THE EMBO JOURNAL

Transgenic mice with expanded neocortex due to human *ARHGAP11B* exhibit increased memory flexibility

Lei Xing, Agnieszka Kubik-Zahorodna, Takashi Namba, Anneline Pinson, Marta Florio, Jan Prochazka, Mihail Sarov, Radislav Sedlacek, and Wieland Huttner
DOI: 10.15252/embj.2020107093

Corresponding author(s): Wieland Huttner (huttner@mpi-cbg.de)

Review Timeline:

Submission Date:	20th Oct 20
Editorial Decision:	13th Nov 20
Revision Received:	13th Feb 21
Editorial Decision:	12th Mar 21
Revision Received:	20th Mar 21
Accepted:	25th Mar 21

Editor: Karin Dumstrei

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 13th Nov 2020

Dear Wieland,

Thank you for submitting your study to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see while referee #1 is not convinced that we gain enough new insight in order to consider publication here, referees #2 and 3 are more positive. Given their positive feedback I would like to consider a revised version should you be able to address the specific concerns raised. I also agree with the referees that it is important to discuss the results of the behavioral tests in a more neutral way. This will not affect the impact the work.

I am happy to discuss the raised points further and maybe it would be most helpful to do so via phone or video.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to discuss your revisions further with you

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:

https://bit.ly/EMBOPressFigurePreparationGuideline

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors:
https://www.embopress.org/page/journal/14602075/authorguide

The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 11th Feb 2021.

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

This study by the Huttner lab extends on a previous series of papers reporting the fundamental role of the human-specific gene ARHGAP11B on the expansion of the human brain during recent evolution. Similar to their previous experimental manipulations, where ARHGAP11B was expressed in mouse cortical progenitor cells by in utero electroporation, here they report the generation of a mouse transgenic line to achieve exactly the same but globally (in all cortical progenitor cells). Not surprisingly, they report some of the same effects of expressing ARHGAP11B as before, namely increased cortical basal progenitors and neurogenesis. There are really only two novel aspects in this study: that the increased neurogenesis phenotype remains in adult animals, and that there is some (very subtle) behavioral phenotype. The study is well conducted, a trademark of the Huttner lab, and the results interesting, but overall the amount of work, degree of novelty, and impact of the findings, are clearly below the standard of EMBO Journal.

In addition to the above mentioned main pillars of criticism to this manuscript, other specific main concerns are as follows:

1- In page 7, line 134, the authors indicate: "Sequential labeling with two thymidine analogs, BrdU at

E13.5 and EdU half an hour prior to sacrifice at E14.5 (Fig 1L), indicated that BPs in 11B mouse embryos showed increased cell cycle re-entry (Fig 1M)". This is not strictly correct, as a difference in cell cycle length without a difference in re-entry would generate the exact same result. To analyze cell cycle re-entry the authors must, for example, give a single pulse of BrdU and, 24hrs later, measure the proportion of BrdU+ cells positive for Ki67. Nevertheless, because BPs most frequently derive from APs, they are born as the result of cell cycle re-entry, and hence this type of analysis is not useful to reveal whether BPs actually re-enter cell cycle themselves, only to measure the abundance of cells in SVZ re-entering cell cycle after either apical or basal division.

2- Page 7, line 138 - "11B mouse embryos show an increased BP abundance that is the result of increased BP proliferation (rather than increased BP generation)". As mentioned in the previous point, this conclusion is not really demonstrated (only partly supported) by the data shown. Increased numbers of BPs may come from BP amplification but also from aRGCs. There is no direct evidence that BPs re-enter the cell cycle (amplify) more in 11B embryos than in controls. They may for example undergo less apoptosis. But in any case, BPs observed by the authors at E14 were generated some time before (E13, E12?). If their conclusion is to be kept in the manuscript, the authors must show conclusive evidence. If their argument is that BPs self-amplify because AP proliferation does not change between Ctrl and 11B embryos, they must demonstrate that this is the case also in the days preceding the stage when there are more BPs (E14.5), when they are being produced. In fact, the increase in outer cortical surface shown later suggests an increase in ventricular surface (more APs) at early stages. The authors have analyzed this only at E18.5, but not at E14.5 nor, most importantly, at E12.5, to see if APs may be self-amplifying prior to producing increased numbers of BPs. If this was the case, it would leave no room for BP self-amplification. Even more conclusively, direct cell lineage tracing analysis showing greater BP self-renewal or amplification would in fact be the most convincing.

3- The analysis of neuron dendritic arbors (page 9, line 174; Figure EV5) is a very nice idea in the context of this study, but it is unfortunately very confusing in this case. The authors indicate that these analyses are performed in "upper layer neurons". What does this exactly mean? Did they focus on spiny stellate neurons in layer 4? Pyramids in layer 4? in layer 3? in layer 2? Dendritic arbor size and structure is completely different across these types. Also, in which cortical area was this measured? Did they analyze together apical and basal dendrites, which are completely different and have very different functional implications?

4- The conclusion that 11B mice have enhanced cognitive function (page 9, line 185) is based on minimal improvement in one of the IntelliCage testing features. I am not sure how one can really test improvement in cognitive function in mice, but this is clearly an overstatement in this report. The authors must tone down their interpertations and conclusions, sized to what these behavioral tests really demonstrate.

5- Interestingly, the authors state that "The lack of persistence of the ARHGAP11B-induced increase in deep-layer neurons may reflect apoptosis during postnatal development due to failure of these "extra-neurons" to wire properly into the cortical circuits" (page 12, line 236). This statement is wildly speculative, and in fact the issue is of sufficient importance in the context of this study that the authors should directly address it. The amount of new data presented here is not so overwhelming that this cannot be included, nor it falls out of the scope of the study.

6- Page 12, line 252 - "in the 11B mice, ARHGAP11B was "only" expressed at a physiological level, which resulted in an increase in cortical neuron numbers that apparently did not surpass the

"threshold" to induce cortical folding as seen previously (Florio et al., 2015)". A similar phenotype was reported in Nonaka-Kinoshita et al. 2013 (EMBO J), where even inducing a quite dramatic megalencephaly in mouse did not cause cortical folding. That phenotype also concurs with increased BP proliferation, and the authors argued that because there is a proportional increase in APs and BPs, the cortex was grown bigger but without folding. Could this also be the case with 11B mice? The Discussion of this study would greatly benefit from including these considerations and comparing the two phenotypes.

Referee #2:

In this paper, titled "Neocortex expansion in adult transgenic mice by human-specific ARHGAP11B enhances cognitive ability," Xing et al. address interesting questions regarding the potential role of the human-specific gene ARHGAP11B, including whether its ectopic expression in mice can induce cortical expansion until adulthood and alter cognitive function. They tackled these questions by replacing one allele of mouse Arhgap11a with a mutated version that encodes the human-specific COOH-terminal sequence of ARHGAP11B. This replacement indeed increased basal progenitor proliferation at E14.5 as well as induced neocortical expansion associated with an increased number of upper layer neurons even at P56. Importantly, the male mutant (11B) mice manifested altered behavioral phenotypes including those related to memory flexibility and anxiety. These results support the hypothesis that the acquisition of ARHGAP11B contributed to the architectural and functional features of the human neocortex. The findings in this paper are thus of great value to our understanding of cortical development and human evolution. However, the authors should address the following points before publication of this paper.

-The authors conclude that neocortical expansion induced by introduction of the human-specific gene ARHGAP11B enhances cognitive ability (as in the title). However, the behavioral alterations apparent in the 11B mice may not be the result of cortical expansion. Functions of ARHGAP11B unrelated to cortical expansion or effects of the protein expressed in noncortical regions, if any, might influence the behavioral outcomes. The authors should therefore avoid overstatements in the title, abstract, and main text.

-The authors conclude that the results of behavioral tests indicate "enhanced (or higher) cognitive abilities" of 11B mice, but this may be misleading. Memory flexibility should be controlled properly in order to show "higher cognitive abilities." For example, excess memory flexibility would have a negative consequence on cognition. It is also unclear whether the reduced anxiety observed in 11B mice contributes to "higher cognitive abilities." I suggest that the authors use more neutral words such as "altered behaviors" instead of "enhanced (or higher) cognitive abilities" for their conclusions. It is of course acceptable to discuss the possible relevance of the altered behaviors to enhanced cognitive abilities in humans.

-The methods, results, and interpretations of the behavioral tests, especially those related to memory flexibility (Figure 4AB), should be described in more detail. Although these results are a highlight of the paper, their description is too superficial. More importantly, it is not clear how the statistical analysis was performed for the experiments shown in Figure 4AB. It is not appropriate to show a p value only for the total experiment. It should be indicated which points were compared between WT and 11B groups (for example: Was the acquisition session included? Were the data for day 1 included for the reversal sessions?).

-The difference between WT and 11B groups in Figure 4AB seems to be subtle and not greater than the difference between reversal sessions for the same groups. I recommend that the authors repeat this experiment with other WT and 11B groups (or at least repeat it with the same WT and 11B groups) to see whether they can reproduce this important result. It would also be desirable to perform another type of memory flexibility task to support the conclusion.

Referee #3:

Xing et al. present a novel transgenic mouse line (11B) in which one allele of the mouse ARHGAP11A gene was converted into a mutant mouse ARHGAP11B gene, which is normally expressed in the human lineage. In adult mice carrying one allele of 11A and one allele of 11B, the neocortex is expanded and the numbers of upper-layer neurons are increased. Interestingly, these mice display behavioral alterations, including increased memory flexibility in the reversal place-learning paradigm.

At first glance, I found the study intriguing. Transgenic expression of a human-specific gene, cortex enlargement and higher cognitive abilities, together make a tantalizing story. However, at closer inspection, several important questions remain that need to be addressed.

1. ARHGAP11A expression is rather widespread in mouse CNS and detectable outside the CNS according to genepaint. Since 11B should match 11A expression, the authors should present ISH or IF data for 11B transcript/protein in the embryo and postnatal brain and other tissues. This is important, because 11B expression outside the neocortex may contribute to the observed behavioral alterations. For example, the observed reduced anxiety of 11B mice may not be caused by 11B expression in the cortex.

2. Related to this, the authors should include a more thorough discussion of the arguments for and against a causal link between the cortex expansion phenotype and the behavioral phenotypes. This reviewer would have been happier to see 11B expression driven by a forebrain-specific promoter leading to cortical expansion in the adult and behavioral changes. It would have been easier to see a causal link. I suggest including a sentence in the discussion like: "Further circuit analysis is required to assess the behavioral consequences of ARHGAP11B-induced cortical expansion."

3. The same group reported that overexpression of ARHGAP11B by in utero electroporation in WT embryos can cause cortex folding. In the present study cortex folding is not observed neither at the embryonic nor in the adult stage. The authors comment "This most likely reflected the fact that in the 11B mice, ARHGAP11B was "only" expressed at a physiological level, which resulted in an increase in cortical neuron numbers that apparently did not surpass the "threshold" to induce cortical folding as seen previously (Florio et al., 2015)." In Florio et al. (2015), there is no quantification of neurons in upper layers, and the claim made in the present study is hard to evaluate. It would be very interesting and important though to evaluate this "threshold" by comparing/estimating the numbers of neurons in 11B mice and in mice where ARHGAP11B is overexpressed. Otherwise, one may have to conclude that the induction of cortical folds in the previous study was unphysiological?

4. In all behavioral tests reported in the paper, the authors did not include the 11A+/- mouse model that they have generated as additional control, only WT mice were used. 11A+/- mice should be

added as controls at least in the tests where the authors observed a difference between WT and 11B mice.

5. In Figure 4A and 4B, the authors present only one graph which includes two procedures (corner alternation and corner reversal). The results of the two procedures should be shown in two separate graphs and the statistical evaluations should be more clearly shown. Which phases of the procedures are significantly different between the experimental groups?

Minor point:
ARHGAP11B is sometimes written AHRGAP11B.

Editorial decision

Thank you for submitting your study to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see while referee #1 is not convinced that we gain enough new insight in order to consider publication here, referees #2 and 3 are more positive. Given their positive feedback I would like to consider a revised version should you be able to address the specific concerns raised. I also agree with the referees that it is important to discuss the results of the behavioral tests in a more neutral way. This will not affect the impact the work.

I am happy to discuss the raised points further and maybe it would be most helpful to do so via phone or video.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to discuss your revisions further with you

Response to Reviewers – Overview of Revision

Item	Panel	Contents	Reviewer
Figure 1	**NEW** Panel N	Quantification of the percentage of BrdU+ cells that are in S-phase 1 day later (BrdU+ / EdU+) in WT and 11B mouse neocortex.	#1
Figure 4	**NEW** Panels A, B, C, D	Previous panels A, B.	#2, 3
	Panels E, F, G, H	Previous panels C, D, E, F.	
NEW Figure EV2	**NEW** Panels A, B, C, D, E	Immunofluorescence for GFP and quantification of the distribution of GFP+ cells in VZ, SVZ, IZ and CP in WT and 11B mouse neocortex at 8 hr, 18 hr, 30 hr and 42 hr post *in utero* electroporation at E13.5, showing that the increase in BPs in embryonic 11B neocortex is not due to increased generation from APs but to BP self-amplification in the SVZ.	#1
Figure EV3 (previous Figure EV5)	**NEW** Panels D, E	Sholl analysis for apical and basal dendrites of pyramidal neurons in layers 2 and 3 of the adult WT and 11B mouse neocortex.	#1
	Panels F, G, H, I	Previous panels E, D, F, G, respectively.	
Figure EV4		Previous Figure EV7.	
Figure EV5		Previous Figure EV6.	
Figure S1 (previous Figure EV2)	**NEW** Panels A, B	Immunofluorescence for 11A and 11B of E12.5 and E14.5 11B-transgenic whole mouse embryos.	#3
	Panels C, C', C'', D, E, F	Previous panels A, A', A'', B, C, D, respectively.	
Figure S2		Previous Figure EV3.	
NEW Figure S3	**NEW** Panels A, B, C	Immunofluorescence for PH3, and quantification of PH3+ APs and PH3+ BPs in WT and 11B mouse neocortex at E12.5, showing mitotic BP but not AP increase also	#1

		at this earlier developmental stage.	
NEW Figure S3	**NEW** Panels D, E, F	Immunofluorescence for Tbr2, and quantification of Tbr2+ cells in VZ and SVZ in WT and 11B mouse neocortex at E12.5, showing lack of newborn BP increase in the VZ but BP increase in the SVZ at this earlier developmental stage.	#1
NEW Figure S3	**NEW** Panels G, H	Immunofluorescence for Tbr2 and active Caspase-3, and quantification of Tbr2+ / Caspase-3+ BPs in E14.5 WT and 11B mouse neocortex, showing lack of reduced apoptosis of BPs in 11B mouse neocortex.	#1
Figure S4		Previous Figure EV4.	
Figure S5		Previous Figure EV8.	
NEW Figure S6	**NEW** Panels A, B, C, C', D, E	Immunofluorescence for Ctip2 and active Caspase-3, and quantification of Ctip2+/Caspase-3+ cells in WT and 11B mouse pups at P0, P2, P4 and P8, showing increased postnatal apoptosis of deep-layer neurons in 11B mice, which showed higher levels of these neurons at the embryonic stage.	#1
NEW Figure S7	**NEW** Panel A	DAPI staining and immunofluorescence for GFP of E18.5 mouse brains electroporated with GFP-expressing and either control or 11B-expressing plasmids at E13.5, showing cortical folding upon 11B overexpression.	#3
	NEW Panel B	Immunofluorescence for GFP and Brn2 of E18.5 mouse brains electroporated with GFP-expressing and either control or 11B-expressing plasmids at E13.5, showing an increase in Brn2+ neurons in the electroporated area upon 11B overexpression.	#3
	NEW Panel C	Quantification of the percentage of GFP+ cells that are Brn2+ in the cortical plate of E18.5 mouse brains electroporated with GFP-expressing and either control or 11B-expressing plasmids at E13.5, showing an increase in the proportion of Brn2+ neurons among the progeny of electroporated cells upon 11B overexpression.	#3

Referee #1:

Reviewer's Comment:
This study by the Huttner lab extends on a previous series of papers reporting the fundamental role of the human-specific gene ARHGAP11B on the expansion of the human brain during recent evolution. Similar to their previous experimental manipulations, where ARHGAP11B was expressed in mouse cortical progenitor cells by in utero electroporation, here they report the generation of a mouse transgenic line to achieve exactly the same but globally (in all cortical progenitor cells). Not surprisingly, they report some of the same effects of expressing ARHGAP11B as before, namely increased cortical basal progenitors and neurogenesis. There are really only two novel aspects in this study: that the increased neurogenesis phenotype remains in adult animals, and that there is some (very subtle) behavioral phenotype. The study is well conducted, a trademark of the Huttner lab, and the results interesting, but overall the amount of work, degree of novelty, and impact of the findings, are clearly below the standard of EMBO Journal.

Authors' response:

We thank the Reviewer for this summary of our findings. We appreciate that the Reviewer finds our study to be well conducted and the results interesting.

Regarding the issue of novelty and impact of our findings, we respectfully disagree with the Reviewer. Specifically, we would like to politely point out that the previous transient *ARHGAP11B* expression in mouse embryos was an overexpression, and hence the results could have been artefacts. In contrast, in the present study, *ARHGAP11B* was expressed in mice at physiological levels, being under the control of the mouse *Arhgap11a* promoter. Our finding that the phenotype induced by this physiological *ARHGAP11B* expression, i.e. the increase in upper-layer neurons and neocortex size, persists into adulthood is very important as it allowed us to assess potential changes in neurobehaviour. As far as we are aware, the increase in memory flexibility observed in the adult 11B mice is the first evidence showing that mice with an expanded neocortex due to the human-specific gene *ARHGAP11B* indeed exhibit altered neurobehaviour. In our humble opinion, these findings are very novel and of great impact.

Regarding the amount of work, we would like to politely mention that the revised manuscript contains 4 main figures, 5 Extended View figures, and 7 supplemental figures, each with multiple panels. The new figures and figure panels added to the revised manuscript are summarized in the Overview of Revision Table above.

Reviewer's Comment:
In addition to the above mentioned main pillars of criticism to this manuscript, other specific main concerns are as follows:

Authors' response:

We hope that we have been able to address the specific main concerns of the Reviewer to the satisfaction of the Reviewer, as is described below.

Reviewer's Comment:
1- In page 7, line 134, the authors indicate: "Sequential labeling with two thymidine analogs, BrdU

at E13.5 and EdU half an hour prior to sacrifice at E14.5 (Figure 1L), indicated that BPs in 11B mouse embryos showed increased cell cycle re-entry (Figure 1M)". This is not strictly correct, as a difference in cell cycle length without a difference in re-entry would generate the exact same result. To analyze cell cycle re-entry the authors must, for example, give a single pulse of BrdU and, 24hrs later, measure the proportion of BrdU+ cells positive for Ki67. Nevertheless, because BPs most frequently derive from APs, they are born as the result of cell cycle re-entry, and hence this type of analysis is not useful to reveal whether BPs actually re-enter cell cycle themselves, only to measure the abundance of cells in SVZ re-entering cell cycle after either apical or basal division.

Authors' response:

In line with the Reviewer's suggestion, we gave a single pulse of BrdU at E13.5 and 23.5 hrs later labeled for 30 min with EdU to label all cells that were again in S-phase, i.e. were cycling and hence had re-entered the cell cycle. We now also express the data as the percentage of BrdU+ cells positive for EdU, which shows that there is an increase in the percentage of BrdU+ cells that are EdU+ in the SVZ, but not in the VZ, of 11B mouse embryos (new panel N of Figure 1). This corroborates our original conclusion that BPs in 11B mouse embryos show increased cell cycle re-entry.

Reviewer's Comment:
2- Page 7, line 138 - "11B mouse embryos show an increased BP abundance that is the result of increased BP proliferation (rather than increased BP generation)". As mentioned in the previous point, this conclusion is not really demonstrated (only partly supported) by the data shown. Increased numbers of BPs may come from BP amplification but also from aRGCs. There is no direct evidence that BPs re-enter the cell cycle (amplify) more in 11B embryos than in controls. They may for example undergo less apoptosis. But in any case, BPs observed by the authors at E14 were generated some time before (E13, E12?). If their conclusion is to be kept in the manuscript, the authors must show conclusive evidence. If their argument is that BPs self-amplify because AP proliferation does not change between Ctrl and 11B embryos, they must demonstrate that this is the case also in the days preceding the stage when there are more BPs (E14.5), when they are being produced. In fact, the increase in outer cortical surface shown later suggests an increase in ventricular surface (more APs) at early stages. The authors have analyzed this only at E18.5, but not at E14.5 nor, most importantly, at E12.5, to see if APs may be self-amplifying prior to producing increased numbers of BPs. If this was the case, it would leave no room for BP self-amplification. Even more conclusively, direct cell lineage tracing analysis showing greater BP self-renewal or amplification would in fact be the most convincing.

Authors' response:

We have performed the following four sets of experiments to address the Reviewer's concerns. All of these new data (please see Overview of Revision Table above) support our conclusion that the increased BP abundance is due to increased BP self-amplification in the SVZ (rather than increased generation of BPs in the VZ or reduced BP apoptosis).

1. We have performed immunofluorescence for Tbr2 and active Caspase-3 (new Figure S3G) and analysed the abundance of apoptotic Tbr2+ BPs in E14.5 WT and 11B embryos. We did not observe any difference between WT and 11B embryos (new Figure S3H), which indicates that the increased abundance of BPs in 11B embryos is not due to *"less apoptosis"*.

2. We have performed immunofluorescence for the mitotic marker phosphohistone H3 (PH3) (new Figure S3A) and analysed apical mitoses and basal mitoses at an earlier embryonic stage as requested by the Reviewer, i.e. at E12.5. We did not observe any changes in apical mitoses (new Figure S3B), but an increase in basal mitoses (new Figure S3C), indicating that also at this earlier developmental stage, the increased BP abundance is due to increased BP self-amplification in the SVZ.
3. We have performed immunofluorescence for Tbr2 (new Figure S3D) and analysed Tbr2+ cells in the VZ and SVZ at E12.5. We did not observe any changes in Tbr2+ cells in the VZ (new Figure S3E), but an increase in Tbr2+ cells in the SVZ (new Figure S3F), indicating (again) that the increased BP abundance is due to increased BP self-amplification in the SVZ and not increased generation of newborn BPs in the VZ.
4. We have performed a cell lineage tracing-like analysis as requested by the Reviewer, by *in utero* electroporating GFP-expressing plasmid to label aRGCs and their progeny at E13.5. We then analysed the distribution of GFP+ cells across the VZ, SVZ, IZ and CP at 8 hr, 18 hr, 30 hr and 42 hr post electroporation (new Figure EV2A-D). We observed an equal appearance of GFP+ progeny in the SVZ at 18 hr post electroporation, followed by an increase in the percentage of GFP+ progeny in the 11B SVZ at 30 hr and 42 hr post electroporation, concomitant with a decrease in the percentage of GFP+ progeny in the intermediate zone (IZ) at 30 hr (new Figure EV2E). Taken together, these data indicate that the generation of BPs from APs is equal between WT and 11B embryos, but that the BPs in 11B mouse embryos, once they are in the SVZ, undergo increased self-amplification (initally at the expense of neuron generation, hence the IZ decrease).

Collectively, these data make it highly unlikely that the increased BP abundance originates from aRGCs, for three reasons. First, there are no differences in the level of APs in mitosis between WT and 11B embryos at both stages analysed, E12.5 and E14.5, as shown in Figure 1C and Figure S3B. Second, if 11B aRGCs were to switch their mode of cell division to increasingly generate BPs (either from 1aRGC –> 2aRGCs to 1aRGC –> 1aRGC + 1BP, or from 1aRGC –> 1aRGC + 1BP to 1aRGC –> 2BPs), one would expect the abundance of aRGCs to decrease and the level of newborn bIPs, which are the Tbr2+ cells in the VZ, to increase. However, this is not the case, as shown in Figure 1G and H and Figure S3E. Third, the cell lineage tracing-like analysis has shown that the percentage of GFP+ cells appearing in the SVZ 18 hr after electroporation was not increased in 11B embryos compared to WT embryos, but was increased at 30 hr and 42 hr after electroporation, and this increase was not accompanied by a decrease of GFP+ cells in the VZ (new Figure EV2E). Hence, these data strongly support our conclusion that the increased BP abundance is due to increased BP proliferation/self-amplification in the SVZ.

Reviewer's Comment:

3- The analysis of neuron dendritic arbors (page 9, line 174; Figure EV5) is a very nice idea in the context of this study, but it is unfortunately very confusing in this case. The authors indicate that these analyses are performed in "upper layer neurons". What does this exactly mean? Did they focus on spiny stellate neurons in layer 4? Pyramids in layer 4? in layer 3? in layer 2? Dendritic arbor size and structure is completely different across these types. Also, in which cortical area was this measured? Did they analyze together apical and basal dendrites, which are completely different and have very different functional implications?

Authors' response:

We apologize for the confusion and for not stating the exact origin of the neurons analysed in the original manuscript. This has now been corrected in both the Results, Figure Legend and Methods. For the analysis of dendritic arbors (Figure EV3, panels F and G), we focused only on pyramidal neurons in layers 2 and 3 in the somatosensory cortex region of the rostral neocortex. As the Reviewer pointed out the different functional implications of apical and basal dendrites, we have now performed Sholl analyses on apical and basal dendrites separately (Figure EV3, new panels D and E).

Reviewer's Comment:

4- The conclusion that 11B mice have enhanced cognitive function (page 9, line 185) is based on minimal improvement in one of the IntelliCage testing features. I am not sure how one can really test improvement in cognitive function in mice, but this is clearly an overstatement in this report. The authors must tone down their interpertations and conclusions, sized to what these behavioral tests really demonstrate.

Authors' response:

We have now toned down our conclusions, as requested by the Reviewer, and throughout the text (including the title) refer to the phenotype observed for adult 11B mice in the IntelliCage tests as increased memory flexibility rather than enhanced cognitive ability. Also, we now use the term "altered neurobehaviour" to refer to the behavioural phenotype of the adult 11B mice.

Reviewer's Comment:

5- Interestingly, the authors state that "The lack of persistence of the ARHGAP11B-induced increase in deep-layer neurons may reflect apoptosis during postnatal development due to failure of these "extra-neurons" to wire properly into the cortical circuits" (page 12, line 236). This statement is wildly speculative, and in fact the issue is of sufficient importance in the context of this study that the authors should directly address it. The amount of new data presented here is not so overwhelming that this cannot be included, nor it falls out of the scope of the study.

Authors' response:

As requested by the Reviewer, we have now directly addressed the question whether the lack of persistence into adulthood of the increased levels of deep-layer neurons observed in 11B embryos reflects postnatal apoptosis of these neurons. Specifically, we have performed immunofluorescence for Ctip2 and active Caspase-3 (new Figure S6A-D) and analysed the abundance of apoptotic deep-layer neurons in WT and 11B pups at postnatal stages P0, P2, P4 and P8 (new Figure S6E). We observed increases in Ctip2+ & Caspase-3+ neurons at P0, P2 and P4, which indicates that there is indeed increased postnatal apoptosis of deep-layer neurons in 11B mice as compared to WT mice, supporting our original – admittedly speculative – suggestion that "*The lack of persistence of the ARHGAP11B-induced increase in deep-layer neurons may reflect apoptosis during postnatal development ... of these "extra-neurons"...*".

Reviewer's Comment:

6- Page 12, line 252 - "in the 11B mice, ARHGAP11B was "only" expressed at a physiological level, which resulted in an increase in cortical neuron numbers that apparently did not surpass the "threshold" to induce cortical folding as seen previously (Florio et al., 2015)". A similar phenotype

was reported in Nonaka-Kinoshita et al. 2013 (EMBO J), where even inducing a quite dramatic megalencephaly in mouse did not cause cortical folding. That phenotype also concurs with increased BP proliferation, and the authors argued that because there is a proportional increase in APs and BPs, the cortex was grown bigger but without folding. Could this also be the case with 11B mice? The Discussion of this study would greatly benefit from including these considerations and comparing the two phenotypes.

Authors' response:

We do not think that the lack of cortical folding in the 11B mice is due to a proportional increase in APs and BPs, because our analyses of 11B embryos indicate a selective increase in BPs without an increase in APs (Figure 1C, D, G, I, J, K). Rather, we think that the strong constitutive CAG promoter used in the previous *ARHGAP11B* overexpression study, in which cortical folding was observed in about half of the embryos (Florio et al. 2015), causes a greater increase in neurons than the more physiological *Arhgap11a* promoter driving *ARHGAP11B* expression in the present 11B mice. In addressing a point raised by Reviewer #3, we have repeated the *ARHGAP11B* overexpression by *in utero* electroporation of the CAG promoter plasmid (new Figure S7). Indeed, this overexpression causes cortical folding (new Figure S7A) and an almost doubling of upper-layer (Brn2+) neurons (new Figure S7C), whereas in the present 11B mice the increase in upper-layer (Satb2+, Brn2+) neurons is only ≤1.2-fold (Figure 2L, Figure 3K, L). These data are consistent with our suggestion that the increase in neurons in the 11B mice does not suffice, i.e. did not surpass the threshold necessary, to cause cortical folding, in contrast to the much greater increase upon *ARHGAP11B* overexpression by *in utero* electroporation using the CAG promoter plasmid.

As suggested by the Reviewer, the revised Discussion now includes a paragraph addresssing the difference between the present 11B mice and the 4D mice of Nonaka-Kinoshita et al. 2013 (EMBO J).

Referee #2:

Reviewer's Comment:
In this paper, titled "Neocortex expansion in adult transgenic mice by human-specific ARHGAP11B enhances cognitive ability," Xing et al. address interesting questions regarding the potential role of the human-specific gene ARHGAP11B, including whether its ectopic expression in mice can induce cortical expansion until adulthood and alter cognitive function. They tackled these questions by replacing one allele of mouse Arhgap11a with a mutated version that encodes the human-specific COOH-terminal sequence of ARHGAP11B. This replacement indeed increased basal progenitor proliferation at E14.5 as well as induced neocortical expansion associated with an increased number of upper layer neurons even at P56. Importantly, the male mutant (11B) mice manifested altered behavioral phenotypes including those related to memory flexibility and anxiety. These results support the hypothesis that the acquisition of ARHGAP11B contributed to the architectural and functional features of the human neocortex. The findings in this paper are thus of great value to our understanding of cortical development and human evolution. However, the authors should address the following points before publication of this paper.

Authors' response:

We thank the Reviewer for this summary of our findings and for stating that our findings are "*of great value to our understanding of cortical development and human evolution"*. We hope that we have been able to address the points raised by the Reviewer to the satisfaction of the Reviewer, as is described below.

Reviewer's Comment:
-The authors conclude that neocortical expansion induced by introduction of the human-specific gene ARHGAP11B enhances cognitive ability (as in the title). However, the behavioral alterations apparent in the 11B mice may not be the result of cortical expansion. Functions of ARHGAP11B unrelated to cortical expansion or effects of the protein expressed in noncortical regions, if any, might influence the behavioral outcomes. The authors should therefore avoid overstatements in the title, abstract, and main text.

Authors' response:

The Reviewer is correct. We have therefore added a sentence to the revised Discussion in which we mention that not all behavioural alterations apparent in the 11B mice, e.g. the reduced anxiety level, may be the result of cortical expansion. However, as one of the behavioural alterations apparent in the 11B mice, that is, the increased memory flexibility, is commonly regarded as a neocortical function, we feel that it is reasonable to relate this particular phenotype to the cortical expansion observed in the 11B mice. Nonetheless, as requested by the Reviewer, we have toned down our interpretations and tried to avoid overstatements in the title, abstract, and main text.

Reviewer's Comment:
-The authors conclude that the results of behavioral tests indicate "enhanced (or higher) cognitive abilities" of 11B mice, but this may be misleading. Memory flexibility should be controlled properly in order to show "higher cognitive abilities." For example, excess memory flexibility would have a negative consequence on cognition. It is also unclear whether the reduced anxiety observed in 11B

mice contributes to "higher cognitive abilities." I suggest that the authors use more neutral words such as "altered behaviors" instead of "enhanced (or higher) cognitive abilities" for their conclusions. It is of course acceptable to discuss the possible relevance of the altered behaviors to enhanced cognitive abilities in humans.

Authors' response:

We have changed our wording and now use more neutral words, notably "altered neurobehaviour", as the Reviewer suggested.

Reviewer's Comment:
-The methods, results, and interpretations of the behavioral tests, especially those related to memory flexibility (Figure 4AB), should be described in more detail. Although these results are a highlight of the paper, their description is too superficial. More importantly, it is not clear how the statistical analysis was performed for the experiments shown in Figure 4AB. It is not appropriate to show a p value only for the total experiment. It should be indicated which points were compared between WT and 11B groups (for example: Was the acquisition session included? Were the data for day 1 included for the reversal sessions?).

Authors' response:

We agree with the Reviewer's comment. We have therefore now added a more detailed description of the behavioural tests, notably of those related to memory flexibility, to the Results, Discussion, and Figure Legend. Also, we have added more detailed information on how the statistical analyses were performed to the Figure Legend. In our opinion, the description of the behavioural tests in Methods was already sufficiently detailed in the original manuscript.

Reviewer's Comment:
-The difference between WT and 11B groups in Figure 4AB seems to be subtle and not greater than the difference between reversal sessions for the same groups. I recommend that the authors repeat this experiment with other WT and 11B groups (or at least repeat it with the same WT and 11B groups) to see whether they can reproduce this important result. It would also be desirable to perform another type of memory flexibility task to support the conclusion.

Authors' response:

We would like to point out that the data presented in our manuscript actually already imply that we have reproduced this result, for the following reason. We have examined a total of 19 WT mice (10 males and 9 females) and 25 11B mice (10 males and 15 females). Obviously, these numbers of mice are too large to be subjected to the behavioural tests in one session. The maximum number of mice one Intellicage can hold is 12. In other words, we had a total of 4 sessions: (i) 5 wt males together with 5 11B males; (ii) 5 other wt males together with 5 other 11B males; (iii) 5 wt females together with 7 11B females; (iv) 4 other wt females together with 8 other 11B females. Not only did the two male sessions (pooled in Figure 4A, C) and the two female sessions (pooled in Figure 4B, D) yield essentially the same results, but (not shown so far) the two male sessions yielded essentially the same results, and the two female sessions yielded essentially the same results. We have stated this now more clearly and precisely in the revised Results and Figure Legend.

Referee #3:

Reviewer's Comment:
Xing et al. present a novel transgenic mouse line (11B) in which one allele of the mouse ARHGAP11A gene was converted into a mutant mouse ARHGAP11B gene, which is normally expressed in the human lineage. In adult mice carrying one allele of 11A and one allele of 11B, the neocortex is expanded and the numbers of upper-layer neurons are increased. Interestingly, these mice display behavioral alterations, including increased memory flexibility in the reversal place-learning paradigm.

At first glance, I found the study intriguing. Transgenic expression of a human-specific gene, cortex enlargement and higher cognitive abilities, together make a tantalizing story. However, at closer inspection, several important questions remain that need to be addressed.

Authors' response:

We thank the Reviewer for this summary of our findings and for stating that our study is intriguing. We hope that we have been able to address the questions raised by the Reviewer to the satisfaction of the Reviewer, as is described below.

Reviewer's Comment:
1. ARHGAP11A expression is rather widespread in mouse CNS and detectable outside the CNS according to genepaint. Since 11B should match 11A expression, the authors should present ISH or IF data for 11B transcript/protein in the embryo and postnatal brain and other tissues. This is important, because 11B expression outside the neocortex may contribute to the observed behavioral alterations. For example, the observed reduced anxiety of 11B mice may not be caused by 11B expression in the cortex.

Authors' response:

We agree with the Reviewer's comment. As requested by the Reviewer, we have now performed immunofluorescence for 11A and 11B on E12.5 and E14.5 11B whole mouse embryos (Figure S1A, B) and adult (P56) wildtype and 11B mouse brain (data not shown). In agreement with the Reviewer's comment, 11A and 11B expression in 11B mouse embryos are indeed widespread in the CNS and detectable outside the CNS (such as in liver, gut and eye, Figure S1A, B). The apparent differences in the pattern of immunofluorescence between 11A and 11B mostly reflect the different subcelluar localization of 11A, which is nuclear, and of 11B, which is in mitochondria and hence in the cytoplasm extending into cell processes. In addition, differences in protein stability may also contribute to the apparent differences in the pattern of immunofluorescence between 11A and 11B. In contrast to 11B mouse embryos, neither 11A nor 11B are detectable in adult mouse brain (data not shown), consistent with the low mRNA levels of both 11A and 11B (Figure EV1F, G).

In light of the expression of 11B in regions of the CNS other than the neocortex in 11B embryos, the Reviewer is correct in stating that 11B expression outside the neocortex during development may contribute to the observed behavioural alterations. We have therefore added a sentence to the revised Discussion in which we mention that certain behavioural alterations of the 11B mice such as the reduced anxiety may be the result of 11B expression in regions of the brain other than the neocortex. However, as one of the behavioural alterations of the 11B mice, that is, the increased

memory flexibility, is commonly regarded as a neocortical function, we feel that it is reasonable to relate this particular phenotype to the cortical expansion observed in the 11B mice.

Reviewer's Comment:

2. Related to this, the authors should include a more thorough discussion of the arguments for and against a causal link between the cortex expansion phenotype and the behavioral phenotypes. This reviewer would have been happier to see 11B expression driven by a forebrain-specific promoter leading to cortical expansion in the adult and behavioral changes. It would have been easier to see a causal link. I suggest including a sentence in the discussion like: "Further circuit analysis is required to assess the behavioral consequences of ARHGAP11B-induced cortical expansion."

Authors' response:

As requested by the Reviewer, we have now included a more thorough discussion of the arguments for and against a causal link between cortex expansion and behavioural phenotypes. We also added the sentence that the Reviewer suggested, and thank the Reviewer for this suggestion.

Reviewer's Comment:

3. The same group reported that overexpression of ARHGAP11B by in utero electroporation in WT embryos can cause cortex folding. In the present study cortex folding is not observed neither at the embryonic nor in the adult stage. The authors comment "This most likely reflected the fact that in the 11B mice, ARHGAP11B was "only" expressed at a physiological level, which resulted in an increase in cortical neuron numbers that apparently did not surpass the "threshold" to induce cortical folding as seen previously (Florio et al., 2015)." In Florio et al. (2015), there is no quantification of neurons in upper layers, and the claim made in the present study is hard to evaluate. It would be very interesting and important though to evaluate this "threshold" by comparing/estimating the numbers of neurons in 11B mice and in mice where ARHGAP11B is overexpressed. Otherwise, one may have to conclude that the induction of cortical folds in the previous study was unphysiological?

Authors' response:

We thank the Reviewer for this constructive comment. In Florio *et al.* 2015, the neocortical folds observed in about half of the embryos were induced by *ARHGAP11B* overexpression, using the strong constitutive CAG promotor. This can indeed be regarded as “unphysiological”. To investigate the issue of a potential “threshold”, and in line with the Reviewer's request, we have now repeated the *ARHGAP11B* overexpression by *in utero* electroporation (IUE) at E13.5, followed by quantification of Brn2+ upper-layer neuron abundance at E18.5 (new Figure S7). We observed, again, cortical folding (new Figure S7A), and found almost a doubling (1.8-fold increase; 26% upon control IUE and 47% upon 11B IUE) of the percentage of GFP+ cells in the cortical plate that were positive for the upper-layer neuron marker Brn2 (new Figure S7B, C). Hence, the increase in upper-layer neurons upon 11B overexpression by IUE is indeed substantially greater than the increase in upper-layer neurons in 11B transgenic mice (≤1.2-fold increase in upper-layer neurons (Figure 2L, Figure 3K, L)).

Reviewer's Comment:

4. In all behavioral tests reported in the paper, the authors did not include the 11A+/- mouse model

that they have generated as additional control, only WT mice were used. 11A+/- mice should be added as controls at least in the tests where the authors observed a difference between WT and 11B mice.

Authors' response:

We do not think it is necessary to carry out the behavioural tests with the 11A+/– mice as controls, for the following two reasons. (Also, this would constitute a huge amount of additional work as the behaviour of the 11A+/– mice would have to be compared with WT mice and 11B mice.)

1. We have investigated a potential effect of the decrease in Arhgap11a protein level on apical and basal progenitor mitosis and abundance as well as on the overall cytoarchitecture of the cortical wall, by examining the 11A+/– mouse embryos, and observed no effect, i.e. no difference to WT (Figure S2E-K). This allowed us to conclude that the phenotypes observed in the adult 11B mice, which resulted from the increased basal progenitor levels in the 11B mouse embryos, i.e. the increase in upper-layer neurons and the neocortex expansion, are not due to the Arhgap11a decrease but the expression of ARHGAP11B.

2. Neither Arhgap11a nor ARHGAP11B are expressed in the adult WT or 11B mouse brain (data not shown). We therefore conclude that the differences between WT and 11B mice in the behavioural tests reported in this study are most likely the consequence of differences between WT and 11B mice during embryonic brain development. Since, as stated above, we did not observe any difference between WT and 11A+/– mouse embryos with regard to key aspects of brain development, we in turn conclude that adult 11A+/– mice would behave like WT and would not exhibit the behavioural alterations attributed to 11B expression.

Reviewer's Comment:
5. In Figure 4A and 4B, the authors present only one graph which includes two procedures (corner alternation and corner reversal). The results of the two procedures should be shown in two separate graphs and the statistical evaluations should be more clearly shown. Which phases of the procedures are significantly different between the experimental groups?

Authors' response:

As requested by the Reviewer, we have now separated the data of the previous two panels A and B of Figure 4 into the four panels A, B, C, D of the revised Figure 4. We also specified in the revised Figure 4A-D which tests yielded statistically significant differences between WT and 11B mice. Moreover, we added a more detailed description of the statistical analyses of the data to the Figure Legend.

Reviewer's Comment:
Minor point:
ARHGAP11B is sometimes written AHRGAP11B.

Authors' response:

We apologize for these typos, which we have corrected.

1st Revision - Editorial Decision 12th Mar 2021

Dear Wieland,

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by referee #3. As you can see from the comments below the referee appreciates the introduced changes and supports publication here.

I am therefore very pleased to let you know that we will accept your manuscript for publication here. There are just a few editorial points that we need to sort out before I can send you the formal acceptance letter.

When you submit the revised manuscript will you take care of the following points

We need 3-5 keywords

We don't allow data not shown (pgs 12 + 14). Please rephrase or add the data

There is a callout to Fig EV1G but the panel is missing from the figure.

Figures S1-S7 should be part of the appendix please also fix callouts to the figure and Table in the appendix should have the word 'Appendix' in it. The appendix is missing a ToC with page numbers

I have asked our publisher to do their checks on the paper. They will send me the file within the next few days. Please wait to upload the revised version until you have received their comments.

Please include a point-by-point response when you resubmit

That should be it! Congratulations on a nice study

Best Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
https://bit.ly/EMBOPressFigurePreparationGuideline

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).

- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors:
https://www.embopress.org/page/journal/14602075/authorguide

The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 10th Jun 2021.

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #3:

The authors have addressed all my concerns adequately. Most importantly, they have repeated the ARHGAP11B overexpression by IUE and quantified upper-layer neuron abundance. They found that overexpression by IUE led to a greater increase in upper-layer neuron abundance (1.8-fold) than the increase found in 11B transgenic mice (1.2-fold). Hence, ARHGAP11B overexpression by IUE may have crossed the threshold for cortical folding.
I would be ok with the authors not repeating the behavioral tests with the 11A+/- mice as controls.

I respectfully disagree with referee 1 on his/her assessment that "the amount of work, degree of novelty, and impact of the findings, are clearly below the standard of EMBO Journal." As the authors state in their rebuttal, this story is the first in its kind showing that mice with an expanded neocortex due to the human-specific gene ARHGAP11B exhibit altered behavior.

Point-by-point response to Editor's and Reviewer's comments

Response to Editor

Editor's comment:

Dear Wieland,

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by referee #3. As you can see from the comments below the referee appreciates the introduced changes and supports publication here.

I am therefore very pleased to let you know that we will accept your manuscript for publication here. There are just a few editorial points that we need to sort out before I can send you the formal acceptance letter.

Authors' response:

Dear Karin, we are extremely pleased about this decision and would like to thank you for your super-professional handling of our manuscript and for your support.

Editor's comment:

When you submit the revised manuscript will you take care of the following points

We need 3-5 keywords

Authors' response:

We have now added five keywords after the abstract.

Editor's comment:

We don't allow data not shown (pgs 12 + 14). Please rephrase or add the data

Authors' response:

We have now deleted the phrase “data not shown” on pages 12 and 14.

Editor's comment:

There is a callout to Fig EV1G but the panel is missing from the figure.

Authors' response:

We have checked our figure, the panel Fig EV1G is there. No panel is missing from any figure.

Editor's comment:

Figures S1-S7 should be part of the appendix please also fix callouts to the figure and Table in the appendix should have the word 'Appendix' in it. The appendix is missing a ToC with page numbers

Authors' response:

We have now added the word "Appendix" when referring to a figure in the Appendix, and a ToC with page numbers.

Editor's comment:

I have asked our publisher to do their checks on the paper. They will send me the file within the next few days. Please wait to upload the revised version until you have received their comments.

Authors' response:

We have revised our manuscript according to the publisher's checks.

Editor's comment:

Please include a point-by-point response when you resubmit

Authors' response:

Our point-by-point response to the Editor's and Reviewer's comments is this file.

Editor's comment:

That should be it! Congratulations on a nice study

Best Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journa

Authors' response:

Thank you again very much!

Response to Reviewer #3

Reviewer's comment:

The authors have addressed all my concerns adequately. Most importantly, they have repeated the ARHGAP11B overexpression by IUE and quantified upper-layer neuron abundance. They found that overexpression by IUE led to a greater increase in upper-layer neuron abundance (1.8-fold) than the increase found in 11B transgenic mice (1.2-fold). Hence, ARHGAP11B overexpression by IUE may have crossed the threshold for cortical folding.
I would be ok with the authors not repeating the behavioral tests with the 11A+/- mice as controls.

Authors' response:

We greatly appreciate this evaluation by the Reviewer.

Reviewer's comment:

I respectfully disagree with referee 1 on his/her assessment that "the amount of work, degree of novelty, and impact of the findings, are clearly below the standard of EMBO Journal." As the authors state in their rebuttal, this story is the first in its kind showing that mice with an expanded neocortex due to the human-specific gene ARHGAP11B exhibit altered behavior.

Authors' response:

We also greatly appreciate this assessment by the Reviewer.

2nd Revision - Editorial Decision 25th Mar 2021

Dear Wieland,

Thank you for submitting your revised manuscript. I have now had a chance to take a close look at everything and all looks good!

I am therefore very pleased to accept your manuscript for publication here.

Congratulations! I think this is a super nice study and I am very happy to see it published here.

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here: https://emboj.embopress.org/about#Transparent_Process

Your manuscript will be processed for publication in the journal by EMBO Press. Manuscripts in the PDF and electronic editions of The EMBO Journal will be copy edited, and you will be provided with page proofs prior to publication. Please note that supplementary information is not included in the proofs.

Should you be planning a Press Release on your article, please get in contact with embojournal@wiley.com as early as possible, in order to coordinate publication and release dates.

If you have any questions, please do not hesitate to call or email the Editorial Office. Thank you for your contribution to The EMBO Journal.

** Click here to be directed to your login page: https://emboj.msubmit.net

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Wieland B. Huttner
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2020-107093

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- ➔ the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ➔ figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- ➔ graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- ➔ if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be iustified
- ➔ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- ➔ a specification of the experimental system investigated (eg cell line, species name).
- ➔ the assay(s) and method(s) used to carry out the reported observations and measurements
- ➔ an explicit mention of the biological and chemical entity(ies) that are being measured.
- ➔ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- ➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- ➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- ➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
- ➔ definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - 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.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No power analysis was performed for determining sample size. Sample size was chosen based on previously published studies and pilot studies.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Due to the uncertainty of the number of wildtype and transgenic embryos obtained from pregnant mice, for all experiments carried out with embryos, we used embryoes derived from at least 3 litters. For experiments carried out with adult mice, we used at least 3 animals per group. For all behavioural tests, analyses were carried out using at least 7 animals per group/genotype, and tests were repeated in mutiple cohorts and for both genders.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples/animals were excluded from any analysis.
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.	In all experiments, both wildtype and transgenic animals/samples were allocated to the same treatment.
For animal studies, include a statement about randomization even if no randomization was used.	N/A
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	All analyses were done blindly (without knowing the genotype of the sample).
4.b. For animal studies, include a statement about blinding even if no blinding was done	All analyses were done blindly (without knowing the genotype of the sample).
5. For every figure, are statistical tests justified as appropriate?	Yes, the statistical tests used are indicated in the Methods and Figure Legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. Data normality was tested using Shapiro-Wilk and KS normality tests. Normally distributed data were subjected to Student's t-test. If data were not normally distributed, Mann Whitney test was used instead.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is there an estimate of variation within each group of data?	Yes, variation within each group of data was reported in each group of data as SD.
Is the variance similar between the groups that are being statistically compared?	Yes, F test was used to test homogeneity of variances.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Antibodies used in this study are listed in the Methods, with related information including species, catalog number, company, RRID, dilution used during experiment and citations. We refer to the manufacturer's instructions for antibody validation.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N/A

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	C57BL/6N mice were used throughout. Gender, age and genetic modification status are reported in the Methods and Figure Legends. All animals used for this study were kept in standardized pathogen-free conditions at the Biomedical Services Facility (BMS) of MPI-CBG and at the Czech Centre for Phenogenomics of the Institute of Molecular Genetics of the Czech Academy of Sciences with free access to food and water. Animals were kept with the following light/dark cycle: 12 h / 12 h (mice)
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experimental procedures were designed and conducted according to the European directive 2010/63/EU and in agreement with the German Animal Welfare Legislation and the institutional guidelines of the Animal Care Committee of the Institute of Molecular Genetics. The license number pertaining to the present experiments with mice is WH-19-ARGHAP11B Gehirnentwicklung (9/2019) and (62/2016; 45/2017) (BIOCEV/IMG).
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	All relevant aspects of animal studies are adequately reported.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Fetal human brain tissue was obtained from the Human Development Biology Resource (HDBR), with the fetal human material being provided by the Joint MRC/Wellcome Trust (MR/R006237/1) Human Developmental Biology Resource (http://www.hdbr.org).
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Informed consent was obtained (HDBR) and experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	No datasets were generated in this study.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A