PM localization of LDLR is relatively unaffected in SNAP23-depleted RGCs (Fig. 5, C and D), and we speculated that LDLR is delivered to the PM by SNAP23-independent pathways. We used the transmembrane domain (TMD) and cytoplasmic region of LDLR because these domains are necessary for the trafficking of LDLR. We made a chimeric construct in which the TMD and a part of the cytoplasmic region between the TMD and p120-catenin-binding region of N-cadherin were replaced with the TMD and a part of the cytoplasmic region of LDLR (referred to as N-cadherin-LDLR) (Fig. 5E). After electroporation of N-cadherin-LDLR-mCherry and Cas9 with the SNAP23 sgRNA plasmid into the cerebral cortex, the formation of AJCs and the shape of the ventricular wall were rescued (Fig. 5G). Further, the expression of N-cadherin-LDLR also rescued the abnormal polarity, proliferation, and differentiation of the RGCs in the SNAP23-depleted cortex (Fig. 6). We thank this reviewer because we can now show the selectivity of SNAP23 in the delivery of N-cadherin in the process of addressing this comment. Considering these results, we conclude that the decreased localization of N-cadherin to the apical PM is a primary cause of the severe dysplasia in NcKO mice.

The results are described in the manuscript from p. 14, line 10 to p. 16, line 11.

2. The finding that SNARE complexes regulate fusion of vesicles carrying specific cargoes is not new. What is missing here is some insight into how the specificity is achieved. Is SNAP23 required for all Golgi to plasma membrane transport in the apical progenitors? (SNAP25 appears not to be expressed in the

apical progenitors).

As described above, the localization of some membrane proteins (N-cadherin, β 1-integrin, and Ephrin-B1) was influenced by SNAP23 depletion; however, we found that the localization of the other proteins, namely, LDLR and Na^+/K^+ -ATPase, was unaffected (Fig. 5, C and D), suggesting cargo selectivity of SNAP23. We consider that the other SNAP25 family protein, SNAP29 or SNAP47, may be involved in the trafficking of these proteins; however, we apologize that we could not determine the mechanism of the cargo selectivity of the SNARE proteins within the revision period. However, our rescue experiments using N-cadherin-LDLR suggest that the TMD and/or sorting motif in the cargo proteins may be important for cargo selection by SNARE proteins.

This description was added in the “Discussion” section, p. 25, lines 5-13.

Is VAMP8 enriched on secretory vesicles containing N-cadherin and not on secretory vesicles containing other cargo?

To address this comment, we used an additional RUSH reporter plasmid, streptavidin-KDEL_SBP-EGFP/mCherry-GPI (Addgene #65294/#65295), and examined the colocalization of SBP-EGFP-N-cadherin or SBP-EGFP-GPI and mCherry-VAMP8 or mCherry-VAMP2 in the COS7 cells. We found that a number of SBP-EGFP-N-cadherin transport vesicles colocalized with mCherry-VAMP8 but few colocalized with mCherry-VAMP2 (Fig. 9, B, C, and F). In contrast, many SBP-EGFP-GPI transporting vesicles were colocalized with mCherry-VAMP2, but few were colocalized with mCherry-VAMP8 (Fig. 9, D-F). These results suggest that VAMP8 preferentially localizes to transport vesicles containing N-cadherin but not vesicles containing GPI-anchor proteins.

The results are described from p. 20, line 6 to p. 21, line 5 and from p. 21, lines 9-17.

Do Stx1B and SNAP23 localize to the AJCs or are they distributed along the basal process?

To address this comment, we confirmed the localization of SNAP23, VAMP8,

and Stx1B in the WT cerebral cortex at E13.5 (Fig. S1). Immunostaining images of these SNARE proteins showed similar localization. All three SNAREs were enriched on the apical side of the RGCs and were also weakly localized to basal processes (Fig. S1A). In the apical processes, these SNAREs colocalized with N-cadherin (Fig. S1C).

The results are described on p. 8, lines 11-13 and p. 19, lines 7-9.

Minor points:

1. The cerebellar defect may be better illustrated by sagittal sections.

To address this comment, we added figures showing sagittal sections of the cerebellum at E16.5 and P0 (Fig. 1, E and H).

2. p12. It is not valid to conclude that increased neuronal death seems to be the cause of severe brain malformation. The increased death may increase the severity of the phenotype and small brain size, but the malformations seen are common to mutations that affect progenitor polarity.

According to this valuable comment, we rewrote the sentence as follows. “Thus, increased neuronal cell death seems to be the cause of the reduced brain size in the NcKO mice” on p. 12, lines 3-4.

3. Figs 7C, 8E, F would benefit from including data for another biotinylated cell surface protein. Is all surface protein transport inhibited in SNAP23-depleted NPCs?

Please see the response to the major point 1.

4. The RUSH experiments are interesting and show nicely that SBP-EGFP-N-cadherin co-localizes with mCherry-VAMP8. This assay could be taken further - eg, how about mCherry-VAMP2? SBP-EGFP-E-cadherin?

We appreciate the reviewer's comment. As previously described in response to

major point 2, we found that SBP-EGFP-N-cadherin transport vesicles colocalized extensively with mCherry-VAMP8 but not with mCherry-VAMP2 (Fig. 9, B, C, and F). In contrast, SBP-EGFP-GPI transporting vesicles colocalized extensively with mCherry-VAMP2 but not with mCherry-VAMP8 (Fig. 9, D-F). We also found that most SBP-mCherry-E-cadherin colocalized with SBP-EGFP-N-cadherin; however, we were not able to show the data in the manuscript due to limited space

In addition, the traffic was not followed to the surface. With some modifications, this approach could be used to follow the kinetics of SBP-EGFP-N-cadherin transport from the ER to the surface in NPCs lacking Stx1B or SNAP23.

According to this valuable comment from the reviewer, we stained nonpermeabilized COS7 cells with a GFP antibody 60 min after biotin treatment. We were able to detect the cell surface localization of SBP-EGFP-N-cadherin. Cell surface staining was clearly decreased in the SNAP23-, VAMP8-, or Stx1B-depleted COS7 cells compared to the control cells, suggesting that the transport of SBP-EGFP-N-cadherin was decreased in these cells (Fig. S3A).

The results are described from p. 20, lines 13-17.

5. Fig 10 would benefit from more negative controls: eg, guide RNA against SNAP25.

To address this comment, we performed CRISPR/Cas9 KO of SNAP25 through the IUE of Cas9 and sgRNA plasmids at E13.5. Two days after electroporation, the apical staining of Par3 was intact (Fig. S5A), suggesting that SNAP25 is not involved in AJC formation. The depletion efficiency of sgRNA was confirmed in the neuronal layer of the cortex (Fig. S5B), as indicated by SNAP25 expression at much lower levels in the RGCs (Fig. S1B).

The results are described on p. 22, lines 9-11.

Reviewer #2

Minor points:

The manuscript is too heavy, contains ten main figures and seven

supplementary. I would suggest rearranging the figures, fusing some of them and transferring some phenotype description figures into the supplement.

We appreciate the comment from this reviewer. Although we made every effort to shorten the manuscript as much as possible, additional experiments were necessarily performed to meet the requests from other reviewers. We apologize for our lengthy manuscript, but we are following the submission rules of JCB with regard to the length of the manuscript and number of figures.

I would suggest taking data on Notch and beta-catenin signaling as well as the final diagram from the supplement into the main figures.

According to this valuable suggestion, we moved the data of Notch and β -catenin signaling from Fig. S4 to a new Fig. 7 and the final diagram from Fig. S5 to a new Fig. 10C.

-I suggest using more conventional terminology, for example "ventral or dorsal cerebral cortex" terms are usually used for primate brain. For the mouse it is more appropriate, to call respective regions lateral or medial cortex.

According to this valuable suggestion, we deleted “dorsal” and changed “ventral” to “medial”.

-It would be also useful for PubMed searchability to also use radial glia cells alone with NPC.

We appreciate the reviewer’s suggestion. We changed “NPC” to “radial glial cell (RGC)”.

-"The primordium of the hippocampus was completely absent" (Fig1 for P0 brain) - typically we call primordium of the hippocampus the structure that we see earlier, at E15-16, but not at P0

We deleted “primordium” according to the suggestion from the reviewer.

-Statement that "SNAP23 was localized to the apical side of NPCs (Fig. 2C, arrows) " might be too strong, as it can be understood as it is restricted to the apical side, while it looks like it is on the the entire cell membrane.

According to the suggestion from the reviewer, we rewrote the sentence as follows. “In RGCs, SNAP23 was localized to both the apical and basal processes but favored the apical side (Fig. S1, A and B, arrows).”

Please look at p. 8, lines 12-13.

-may be it would be more appropriate to describe first the expression of SNAPs and then the phenotype of the mutants

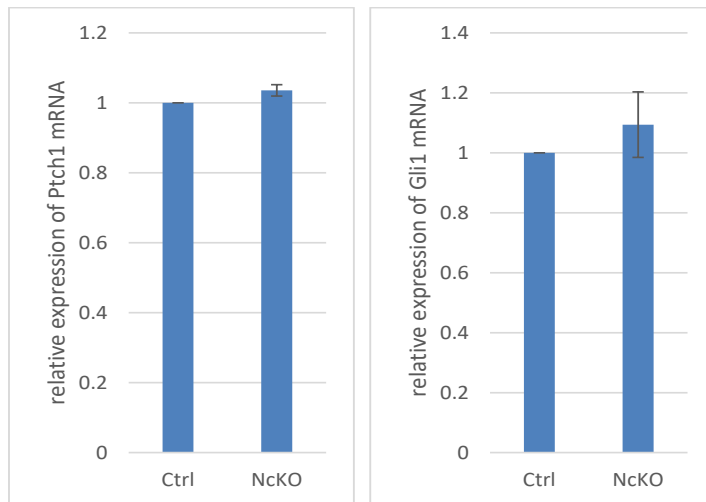
We appreciate this comment. Restructuring the manuscript in addition to the revision would require extensive time, and we deeply apologize for not being able to meet this request.

-Fig 3 AB do not contain markers used for IHC.

We appreciate the reviewer for pointing this out. We added marker information to the pictures.

-cilium seems to be affected in the mutant. Did the authors consider looking at Shh pathway?

We appreciate this valuable question. To answer this question, we determine the expression of some target genes of the Shh pathway using quantitative real-time PCR. We found that the expression of Ptch1 and Gli1 was comparable between the Ctrl and NcKO brains (n= 4), suggesting that Shh signaling was unaffected. We apologize that we could not include these data in the figure because of space limitations.



Reviewer #3

Major point:

1. In their siRNA knockdown experiments for various SNAREs (e.g. in Figures 7, 8 and S2), the authors show the effects of only one siRNA and never attempt any rescue experiments by over-expressing siRNA-resistant SNAREs. A minimal requirement should be 2 independent siRNAs and a gold-standard for this type of experiment is to 'rescue' if possible.

To address this valuable comment, we increased the number of siRNA oligos to obtain additional knockdown RGCs of each SNARE protein. We confirmed the depletion efficiency of the siRNAs by immunoblotting, and the results of the cell aggregation assay were comparable for the two different siRNAs (Fig. S2, A-C). We apologize that we were not able to perform rescue experiments with siRNA-resistant SNAREs within the revision period.

The results are described on p. 19, lines 4-7.

2. Other than for SNAP23 in Figure 7, SNARE knockdown is only shown at the level of mRNA by RTPCR (see Figure 2) rather than demonstrating protein depletion by immunoblotting. For VAMP8 and Stx1B, the authors do have antibodies which they have used for immunofluorescence microscopy in Figure 8A

and for other SNAREs knocked down in Figure S2B there are commercially available antibodies that are claimed to react with the mouse as well as human VAMP. It is not clear why immunoblotting has not been used to show SNARE protein depletion other than for SNAP23.

To address this comment, we confirmed the depletion efficiency of the siRNAs by immunoblotting (Fig. S2A).

3. In Figure S2B, the authors show the effects of siRNAs to several SNAREs on cell aggregation. However, the data are not quantified. This should be done in the same way as for the knockdown of SNAP23 in Figure 7F.

In response to this comment, we quantified the density of the aggregates in each SNARE-depleted RGC group based on 3 independent analyses (Fig. S2C).

4. In Figure 8, the authors show the effects of knocking down VAMP8 and Stx1B on delivery of N-cadherin to the cell surface. This experiment should have further controls e.g. the knockdown of VAMP3 and the knockdown of Stx1A. These controls are very important given that only 1 peptide for Stx1B was found in the mass spec of the immunoprecipitates of SNAP23 (Table 1) i.e. fewer than for VAMP3 and the authors speculate on page 24 that Stx1A may compensate for loss of Stx1B. Given the presence of only 1 Stx1B peptide in the mass spec analysis of the SNAP23 immunoprecipitate, it may also be useful to blot the immunoprecipitate with an antibody to Stx1A to see if it is present.

We appreciate the critical comments from this reviewer. To address these comments, we first observed the localization of N-cadherin in VAMP3-, VAMP4-, or VAMP5-depleted RGCs. We found that the PM localization of N-cadherin was unaffected in each RGC knockdown, suggesting that these VAMPs are not involved in the transport of N-cadherin in RGCs (Fig. S2D). Stx1A was found to coimmunoprecipitate with SNAP23 in the lysate of the RGCs, as shown by immunoblotting (Fig. S5C). This result suggested that Stx1A might also be involved in the localization of N-cadherin to the PM in RGCs. However, when we knocked out Stx1A in the cerebral cortex through IUE, apical staining of Par3 remained intact (Fig.

S5D). This result suggests that Stx1B, but not Stx1A, is a major Qa-SNARE that is involved in the localization of N-cadherin to the PM in RGCs.

The results are described on p. 19, lines 13-14, and from p. 22, line 12 to p. 23, line 7.

5. The RUSH experiments on COS7 cells shown add very little if anything to the present manuscript. Whilst evidence is presented for co-localization of tagged N-cadherin and tagged VAMP8 in post-Golgi vesicles migrating to the cell surface there is no real evidence that in this cell type the pathway has similar characteristics/requirements to the pathway in cultured NPCs. For example, in COS7 cells can the delivery of N-cadherin to the cell surface be inhibited by depletion of SNAP23, VAMP8 and Stx1B?

We appreciate the critical comment from the reviewer. To address this comment, we performed immunoblotting to confirm the expression of N-cadherin in the COS7 cells. We found that, similar to the findings with the RGCs, N-cadherin is abundantly expressed in the COS7 cells, but E-cadherin is not (Fig. 8H). Immunofluorescence images showed that N-cadherin was localized to the PM of the COS7 cells (Fig. 8I). This localization was greatly decreased, and cell-cell contact was lost in SNAP23-, VAMP8-, or Stx1B-depleted COS7 cells (Fig. 8, I-K). The PM localization of exogenously expressed SBP-EGFP-N-cadherin was also decreased in the SNAP23-, VAMP8-, or Stx1B-depleted COS7 cells (Fig. S3A). These results suggest that N-cadherin is likely delivered in a similar manner in RGCs and COS7 cells.

The results are described from p. 19, line 18 to p. 20, line 17.

Minor point:

1. P8. Bottom paragraph. The second sentence in this paragraph reports disorganization of ventricular walls in the dorsal cortex and cerebellum in the NcKO E13.5 embryos. The third sentence says that SNAP23 was expressed in NPCs and neurons at E13.5. Presumably this refers to Figure 2C. The authors should make it clearer in the text that this expression is in the wild type/control embryos.

According to the comment from this reviewer, we rewrote the sentence as follows.

“SNAP23 was expressed in both RGCs and neurons in the control cortex at E13.5.”

Please see p. 8, lines 11-12.

After making the revisions, we consider our manuscript to be significantly improved compared with the previous manuscript, and we hope that it satisfies the suggestions and addresses the uncertain points identified by you and the reviewers. During the revision, we added an author, Satoshi Kanda, for his contribution.

We apologize for spending substantial time while performing additional experiments for use in revising the manuscript, and we greatly appreciate your patience in waiting to receive our revision under this COVID-19 pandemic situation.

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Thank you for your kind consideration.

Sincerely yours,

Akihiro Harada, MD, PhD
Department of Cell Biology
Graduate School of Medicine
Osaka University

October 8, 2020

RE: JCB Manuscript #201910080R

Prof. Akihiro Harada
Osaka University
2-2 Yamadaoka
Suita, Osaka 565-0871
Japan

Dear Prof. Harada:

Thank you for submitting your revised manuscript entitled "SNAP23 deficiency causes severe brain dysplasia through the loss of radial glial cell polarity". The paper has now been assessed again by the original reviewers and, as you'll see below, they all recommend acceptance. Therefore, we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, <https://jcb.rupress.org/submission-guidelines#revised>. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles and Tools is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends. At this time, you are well below this limit but please bear it in mind when revising.

2) Figures limits: Articles and Tools may have up to 10 main text figures. You currently meet this limit but please bear it in mind when revising.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, since you used parametric tests in your study (e.g. t-tests, ANOVA, etc.), you should have first determined

whether the data was normally distributed before selecting that test. In the stats section of the methods, please indicate how you tested for normality. If you did not test for normality, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

6) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies.

7) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:

- a. Make and model of microscope
- b. Type, magnification, and numerical aperture of the objective lenses
- c. Temperature
- d. imaging medium
- e. Fluorochromes
- f. Camera make and model
- g. Acquisition software
- h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

8) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

9) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental figures. At the moment, you meet this limit but please bear it in mind when revising.

Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

10) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

11) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (<https://casrai.org/credit/>).

12) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and video files: See our detailed guidelines for preparing your production-ready images, <https://jcb.rupress.org/fig-vid-guidelines>.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.

The license to publish form must be signed before your manuscript can be sent to production. A link to the electronic license to publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements before choosing the appropriate license.

Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days. If complications arising from measures taken to prevent the spread of COVID-19 will prevent you from meeting this deadline (e.g. if you cannot retrieve necessary files from your laboratory, etc.), please let us know and we can work with you to determine a suitable revision period.

Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Yukiko Gotoh, PhD
Monitoring Editor
Journal of Cell Biology

Tim Spencer, PhD
Executive Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The authors took my concerns seriously and came up with a very creative and original way to rescue N-cadherin traffic in the SNAP23 knockout cells. This was a brave step and paid off. In addition the authors have nicely shown that VAMP8 co-localizes in vesicles with N-cadherin but not with GPI anchor proteins, which instead co-localize with VAMP2, and used the RUSH assay to show specific delays in N-cadherin reaching the surface when SNAP23, VAMP8 or Stx1B was inhibited. Taken together with the strong phenotypic characterization in the original submission, this paper now represents a major advance in understanding, not only of cortical development and N-cadherin traffic, but also of cargo selection by different VAMP/SNAP proteins and cadherin traffic in non-neuronal cells.

Reviewer #2 (Comments to the Authors (Required)):

The authors addressed all my (minor) comments

Reviewer #3 (Comments to the Authors (Required)):

The authors have appropriately addressed all of the comments I previously made and I have no further comments.