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Regional heterogeneity of astrocyte morphogenesis dictated by the formin protein, Daam2, modifies circuit function

Juyeon Jo, Junsung Woo, Carlo Cristobal, Jong Min Choi, Chih-Yen Wang, Qi Ye, Joshua Smith, Kevin Ung, Gary Liu, Diego Cortes, Sung Yun Jung, Benjamin R Arenkiel, and Hyun Kyoung Lee
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Corresponding author(s): Hyun Kyoung Lee (hyunkyol@bcm.edu)

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1st Editorial Decision **31st May 2021**

Dear Dr. Lee

Thank you for the transfer of your manuscript to EMBO reports. We have now received the enclosed referee reports on it.

As you will see, all referees acknowledge that the data are novel, of good overall quality and interesting. However, they also point out that significant revisions will be required to strengthen the study. I think all referee concerns are reasonable and should be addressed. Please let me know in case you disagree, so that we can discuss the revisions further, also in a video chat, if you like.

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

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

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

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:
1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.
2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

3) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

4) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

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

6) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript

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

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at <https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

9) Our journal also encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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

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

Kind regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

The authors investigated regional heterogeneity of astrocyte morphology by targeting a formin protein, Daam2. Authors revealed that loss of Daam2 in astrocytes resulted in increased morphological complexity and increased GFAP expression in the cortex and olfactory bulb. Authors also revealed that loss of Daam2 resulted in distinct effects on calcium dynamics of astrocytes and synaptic activities in these two regions, which led to change in olfactory behavior in Daam2 cKO mice. By proteomics profiling for Daam2 cKO mice, authors found Slc4a4 as an interactor of Daam2 and knocking down Slc4a4 in astrocytes of Daam2 cKO mice restored morphological properties of astrocytes. Overall, the findings regarding Daam2 regulating morphological complexity of astrocytes and affecting circuit function of olfactory bulb are novel and of interest in the field. The experiments are well designed and well performed. The paper is clearly and well written and the figures are compelling. However, this reviewer has some comments for clarification as listed below.

1) Authors did not show detailed information about statistical analyses for their data. The methods section must contain a detailed section on statistics, how particular tests were chosen and how significance was declared. Further details on calcium imaging analyses are also needed. N numbers should be reported in the figure legends for every data set.

2) Authors clearly showed Daam+ astrocytes in multiple regions or at multiple time points during development (Fig1A-C). However, there is less evidence about whether Daam2 expression is specific to astrocytes or not. Regarding to Daam2 cKO experiments, Aldh1l1 or GfaABC1D promoter-dependent conditional knocking out potentially affects Daam2 in neuronal progenitor cells to some extent if Daam2 exists in those cells. To estimate cell-type specificity of Daam2, authors should carefully analyze CRISPR-Cas9 based Daam2 knock-in mice by detecting Flag tag and seeing which cells express it (FigS5G). If they find some in neuronal progenitors, then this does not diminish the current study and the authors could simply discuss the implications in an expanded discussion.

3) Authors claimed that morphological alteration by knocking down Daam2 in olfactory bulb modified circuit function. However, relationships among morphological alteration, changes in synaptic activity, and olfactory behavioral deficits remain unclear. Since authors found Slc4a4 as an interactor of Daam2 in olfactory bulb and knocking down Slc4a4 in astrocytes of Daam2 cKO mice restored morphological properties of astrocytes, they should discuss about the possibility that knocking down Slc4a4 in astrocytes of Daam2 cKO mice might restore changes in synaptic activity and olfactory behavioral deficits as well. Again, I am not suggesting these experiments need to be performed as they may require > 1 year of work. However, they could point the reader in the right direction with expanded discussion along these lines.

4) Authors claimed that interaction between Daam2 and Slc4a4 was specific to olfactory bulb (Fig8D). Perhaps they can speculate about the underlying molecular mechanism for this region specific biological phenomenon in astrocytes.

5) Fig7C and 7D: There is no detail about scale of heatmaps (fold change etc.).

6) Fig7D: Authors could show additional information about expressions of K+ channels and other neurotransmitter transporters etc. from their proteomics data as well as Slc1a2 and Slc6a11. This would be useful to see.

7) The short title should be modified (currently it is odd to read and missing some words)

Overall, a very nice paper that needs some straightforward revisions.

Referee #2:

The manuscript "Regional heterogeneity of astrocyte morphogenesis dictated by the formin protein, Daam2, modifies circuit function" utilizes an array of techniques from molecular manipulation to proteomic analysis to identify how the cytoskeleton remodeling-related protein, Daam2, influences astrocyte morphology and may be a region specific influencer of circuit development and behavior. Specifically, astrocyte specific knockout of Daam2 increases morphological complexity across regions while differentially influencing calcium dynamics and synaptic activity in the cortex and olfactory bulb. In the olfactory bulb, it was found that these alterations result in deficits in olfactory behavior tasks in food finding and odor discrimination. Mass-spec proteomic profiling and GoTerm analysis also reveal olfactory and cortical regional differences in differentially expressed genes. Mass-spec data was also used to identify a target, Slc4a4, that may, in part, mediate the effects of cortical observations found with Daam2 manipulations. The data appear high quality and the role of Daam2 is astrocyte biology is novel. Few studies have aimed at identifying underlying molecular mechanism that regulate the complex morphology of astrocytes, thus this article is likely to be of interest to glia and neurodevelopmental biologists. However, there are concerns with the manuscript in it current form. These are listed below.

Major Concerns:
1. The authors demonstrate a robust increase in astrocyte process length and branching with no change in astrocyte cell number. Given that typically in the adult organism astrocytes tile the brain in non-overlapping domains, this would suggest that astrocytes display significant overlap of their processes in daam2 cKO mice. Can the domain overlapped be measured the images captured by the authors?
2. Several of the phenotypes the authors report, more astrocyte branches, longer branches, what appears to be larger somas in (figure 2, although not quantified), potentially enhanced overlap of primary branches and elevated GFAP staining are indicative of reactive glia- perhaps KOing daam2 early in development results in generating reactive astrocytes. This could also describe changes in neuronal electrophysiology and changes in behavior. The authors should evaluate the reactive status of astrocyte sin cKO mice. The proteomics might be a good place to start. There are hundreds of publications the authors can use as a resource to identify morphological features, genes and proteins that are dysregulated in reactive astrocytes.
3. Is astrocyte volume increasing? Can this be calculated with the authors' current images using 3D Imaris reconstruction for volume measures?
4. The in vitro data supporting the heading 'Daam2 modulates astrocyte morphology by altering GFAP and F-actin' is not strong. Consider adding more comprehensive measures of F-actin/cytoskeleton/g-actin/f-actin stress fibers. There are commercially available kits that allow for this (Cytoskeleton Inc., #BK037). Alternatively, this aspect of the story can be removed. It does not add much and is not followed up on in later parts of the study.
5. Please clarify/justify the use of cHET mice as controls- are the controls Cre negative floxed hets, cre positive floxed hets, or some other combination?

6. There are instances in the body of the manuscript where the justification or rational for an experiment is not well delineated, for example it is not totally clear why the authors chose to examine the olfactory bulb or focus on SlC4a4. Please provide a more clear justifications of the reasoning for examining these region and protein targets.
7. There are other instances where it is not clear what the control for a particular experimental group is. Specific examples below but the entire results sections and all the figure legends should be edited for clarity and transparency
• Figure 2: Daam2 AAV overexpression experiment - these aspects of the manuscript require editing for clarity. How was the analysis performed- is it between groups (AAV-Daam2 overexpresses verses GFP) or within groups (AAV-Daam2 reporter astrocytes vs. non-reporting astrocytes).
• Figure 2: It is unclear why WT and Aldh1l1 animals where both used for this experiment.
• Figure 3F-H similarly lacks clarity. Arrows are presented that have no description as to their purpose. Not clear which image corresponds to treatment or control groups.
• Figure 3: There is also a statement that morphological complexity is reduced in Aldh1l1 reporter mice after Daam2 overexpression, but there is no analysis similar to those done in figure 1 and 2 that justifies this claim.
• Figure 3: Indicated F-actin is altered without provision of quantitative data.
• Figure 8: Similar morphological assessment as shown in Figure 2 should be provided

Minor issues:
1. Figure 1C: Figure caption requires clarification if measure is of particular region, somewhat ambiguous given that text mentions multiple regions referencing this figure
a. Are there available supplemental data that has separate analysis by region?
2. Figure 1 E-F: Figure caption requires clarification if measure is of particular region, somewhat ambiguous given that text mentions both olfactory bulb and cortex as referencing this figure
a. Are there available supplemental data that has separate analysis by region?
3. Figure 2: In text citation makes reference to cortical analyses by layer as well as to measures of neurogenesis changes in the olfactory bulb in DAAM2 cKO animals
a. Was reference to OB analyses meant to be cited as S2?
4. Figure 3C: Figure caption could benefit from reminder that the signal intensity measure in vivo was only measured for GFAP signal
5. On page 12 animals are referred to as Daam2 KO mice and Daam2 Het controls are these the same cKO mice used throughout?
6. Whole cell capacitance is a measure of membrane area-it is notable that this value is not different between groups and possibly speaks to low input resistance and thus poor voltage clamp on these leaky cells- this should be commented on in the discussion, particularly if it is found that membrane cell volume is increased (major concern #3).

Referee #3:

In the manuscript of "Regional heterogeneity of astrocyte morphogenesis dictated by the formin protein, Daam2, modifies circuit function", Jo et al., report a role of Daam2 in controlling astrocyte growth and maintenance in the mouse CNS with different effects on modulating astrocyte-neuron communications in two brain regions. They demonstrate that loss of Daam2 in astrocytes increases the morphological complexity of astrocytes in the cortex and the olfactory bulb, and likely mediate through the regulation of GFAP and F-actin. Interestingly, such increased astrocyte complexity leads to opposite phenotypes of astrocyte intracellular Ca2+ and excitatory/inhibitory synaptic transmissions in the cortex versus the olfactory bulb. Functionally, they show that astrocyte-specific deletion of Daam2 results in impaired olfactory behaviors. They further employed proteomic approaches to identify the distinct regulation of Slc4a4 level is responsible for Daam2 mutant astrocyte phenotypes in the cortex. Overall, these findings provide a new molecule, Daam2, in mediating astrocyte morphogenesis in vivo, and present a region-specific functional requirement of Daam2 in modulating circuit function. There are a few points need to be addressed to strengthen the findings.

Major points:
1) The authors initially assessed the morphological complexity of astrocytes via Aldh1l1-EGFP reporter. However, it is possible the changes of Aldh1l1-EGFP level (i.e. increased EGFP levels) in Daam2 cKO lead to a similar conclusion. It is therefore encouraged to compare the EGFP levels in cHet versus cKO by qPCR or Western blots to rule out this possibility.

2) Based on the Method section, the morphological analysis of astrocytes was carried out using the max projection of data, which means analyzing in 2D throughout the figures. 3D analysis of astrocyte morphology is a more accurate way to examine astrocytes (Stogsdill J., et al., 2017; Lanjakornsiripan D., et al., 2018). With sparse labeling (Fig. 2) and IMARIS software, the authors should analyze astrocyte morphology in 3D to more accurately represent the complexity of astrocytes.

3) In Fig. 4 and Fig. 5, the experiments in the olfactory bulb were performed in the cKO (Aldh1l1-Cre; Daam2F/F) mice; However, given the neurogenesis in the olfactory bulb (neural stem cells also labeled by Aldh1l1-Cre; Foo L., et al., 2013), it is possible the differences the authors observed between the cortex and the olfactory bulb in Fig. 4 and Fig. 5 are due to secondary effect to SVZ neurogenesis or Daam2 role in neurons. Although the authors provide evidence (Fig. S2) to show that

no difference was found in the markers examined, the authors could adapt an inducible astrocyte-specific Cre-ER line to conditionally knockout Daam2 at a later time point in the olfactory bulb and test the phenomena. As this study highlights the regional heterogeneity of Daam2, this is a key experiment needs to be addressed.

4) In Fig. 8, the genetic interaction between Slc4a4 and Daam2 was only examined by GFAP level. Changes in GFAP level might not necessarily reflect astrocyte morphological changes. The authors should also include experiments to demonstrate other perspectives of astrocyte complexity (i.e. 3D volume, related to point 2). Also, does deletion of Slc4a4 affect astrocyte Ca^{2+} dynamics and/or synaptic transmission in the cortex?

Minor points:
1. In page 7, line 3: Figure 2A-E should be Figure S2A-E.
2. In Fig. 1F,1I,1K and Fig. 2D, when showing total process length of astrocytes, it would be idea to include a representative IMARIS Filament traced process.
3. In Fig. 7 and the corresponding paragraph, it is unclear the proteome profiling was performed using global Daam2 KO or cKO, as the texts are interchanged inbetween.

Reviewer Response for *"Regional heterogeneity of astrocyte morphogenesis dictated by the formin protein, Daam2, modifies circuit function"*

We thank all three reviewers for the constructive and generally positive reviews of our manuscript. In an effort to strengthen our conclusions and provide additional rigor to our studies, we have performed new experiments and revised our manuscript to incorporate the reviewers' suggestions. All new or edited text is shown as highlighted in the revised manuscript. Below, we provide our point-by-point responses to the reviewers' comments.

REVIEWER #1

Comment 1-1: *"Authors did not show detailed information about statistical analyses for their data. The methods section must contain a detailed section on statistics, how particular tests were chosen and how significance was declared. Further details on calcium imaging analyses are also needed. N numbers should be reported in the figure legends for every data set"*

Thank you for the comment. We provided detailed information on statistics and added a separate method section for statistical analysis.

Comment 1-2: *"…To estimate cell-type specificity of Daam2, authors should carefully analyze CRISPR-Cas9 based Daam2 knock-in mice by detecting Flag tag and seeing which cells express it (FigS5G). If they find some in neuronal progenitors, then this does not diminish the current study and the authors could simply discuss the implications in an expanded discussion"*

We thank the reviewer for bringing up this important point. Our previous expression analysis for Daam2 mRNA level via *in situ* hybridization as well as multiple transcriptome profiling experiments by others (Hrvatin et al., 2018; Zeisel et al., 2018; Zhang et al., 2014) have indicated that Daam2 expression is highly restricted to glial cells. However, it is important to address whether the Daam2 protein is expressed in the neural lineage. Our Daam2-Flag-knock-in reporter mice would be great for this purpose. While we were able to detect Daam2 protein expression via immunoblotting, yet immunostaining is not successful to appropriately address this question. Alternatively, we used Daam2 LacZ-reporter mice to define the Daam2 expression in neuronal progenitors in regions with active neurogenesis including the cortical subventricular zone (SVZ) and the rostral migratory stream (RMS) of the olfactory bulb. We performed co-immunostaining of beta-galactosidase with the neuronal progenitor marker, Nestin. Importantly, no Daam2 immunostaining was observed in Nestin-positive neuronal progenitors throughout the SVZ and RMS, suggesting that Daam2 is mainly expressed in glial cells and the phenotype we observed with Daam2 conditional mutant mice (Aldh1l1-Cre derived) is highly likely due to Daam2 loss in astrocytes. Please see the revised **Fig EV1B** and text, **lines 110-112.**

Comment 1-3: *"Authors claimed that morphological alteration by knocking down Daam2 in olfactory bulb modified circuit function. However, relationships among morphological alteration, changes in synaptic activity, and olfactory behavioral deficits remain unclear. Since authors found Slc4a4 as an interactor of Daam2 in olfactory bulb and knocking down Slc4a4 in astrocytes of Daam2 cKO mice restored morphological properties of astrocytes, they should discuss about the possibility that knocking down Slc4a4 in astrocytes of Daam2 cKO mice might restore changes in synaptic activity and olfactory behavioral deficits as well. Again, I am not suggesting these experiments need to be performed as they may require > 1 year of work. However, they could point the reader in the right direction with expanded discussion along these lines"*

We agree that is important to understand whether the morphological alteration of astrocytes is a leading cause or the outcome of the functional deficit. We revised the discussion to provide better insight for readers regarding the relationship between morphological alteration and functional outcome of astrocytes.

With regards to this review comment on Daam2 and Slc4a4 axis, our results show that *Daam2 strongly associates with Slc4a4 in the cortex, but not the olfactory bulb* (**Fig 7D**). In addition, we found

that Slc4a4 loss restores morphological alteration caused by the loss of Daam2 only in the cortex, but not in the olfactory bulb (**Fig 7F-J**). Thank you for the suggestion. Please see the text, **lines 349-352 and 364-365.**

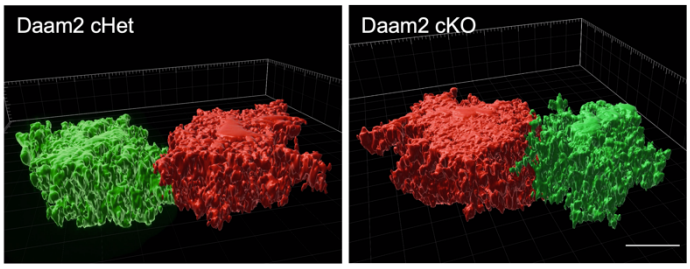
Comment 1-4: *"Authors claimed that interaction between Daam2 and Slc4a4 was specific to olfactory bulb (Fig8D). Perhaps they can speculate about the underlying molecular mechanism for this region specific biological phenomenon in astrocytes"*
As discussed in the above response (Comment 1-3), our current data support the notion that the interaction between Daam2 and Slc4a4 is specific to the cortex, not the olfactory bulb. This has been clarified in the corresponding results section. In addition, we have added discussion of possible molecular mechanisms underlying this region-specific interaction as requested by the reviewer. Please see the revised text, **lines 461-472.**

Minor Comments
(1) Fig7C and 7D: There is no detail about scale of heatmaps (fold change etc).
Thank you for the comment. We added the scale of heat maps.

(2) Fig7D: Authors could show additional information about expressions of K+ channels and other neurotransmitter transporters etc. from their proteomics data as well as Slc1a2 and Slc6a11. This would be useful to see.
Thank you for the comment. We have added transcript expression changes of Slc6a11 from qRT-PCR analysis. Please see the revised **Fig 6E**.

(3) The short title should be modified (currently it is odd to read and missing some words).
Thank you for the comment. It was a typographical error on our end and has been revised.

REVIEWER #2
Comment 2-1: *"The authors demonstrate a robust increase in astrocyte process length and branching with no change in astrocyte cell number…Can the domain overlapped be measured the images captured by the authors?"*
This is an excellent point raised by the reviewer. As single astrocytes display unique non-overlapping domains, labeling individual astrocytes with differentially colored dyes or reporters would be the best way to test if Daam2 deficient astrocytes still maintain distinct domains. Our current data are based on a single-color fluorescent reporter, which cannot address the question regarding the domain overlapping. With our best effort for 3D-volume rendering within neighboring astrocytes, we observed no significant overlapping astrocytes between Daam2 cHet and cKO mice (see **Reviewer's Figure 1**).



Reviewer's Figure 1. 3D-volume rendering of Daam2 cHet and Daam2 cKO astrocytes in cortex. Each astrocyte is pseudo-colored. Scale bar: 20 mm.

There are inconsistent results from different studies regarding the domain overlapping of reactive astrocytes which are known to display highly elaborate morphology (Oberheim et al., 2008; Wilhelmsson et al., 2006). Because morphological changes induced by the loss of Daam2 are not as profound as those seen with astrocyte reactivity (see **Fig EV4C** and **text line 186-188**), we speculate that no significant difference in domain overlapping would be seen in our Daam2 cKO animals when compared with control.

Comment 2-2: *"…The authors should evaluate the reactive status of astrocyte sin cKO mice. The proteomics might be a good place to start"*
This is another great point raised by the reviewer. We have cross-compared previous published genes/resources to our screening data. We performed immunostaining for other known markers of reactive astrocytes such as MaoB, S100b, and AldoC and found no difference compared to control (see **Fig EV4C**), suggesting that other changes noted by the reviewer may not necessarily be indicative of astrocyte reactivity in this context. Please see the revised text, **text line 186-188.**

Comment 2-3: *"Is astrocyte volume increasing? Can this be calculated with the authors' current images using 3D Imaris reconstruction for volume measures?*
Thank you for the comment. Following postnatal electroporation, we performed 3D volume measurement of GFP-labeled individual astrocytes by using high-resolution confocal imaging and IMARIS. We found that depletion of Daam2 in cortical astrocytes significantly enhanced astrocyte volume. Please see the revised **Fig EV2E-F** and text, **lines 144-146.**

Comment 2-4: *"…Consider adding more comprehensive measures of F-actin/cytoskeleton/g-actin/f-actin stress fibers. There are commercially available kits that allow for this (Cytoskeleton Inc., #BK037). Alternatively, this aspect of the story can be removed. It does not add much and is not followed up on in later parts of the study.*
Thank you for the suggestion. We have moved the *in vitro* data to the Figure Extended View. Please see the revised **Fig EV4A-B** and text, **lines 177-180.**

Comment 2-5: *"Please clarify/justify the use of cHET mice as controls- are the controls Cre negative floxed hets, cre positive floxed hets, or some other combination?*
Thank you for the comment. Controls include both Cre positive and Cre negative floxed HET.

Comment 2-6: *There are instances in the body of the manuscript where the justification or rational for an experiment is not well delineated, for example it is not totally clear why the authors chose to examine the olfactory bulb or focus on SlC4a4. Please provide a more clear justifications of the reasoning for examining these region and protein targets.*
Thank you for the comment. We have focused on cortex and OB because these two brain regions showed a clear change in astrocyte morphology in the absence of Daam2. Slc4a4 is targeted because we prioritized candidates with the following distinct criteria: i) enriched or exclusively expressed in astrocytes; ii) differentially regulated between cortex and OB following Daam2 loss; and iii) strong affinity for binding to Daam2. We have revised the manuscript carefully to provide a clear rationale for our selection of brain regions and gene/protein targets. Please see the revised text, **lines 312-324.**

Comment 2-7: *There are other instances where it is not clear what the control for a particular experimental group is. Specific examples below but the entire results sections and all the figure legends should be edited for clarity and transparency:*
• *Figure 2: Daam2 AAV overexpression experiment - these aspects of the manuscript require editing for clarity. How was the analysis performed- is it between groups (AAV-Daam2 overexpresses verses GFP) or within groups (AAV-Daam2 reporter astrocytes vs. non-reporting astrocytes).*
• *Figure 2: It is unclear why WT and Aldh1l1 animals where both used for this experiment.*
We appreciate the reviewer pointing this out. We overexpressed Daam2 using AAV-GfaABC$_1$D-myc-Daam2 in WT mice and injected AAV-GfaABC$_1$D-GFP as a control into separate WT mice. Then we measured GFAP levels by immunostaining only in successfully transduced cells expressing Myc-Daam2 (Daam2 overexpression) or GFP (control vector expression). For astrocyte morphology analysis, we injected AAV-GfaABC$_1$D-myc-Daam2 into Aldh1l1-EGFP reporter mice and compared infected cells (as indicated by Myc staining) and non-infected cells in the same mouse. We have

revised the cognate figure legends accordingly. Please see the revised **Fig 2E-I** and text, **lines 180-186.**

• *Figure 3F-H similarly lacks clarity. Arrows are presented that have no description as to their purpose. Not clear which image corresponds to treatment or control groups.*
We appreciate the reviewer's comment. Please see the revised **Fig 2E-I** and text, **lines 180-186.** Filled arrows indicate normal levels of GFAP labeled branches in controls, and blank arrows denote decreased GFAP expression in Daam2-overexpressing cells. Mis-labeled arrows were removed.

• *Figure 3: There is also a statement that morphological complexity is reduced in Aldh1l1 reporter mice after Daam2 overexpression, but there is no analysis similar to those done in figure 1 and 2 that justifies this claim.*
We thank the reviewer for this excellent comment. We have quantified Daam2-overexpressing astrocytes morphology with IMARIS similar to original figures 1 and 2 (revised Fig 1 and Fig EV2), and found reduced morphological complexity compared to control cells. Please see the revised **Fig 2H-I** and text, **lines 185-186**.

• *Figure 3: Indicated F-actin is altered without provision of quantitative data.*
The original Figure 3D showing quantification of F-actin in primary cultured astrocytes has been moved to the revised **Fig EV4**.

• *Figure 8: Similar morphological assessment as shown in Figure 2 should be provided*
Thank you for raising this point. We have utilized GFAP immunostaining to perform morphological assessment in the original Figure 8 with Sholl analysis and provided quantification. Please see the revised **Fig 7I-J** and text, **lines 364-365**.

Minor Comments
(1) Figure 1C: Figure caption requires clarification if measure is of particular region, somewhat ambiguous given that text mentions multiple regions referencing this figure a. Are there available supplemental data that has separate analysis by region?
Thank you for pointing this out. Figure 1C is a measurement of cortical Daam2-expressing astrocytes during development, reflecting Figure 1B. We revised the figure legend accordingly.

(2) Figure 1 E-F: Figure caption requires clarification if measure is of particular region, somewhat ambiguous given that text mentions both olfactory bulb and cortex as referencing this figure a. Are there available supplemental data that has separate analysis by region?
Thank you for the comments. Figure 1E and 1F show changes of GLAST and NFIA in the cortex only. We have separated cortex and OB Sox9 staining data in the revised **Fig 1E-F**.

(3) Figure 2: In text citation makes reference to cortical analyses by layer as well as to measures of neurogenesis changes in the olfactory bulb in DAAM2 cKO animals a. Was reference to OB analyses meant to be cited as S2?
Thank you. It was meant to the original Figure S2 which is now the revised **Fig EV3**. We have corrected this typographical error.

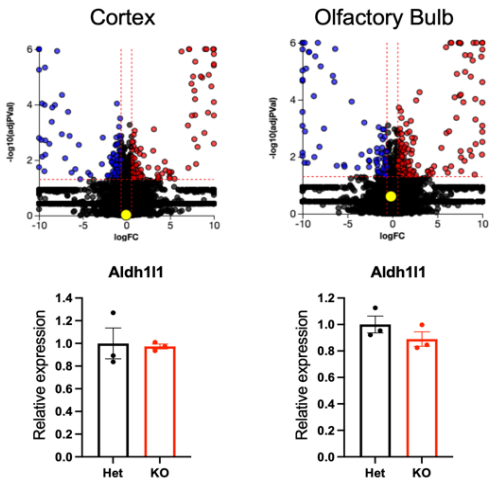
(4) Figure 3C: Figure caption could benefit from reminder that the signal intensity measure in vivo was only measured for GFAP signal
Thank you. We revised the figure accordingly.

(5) On page 12 animals are referred to as Daam2 KO mice and Daam2 Het controls are these the same cKO mice used throughout?
We used constitutive Daam2 KO for the Mass-spec analysis.

(6) Whole cell capacitance is a measure of membrane area-it is notable that this value is not different between groups and possibly speaks to low input resistance and thus poor voltage clamp on these leaky cells- this should be commented on in the discussion, particularly if it is found that membrane cell volume is increased (major concern #3).
Thank you for the comment. Even though we observed the morphological complexity and increased volumes in Daam2 cKO astrocytes, the cell capacitance was unaffected. This could be the result of leaky membrane due to Daam2 loss, leading to poor voltage clamping. Further studies are needed to further understand this phenomenon.

REVIEWER #3

Comment 3-1: *"…it is possible the changes of Aldh1l1-EGFP level (i.e. increased EGFP levels) in Daam2 cKO lead to a similar conclusion. It is therefore encouraged to compare the EGFP levels in cHet versus cKO by qPCR or Western blots to rule out this possibility."*
We thank the reviewer for this important point. Because we used a GFP reporter under the control of the Aldh1l1 promoter, we compared Aldh1l1 gene expression in our proteome analysis and found no difference (see **Reviewer's Fig 2**).



Reviewer's Figure 2. Expression of Aldh1l1 by Daam2 loss. Volcano plots of mass-spec profiling in comparison of Daam2 Het and Daam2 KO in cortex and olfactory bulb. Expression of Aldh1l1 is indicated as yellow dot. P= 0.946 for cortex and 0.247 for OB.

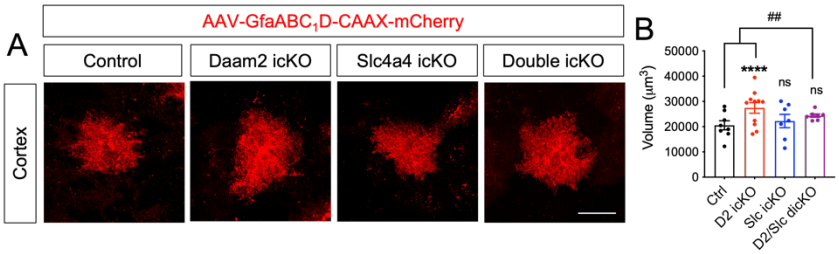
Comment 3-2: *"…With sparse labeling (Fig. 2) and IMARIS software, the authors should analyze astrocyte morphology in 3D to more accurately represent the complexity of astrocytes.*
Thank you for the comment. This is a shared comment also raised by Reviewer 1 (Comment 2-3). We analyzed GFP-labeled individual astrocytes following postnatal electroporation, using high-resolution confocal imaging and IMARIS to measure 3D volume. We found that depletion of Daam2 in cortical astrocytes significantly enhanced astrocyte volume. Please see the revised **Fig EV2E-F** and text, **lines 144-146.**

Comment 3-3: *"…Although the authors provide evidence (Fig. S2) to show that no difference was found in the markers examined, the authors could adapt an inducible astrocyte-specific Cre-ER line to conditionally knockout Daam2 at a later time point in the olfactory bulb and test the phenomena. As this study highlights the regional heterogeneity of Daam2, this is a key experiment needs to be addressed.*
We thank Reviewer 3 for this comment. We agree with the reviewer that inducible and conditional knockout would resolve the potential early neurogenesis issue in Aldh1l1-Cre mice. However, using an Aldh1l1-Cre-ER line would not fully eliminate issues surrounding adult neurogenesis as low levels of Cre expression were detected in neurons of the inducible Cre line as well (Nagai et al., 2019). Alternatively, we provide evidence that Daam2 is not expressed in neuronal progenitor cells, further suggesting the phenotype we observed with Aldh1l1-Cre is most likely due to Daam2 loss in astrocytes. Please see the revised **Fig EV1B** and text, **lines 110-112.** While discussion with an editor for the revision plan, we agreed that repeating our entire battery of electrophysiological and calcium imaging experiments using newly generated inducible Cre-ER lines (3 additional new lines) would not be feasible in the limited time frame provided for the current revisions. However, the point is well taken and future studies will be performed to further address this issue.

Comment 3-4: *"…In Fig. 8, the genetic interaction between Slc4a4 and Daam2 was only examined by GFAP level. Changes in GFAP level might not necessarily reflect astrocyte morphological changes. The authors should also include experiments to demonstrate other perspectives of astrocyte complexity (i.e. 3D volume, related to point 2). Also, does deletion of Slc4a4 affect astrocyte Ca2+ dynamics and/or synaptic transmission in the cortex?*

Thank you for raising this point. We have analyzed morphology of GFAP+ astrocytes in this set of experiments with Sholl analysis and appropriate quantification graph. Please see the revised **Fig 7I-J** and text, **lines 364-365**.

We also performed a new experiment with cortical injection AAV-GfaABC$_1$D-CAAX-mCherry postnatally to visualize and quantify astrocyte morphology in a more rigorous manner. We found the morphological complexity of Daam2 deficient astrocyte is increased. Please see the **Reviewer's Fig 3**.



Reviewer's Figure 3. Double loss of Daam2 and Slc4a4 rescued increased volume of astrocyte by single loss of Daam2. Representative images (A) and quantification (B) of cortical astrocytes with AAV-GfaABC$_1$D-CAAX-mCherry at P28. Scale bar: 20 mm. n=7-11 cells, N=2-3 mice per genotype. Two-way ANOVA is used for statistics. [*] and [#] indicates comparison with control and double icKO respectively. p****<0.0001, $^{\#\#}$p<0.01.

Minor Comments

(1) In page 7, line 3: Figure 2A-E should be Figure S2A-E.

Thank you for the comment. We have revised it.

(2) In Fig. 1F,1I,1K and Fig. 2D, when showing total process length of astrocytes, it would be idea to include a representative IMARIS Filament traced process.

Thank you for the suggestion. We have added representative trace of astrocyte of each genotype in **Appendix Fig 1** and text, **line 139-142**.

(3) In Fig. 7 and the corresponding paragraph, it is unclear the proteome profiling was performed using global Daam2 KO or cKO, as the texts are interchanged in between.

We used constitutive Daam2 KO for the Mass-spec analysis.

References

Hrvatin, S., Hochbaum, D. R., Nagy, M. A., Cicconet, M., Robertson, K., Cheadle, L., Zilionis, R., Ratner, A., Borges-Monroy, R., Klein, A. M., et al. (2018). Single-cell analysis of experience-dependent transcriptomic states in the mouse visual cortex. *Nat. Neurosci.* 21,.

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Zhang, Y., Chen, K., Sloan, S. A., Bennett, M. L., Scholze, A. R., O'Keeffe, S., Phatnani, H. P., Guarnieri, P., Caneda, C., Ruderisch, N., et al. (2014). An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. *J. Neurosci.* 34,.

Dear Dr. Lee,

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from the referees and I am happy to say that all support its publication now. Only referee 2 still has a minor suggestion that I would like you to incorporate before we can proceed with the official acceptance of your manuscript.

A few other editorial changes are also required:

- Callouts for Appendix Fig. S2 panels are missing, please add.

- The APPENDIX FILE is missing a table of content with page numbers, please add.

- Figure 1B, 3A, 3E and EV2A need scale bars.

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

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I would like to suggest a few minor changes to the abstract that needs to be written in present tense. Please let me know whether you agree with these changes:

Astrocytes display extraordinary morphological complexity that is essential to support brain circuit development and function. Formin proteins are key regulators of the cytoskeleton; however, their role in astrocyte morphogenesis across diverse brain regions and neural circuits is unknown. Here we show that loss of the formin protein Daam2 in astrocytes increases morphological complexity in the cortex and olfactory bulb, but elicits opposing effects on astrocytic calcium dynamics. These differential physiological effects result in increased excitatory synaptic activity in the cortex and increased inhibitory synaptic activity in the olfactory bulb, leading to altered olfactory behaviors. Proteomic profiling and immunoprecipitation experiments identify Slc4a4 as a binding partner of Daam2 in the cortex, and combined deletion of Daam2 and Slc4a4 restores the morphological alterations seen in Daam2 mutants. Our results reveal new mechanisms regulating astrocyte morphology and show that congruent changes in astrocyte morphology can differentially influence circuit function.

I look forward to seeing a new revised version of your manuscript as soon as possible. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

My previous comments have been addressed in the revised manuscript, which is both clearer and stronger as a result. I have no additional comments.

Referee #2:

The authors have addressed nearly all of my concerns adequately, except one.
It may just be a matter of clarifying text regarding their controls.
question:
"Please clarify/justify the use of cHET mice as controls- are the controls Cre negative floxed hets, cre positive floxed hets, or some other combination?
Response

Thank you for the comment. Controls include both Cre positive and Cre negative floxed HET.
Comment: Cre+ floxed HET mice injected with tamoxifen would have some knockdown of protein as opposed to Cre- mice. This is an unusual control. Was protein, mRNA, volume reconstructions etc in these mice compared to Cre- controls. Please justify the use of these animals as controls and include in the discussion how you expect this to impact your results.

Referee #3:

The authors have addressed my previous comments. The revised manuscript is suitable to publish in this journal.

The authors have addressed all minor editorial requests.

Hyun Kyoung Lee
Baylor College of Medicine
Pediatrics and Neuroscience
1250 Moursund Street
Houston, Texas 77030
United States

Dear Dr. Lee,

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The data shown in figures should satisfy the following conditions:

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- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
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- a statement of how many times the experiment shown was independently replicated in the laboratory.
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  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

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1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The sample size was chosen based on previous experience and literatures for each experiment to yield high power to detect specific effects. No statistical methods were used to predetermine sample size.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Animal was chosen based on genotype and each experiment contained animals from at least two different litters to validate observed phenotype. Sex-specific differences were eliminated by including both sexes for all studies.
For animal studies, include a statement about randomization even if no randomization was used.	NA
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4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Only common but proper tests were used and described in figure legends and method section with p-values.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	F test for student-t test and strandarized residual is performed for two-way ANOVA.
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9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All mice were maintained and studied according to protocols approved by the Institutional Animal Care and Use Committee of Baylor college of Medicine
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