nature portfolio

Peer Review File

Syngap1 and the development of murine neocortical progenitor cells

Corresponding Author: Dr Richard Huganir

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this study, the authors investigate whether mutations in the neurodevelopmental disorder (NDD) risk gene Syngap1 affect cortical neurogenesis in mice. While SynGAP was originally identified as a major component of the post-synaptic density and a synaptic regulator, recent studies have suggested that it may also regulate early neurodevelopment. With the advent of human brain organoid modeling, many studies have demonstrated alterations in neuronal differentiation and/or maturity in organoids with mutations in NDDs or other psychiatric disorder risk genes. This is somewhat unexpected given that the brains of individuals with these disorders is thought to be grossly normal (i.e. no major cortical developmental abnormalities have been reported). It is therefore important to validate these findings in other systems, including in animal models. Here the authors find no evidence for defects in corticogenesis resulting from Syngap1 loss of function mutations in mice. This important analysis demonstrates that there are either species differences in how these mutations affect brain development and function and/or that brain organoid models may be overly sensitive in detecting abnormalities and that perhaps, in vivo, compensatory systems are engaged to normalize development.

Strengths:
The experiments are well done and are appropriate to address the question. It is appreciated that multiple embryonic and post-natal time points were assessed. It is also a strength that multiple Syngap1 mouse models were investigated. The findings are important and provide a counterpoint to recent studies showing large changes in early neurodevelopment resulting from Syngap1 mutations.

Weaknesses:
The only weakness I see with this study is that it is somewhat limited in scope. While showing that there are not major changes in NPC proliferation/differentiation in mouse models of Syngap1 deficiency is an important conclusion, the scope of the experiments and analyses is limited. This is an editorial call, but if the authors wanted to expand the scope, they could consider following up on the potential migration defect they observed and/or consider performing scRNA-seq as this is a very sensitive method for detecting changes in gene expression during development. If Syngap1 does play any role in early development or cell type proportions, it should be detectable by scRNA-seq. If not, then it strongly rules out changes in development in mice as contributing to their phenotypes.

Specific points:
I appreciate that this study was motivated by two prior studies by Willsey and Birtele and that the conclusions of this study differ from those. The authors have done a good job of presenting their opposing view without being overly confrontational. That said, a minor suggestion would be to not refer to the first authors' names when referring to these studies. Rather, the authors might consider refer to them as the study in Xenopus or the study in brain organoids, so as not to single out those authors in particular.

The authors have focused on somatosensory cortex, but it is possible that other cortical regions could be affected by Syngap1 loss. Examining one other cortical region at one of the time points would strengthen their argument.

Were both male and female mice used in this study? What was the sex of the cells from which the brain organoids were derived?

Minor points:
Line 41: The authors write “Research in knockout mice…”. Perhaps just say “Research in Syngap1 heterozygous loss-of-function mice…”

It is unclear why the authors start with E18.5 and then go back to E14.5, it seems more logical to start with the earliest time point and then present later time points. Related to this, it would be helpful if the authors could comment on the correspondence in developmental timing between their mouse models and the human brain organoids in the brain organoid study (Birtele et al).

Reviewer #2

(Remarks to the Author)
This is an important follow-up manuscript published by Giorgio Quadrato and colleagues in 2023 describing a potential non-synaptic function for SYNGAP1. As stated by the authors of this manuscript, SYNGAP1 was discovered by the senior author as one of the most abundant synaptic proteins over 20 years ago. Mutations in the SYNGAP1 gene cause a developmental and epileptic encephalopathy in humans. The manuscript by Quadrato used primarily human organdies to suggest that neurogenesis is abnormal with mutations in SYNGAP1 and then reported a similar finding in mice. In this manuscript by Huagnir and colleagues, the authors deeply investigate this finding in mice and come to a differing conclusion that neurogenesis in both Syngap1 haploinsufficient and complete loss of function have no clear deficits in neurogenesis. The experiments are very well done, data well presented and manuscript well written. The authors for the most part conclude that either differences between humans and mice or differences in experimental design lead to the differing results. I have only 2 major point that need to be addressed and a few minor observations.

Major points:
1. In the discussion, the authors state on the bottom of page 9 to beginning of page 10 that "the observed differences may have resulted from a comparison between different regions of the rostral-caudal axis in control and Syngap1 KO brains" in reference to the mouse findings of Quadrato and colleagues. This is the only mention of a methodological issue with the Quadrato manuscript and does not seem to be supported by any data that is presented. Is this merely speculation or is there evidence to support this. If only speculation, then it should either be taken out or very clearly stated as mere speculation. If there is evidence of this, it should be provided.

2. In extended Figure 2, part C, the authors present evidence that there is no difference in cortical thickness between CT, GAP/GAP and GAP/WT. However in closely examining the data and statistical analysis, it seems there may in fact be a difference for which this experiment was underpowered to detect. I would like to see this experiment repeated following a power analysis to determine the number of mice needed to detect the 10-15% difference that appears to be present.

Minor points:
1. In the last line of the abstract, "SYNGAP" is used. I think the authors want to change this to SYNGAP1.
2. On page 33, in extended Figure 2C figure legend, the authors say "T vs GAP*/GAP*..." I believe the T should be CT.

Thank you for the opportunity to review this manuscript.

Reviewer #3

(Remarks to the Author)
In this study, Barão et al. test the hypothesis that non-synaptic mechanisms of SYNGAP1-deficiency affect brain development in mice, as has been suggested by recent studies in other organisms. In three figures, the authors quite convincingly demonstrate that there are no changes in the number of neuroprogenitors, proliferation of neuroprogenitors, and the numbers of neurons and glia generated in the Syngap1 knockout mice. These studies are clearly described and well-controlled.

One question is whether there is an alternative transcript of Syngap1 that is not dependent on the deleted exons and continues to be expressed in the knockout animals. Discussion of known alternative transcripts and other possible starts might be helpful. Another possibility would be to examine RNA sequencing data from these animals. The investigators could evaluate the transcripts originating from this locus and determine whether this is a possibility.

The other question is whether this is a true species difference or is an unrelated effect of the techniques used in the other studies. The authors might be able to analyze the trajectory of SYNGAP1 in human and mouse neuroprogenitors from available expression data to determine whether there might be a difference that accounts for this discrepancy.

Third, there appears to be some differences between the mouse background strains used in this manuscript vs those studied in Birtele et al paper. It is possible, and in fact common, that some phenotypes of a pathogenic variant (in this case in Syngap1) can only be detected in a certain mouse strain background but not in others. Adding more details of the mixed background used in this paper to provide a more detailed way to compare the differences between the two papers and adding this possibility to the discussion would be helpful.

Finally, the literature on the brain/head size of patients with SYNGAP1-related intellectual disability (SYNGAP1-ID) is mixed. There appears to be a subset of patients with microcephaly while this is not a uniform manifestation of this condition.

It would be helpful to add some comments related to the significance of these mouse findings in the setting of the clinical picture.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed my main comments and I believe the data are convincing. The inclusion of the sequencing analyses bolsters the conclusions that Syngap1 mRNA is lowly expressed during embryonic development and that there are not major changes in cortical cell type proportions as determined by levels of key cell type marker genes. I just have a few minor comments:
1) One of the other reviewers mentioned the possibility of another isoform of Syngap1 being expressed in the mouse models that could account for the lack of effect on early development. The authors provided a thorough response in the rebuttal but I think it would be worth discussing this more in the manuscript itself.
2) There may be a word missing in the sentence in lines 199-200: do the authors mean "undergoing differentiative division"?
3) In line 200 – perhaps remove this part of the statement "…properties of their neocortical progenitor cells" since the properties of the cells were not directly addressed here.
4) In lines 217-218 the authors state that "only a reduced subset of human SYNGAP1 haploinsufficiency patients are diagnosed with microcephaly or structural brain abnormalities". It would be helpful to be more specific here, i.e. approximately what percentage of SYNGAP1 patients have microcephaly?
5) The authors sometimes use "SYNGAP1" when referring to the mouse gene (instead of "Syngap1")
6) In lines 219-220, the authors state that the phenotypes likely arise due to SynGAP's synaptic funcitons. Perhaps this could be broadened to say SynGAP's roles in neuronal physiology (including synaptic and intrinsic excitability changes)

Reviewer #3

(Remarks to the Author)
The authors have addressed all my comments.

NCOMMS-25-05776_Response to reviewer comments

We thank the reviewers for their insightful comments and the valuable time they invested in their reviews. We are pleased to report that the resulting changes and additions have strengthened the manuscript and have contributed to a better clarification of the role of SYNGAP1 in murine neocortical progenitor cells. Below we provide a detailed response to the reviewer comments.

Summary of the main changes to the original manuscript:

- The text was adapted to not include the name of the authors of the previous studies.
- Analysis of $PAX6^+$ progenitor cells in the motor cortex were included in **Supplementary Figure 2b**. These results further strengthened our findings showing that the number of $PAX6^+$ progenitor cells also remains unaltered in the motor cortex of these *Syngap1* mouse models.
- Single-cell RNA sequencing analyses were done using previously published datasets from mouse (Di Bella et al. 2021)[31] and human neocortex (Polioudakis et al. 2019)[32] to better represent the expression patterns of *Syngap1* in the two species and support the discussion of the manuscript. These analyses are now part of **Supplementary Figure 1**. Overall, the expression of *Syngap1* is extremely low during embryonic development in both mouse and human neocortex.
- Whole-brain RNA sequencing analyses to characterize the expression of neurodevelopmental marker genes were done using a previously published dataset from two different *Syngap1* haploinsufficiency mouse models originally reported in Araki et al., 2023[16]. These analyses are now included in **Supplementary Figure 3**. In line with the results we reported, this analysis shows that an extensive list of cortical layer and cellular markers are unaltered in $Syngap1^{+/-}$ and $Syngap1^{+/c.3583\text{-}9G>A}$ haploinsufficient adult mice.
- We have confirmed that all analyses passed the normality tests. Two analyses had to be improved: In **Figure 2c**, the number of cells per animal was set to a minimum of 20 to guarantee the normality of the analysis; In **Supplementary Figure 2d** (previous Supplementary Figure 2c) and following the remark number 2 from reviewer #2, the number of animals analyzed per group was increased to guarantee the normality of the analysis and make sure we could detect a 10-15% difference if present. Similar to what we have reported in the original manuscript, no statistical differences of cortical thickness were found for $Syngap1^{GAP*/GAP*}$ or $Syngap1^{+/GAP*}$ when compared to the control.
- In the new version of the manuscript, we have replaced the multiple unpaired t-test by the one-way ANOVA followed by Dunnett's post-hoc test when we analyzed more than two groups to guarantee a statistically rigorous comparison of each mutant to the control group. This approach did not change any of our previous conclusions. The methods, the figure legends, and the DATA source file were updated accordingly.

Response to reviewer comments

Reviewer #1 (Remarks to the Author):

In this study, the authors investigate whether mutations in the neurodevelopmental disorder (NDD) risk gene Syngap1 affect cortical neurogenesis in mice. While SynGAP was originally identified as a major component of the post-synaptic density and a synaptic regulator, recent studies have suggested that it may also regulate early neurodevelopment. With the advent of human brain organoid modeling, many studies have demonstrated alterations in neuronal differentiation and/or maturity in organoids with mutations in NDDs or other psychiatric disorder risk genes. This is somewhat unexpected given that the brains of individuals with these disorders is thought to be grossly normal (i.e. no major cortical developmental abnormalities have been reported). It is therefore important to validate these findings in other systems, including in animal models. Here the authors find no evidence for defects in corticogenesis resulting from Syngap1 loss of function mutations in mice. This important analysis demonstrates that there are either species differences in how these mutations affect brain development and function and/or that brain organoid models may be overly sensitive in detecting abnormalities and that perhaps, in vivo, compensatory systems are engaged to normalize development.

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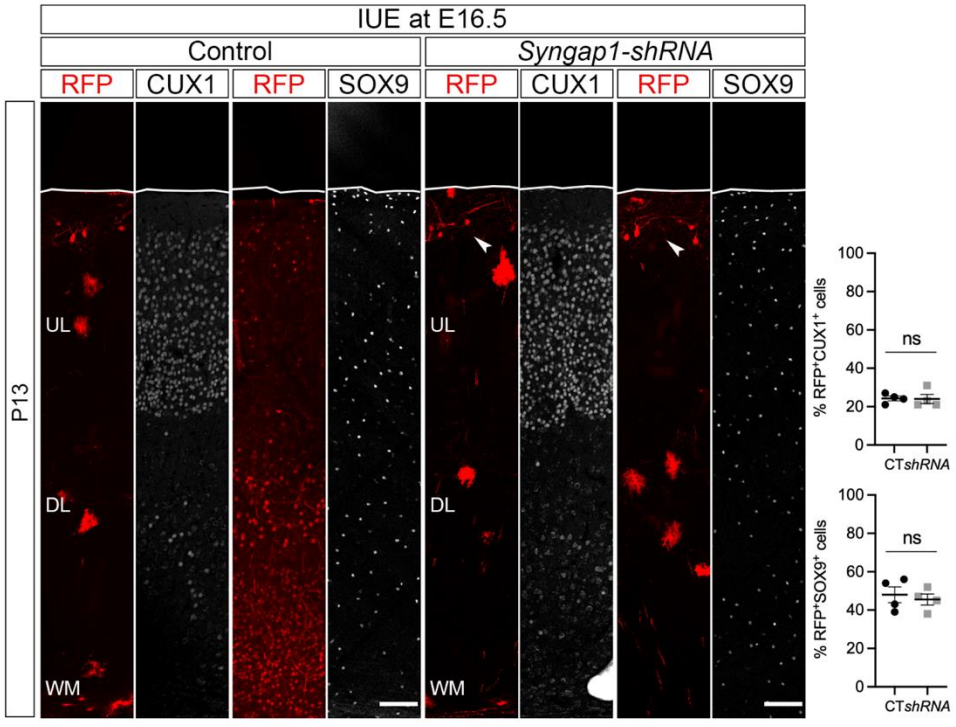
We have taken multiple approaches to address this concern. First, although we fully agree that a single-cell RNA sequencing experiment at different developmental stages in *Syngap1* haploinsufficient mice would be a more elegant way to clarify SYNGAP1's role in neurodevelopment, we re-analyzed the whole mouse brain bulk RNA-sequencing results from Araki et al., 2023[16] in two different *Syngap1* mouse models (**Supplementary Figure 3**). In line with the results we obtained in this manuscript, this analysis shows that an extensive list of cortical layer and cellular markers are unaltered in *Syngap1*$^{+/-}$ and *Syngap1*$^{+/c.3583\text{-}9G>A}$ haploinsufficient adult mice, supporting our results that *Syngap1* loss-of-function does not affect the specification of the different cell types during early neurodevelopment.

Second, to confirm the migration phenotype we observed in *Syngap1KO* and *Syngap1*$^{GAP*/GAP*}$ mice, we knocked down *Syngap1* by in utero electroporation with *Syngap1* shRNA in mice at E16.5 and analyzed the cell fate of the electroporated cells at P13. *Syngap1*-deficiency did not affect the ratio of RFP-positive ULNs (RFP$^+$CUX1$^+$ cells) or RFP-positive glia cells (RFP$^+$SOX9$^+$ cells) in mice (**Revision Figure 1**). In these experiments, we did not observe migration defects in the electroporated cells, suggesting SYNGAP1's role in migration is non-cell-autonomous or developmental stage specific (arrowheads). Nonetheless, more studies are needed to fully understand the role of SYNGAP1 in neuronal migration.



Revision Figure 2. *Syngap1* knocked down by in utero electroporation of progenitor cells did not change their neuron/glia ratio or migration. Cortical cell identities of the control and *Syngap1*-shRNA condition immunolabeled for RFP (red), CUX1 or SOX9 (grey) at P13 after in utero electroporation (IUE)

of wild-type mice at E16.5 (n=4 CT, n=4 *Syngap1*-shRNA; two-sided unpaired t-test: CUX1 p=0.9289; SOX9 p=0.6387). Arrowheads highlight the correct position of the RFP^+ neurons. DL, Deep Layer; UL, Upper Layer; WM, White Matter. Values are mean ± SEM; ns, not significant. Scale bars: 100 μm (P13).

Specific points:

I appreciate that this study was motivated by two prior studies by Willsey and Birtele and that the conclusions of this study differ from those. The authors have done a good job of presenting their opposing view without being overly confrontational. That said, a minor suggestion would be to not refer to the first authors' names when referring to these studies. Rather, the authors might consider refer to them as the study in Xenopus or the study in brain organoids, so as not to single out those authors in particular. The text was changed to incorporate the reviewer's suggestion along the manuscript.

The authors have focused on somatosensory cortex, but it is possible that other cortical regions could be affected by Syngap1 loss. Examining one other cortical region at one of the time points would strengthen their argument. To address this concern, we have quantified the number of $PAX6^+$ cells in the motor cortex of Control (CT), heterozygous (+/-) and homozygous (-/-) *Syngap1KO* brains at E18.5. These results were incorporated in **Supplementary Figure 2** – now panel b. The results further strengthened our findings showing that the number of $PAX6^+$ progenitor cells also remain unaltered in the motor cortex of these *Syngap1* mouse models.

Were both male and female mice used in this study? What was the sex of the cells from which the brain organoids were derived? We now report on gender in the reporting summary. Briefly, we have not determined sex for embryonic or postnatal day 0 samples. For P13 analysis, since no differences have been reported between males and females for the analyzed markers in our previous studies, we have included both sexes in these analysis - **Figure 3c and 3d**: control = 3 males and 1 female; $Syngap1^{+/-}$ = 2 males and 2 females. Birtele et al.[22] used iPSCs derived from mutant and control males to generate the human organoids.

Minor points:

Line 41: The authors write "Research in knockout mice…". Perhaps just say "Research in Syngap1 heterozygous loss-of-function mice…" The text was changed to incorporate the reviewer's suggestion in the revised manuscript.

It is unclear why the authors start with E18.5 and then go back to E14.5, it seems more logical to start with the earliest time point and then present later time points. Related to this, it would be helpful if the authors could comment on the correspondence in developmental timing between their mouse models and the human brain organoids in the brain organoid study (Birtele et al). It is still challenging to establish the exact age equivalent between the time of the organoid cultures and the developmental time of the human brain and even more so to link it to the developmental time of the mouse brain. A 2-month-old brain organoid used by Birtele et al.[22] roughly corresponds to the early to mid-fetal brain development stage in humans. Specifically, it may be comparable to prenatal stages in human fetal brain development (10-38 weeks post-conception). This is a vast period for human brain development and therefore also very imprecise when compared to mouse brain development but roughly corresponds to the end stage of embryonic development in mice (E17-E18). Taking this into consideration, we focused the manuscript mainly on the study of E18 brains similar to the age presented by Birtele et al. for the mouse DATA[22]. We then included E14.5 experiments to understand whether *Syngap1* would play a role in the neurogenesis of upper layer neurons and whether in those progenitors its role could be more evident. To address the reviewer concern, we have added modifications in the manuscript to make this reasoning clearer.

Reviewer #2 (Remarks to the Author):

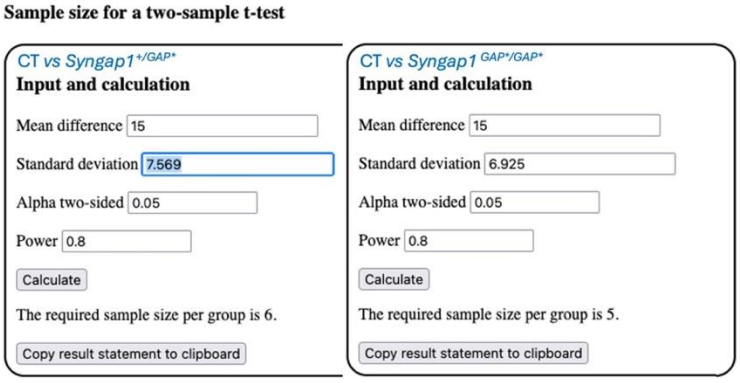
This is an important follow-up manuscript published by Giorgio Quadrato and colleagues in 2023 describing a potential non-synaptic function for SYNGAP1. As stated by the authors of this manuscript, SYNGAP1 was discovered by the senior author as one of the most abundant synaptic proteins over 20 years ago. Mutations

in the SYNGAP1 gene cause a developmental and epileptic encephalopathy in humans. The manuscript by Quadrato used primarily human organdies to suggest that neurogenesis is abnormal with mutations in SYNGAP1 and then reported a similar finding in mice. In this manuscript by Huagnir and colleagues, the authors deeply investigate this finding in mice and come to a differing conclusion that neurogenesis in both Syngap1 haploinsufficient and complete loss of function have no clear deficits in neurogenesis. The experiments are very well done, data well presented and manuscript well written. The authors for the most part conclude that either differences between humans and mice or differences in experimental design lead to the differing results. I have only 2 major point that need to be addressed and a few minor observations.

Major points:

1. In the discussion, the authors state on the bottom of page 9 to beginning of page 10 that "the observed differences may have resulted from a comparison between different regions of the rostral-caudal axis in control and Syngap1 KO brains" in reference to the mouse findings of Quadrato and colleagues. This is the only mention of a methodological issue with the Quadrato manuscript and does not seem to be supported by any data that is presented. Is this merely speculation or is there evidence to support this. If only speculation, then it should either be taken out or very clearly stated as mere speculation. If there is evidence of this, it should be provided. Because the brain sections shown in Figure 5 panels e and f of Birtele et al.[22] are clearly taken from different rostral-caudal levels in control and *Syngap1*KO brains, we can speculate that this discrepancy contributed to the statistical differences reported in panels g and h. Notably, when sections from comparable rostral-caudal levels were analyzed (Extended Data Figure 6, panels h–j), Birtele et al.[22] reported no significant differences. Nonetheless, we fully agree with the reviewer's point, and given that our original statement was speculative, we have removed it from the manuscript.

2. In extended Figure 2, part C, the authors present evidence that there is no difference in cortical thickness between CT, GAP/GAP and GAP/WT. However, in closely examining the data and statistical analysis, it seems there may in fact be a difference for which this experiment was underpowered to detect. I would like to see this experiment repeated following a power analysis to determine the number of mice needed to detect the 10-15% difference that appears to be present. Taking into consideration this concern, we have confirmed the normality for all the analysis presented in the manuscript and have increased the n in few analyses to guarantee the statistical tests passed the normality test – **Figure 2c**: number of cells per condition was set to a minimum of 20 to guarantee the normality of the presented analysis; **Supplementary Figure 2d** (previous Supplementary Figure 2c): number of animals analyzed per group was increased to guarantee the normality of the analysis and make sure we can detect a 10-15% difference if present. In the experiment that the Reviewer 2 mentioned, after power analysis we determined we needed to increase the n for all the groups to be able to confidently detect a 10-15% difference if present (see power analysis reported below – **Revision Figure 2**). The new analysis is now included in **Supplementary Figure 2d** (previous Supplementary Figure 2c). Similar to what we have reported before, no statistical differences of cortical thickness were found for *Syngap1*$^{GAP*/GAP*}$ or *Syngap1*$^{+/GAP*}$ when compared to the control.



Revision Figure 2. Power analysis for a two-sample t-test based on the results previously reported in Supplementary Figure 2c

Minor points:

1. In the last line of the abstract, "SYNGAP" is used. I think the authors want to change this to SYNGAP1. The text is now corrected in the revised manuscript.

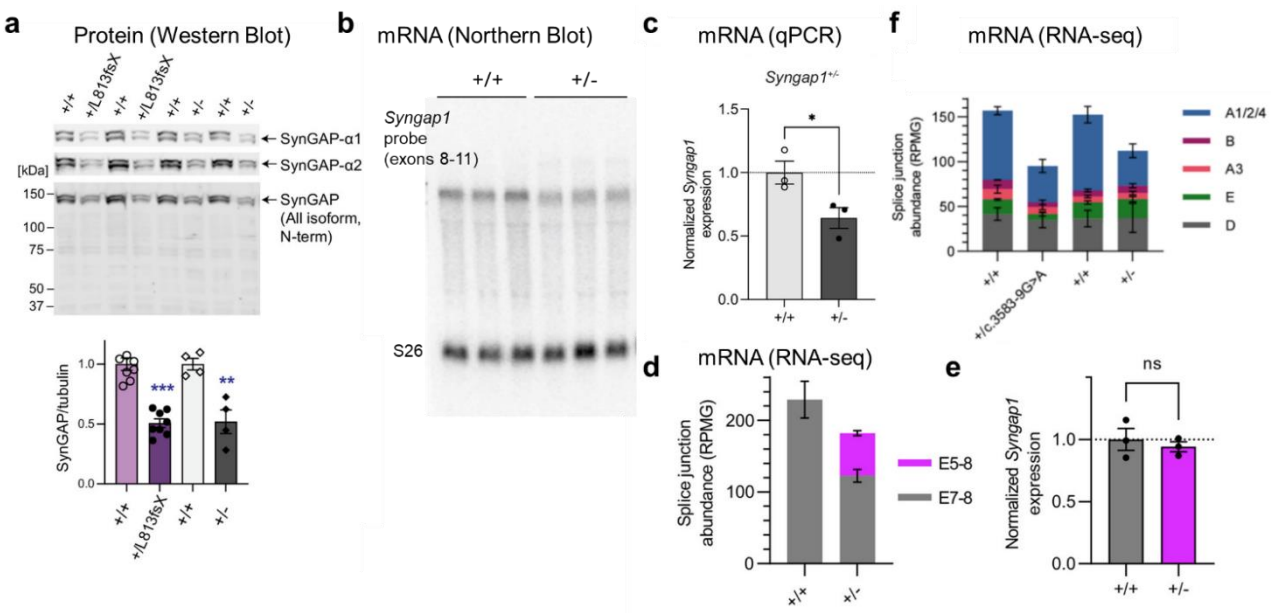
2. On page 33, in extended Figure 2C figure legend, the authors say "T vs GAP*/GAP*..." I believe the T should be CT. The text is now corrected in the revised manuscript.

Reviewer #3 (Remarks to the Author):

In this study, Barão et al. test the hypothesis that non-synaptic mechanisms of SYNGAP1-deficiency affect brain development in mice, as has been suggested by recent studies in other organisms. In three figures, the authors quite convincingly demonstrate that there are no changes in the number of neuroprogenitors, proliferation of neuroprogenitors, and the numbers of neurons and glia generated in the Syngap1 knockout mice. These studies are clearly described and well-controlled.

One question is whether there is an alternative transcript of Syngap1 that is not dependent on the deleted exons and continues to be expressed in the knockout animals. Discussion of known alternative transcripts and other possible starts might be helpful. Another possibility would be to examine RNA sequencing data from these animals. The investigators could evaluate the transcripts originating from this locus and determine whether this is a possibility.

We examined the transcript expression of *Syngap1*$^{+/-}$ mice extensively previously[16] and here to address the reviewer's question. Overall, there is likely little protein arising from the deleted allele. *Syngap1*$^{+/-}$ mice were previously generated by deleting the core exons 6 and 7, which shifts the reading frame and leads to reduction of Syngap1[10]. Western blotting of protein extracted from the whole brain of *Syngap1*$^{+/-}$ mice revealed approximately 50% less Syngap1 protein expression (**Revision Figure 3a**). Northern blots with a *Syngap1* probe targeting exons 8-11 (and a control S26 RNA probe) show a similar reduction of *Syngap1* expression and no significant other isoform bands (**Revision Figure 3b**). Quantitative PCR (qPCR) using exon-exon junction assays on whole-brain mRNA extracts from *Syngap1*$^{+/-}$ mice revealed a ~40% reduction of mRNA in comparison to wild-type controls (**Revision Figure 3c**), suggesting that mutant *Syngap1* mRNA is subject to nonsense-mediated decay (NMD) as expected due to the premature stop codon. We verified in bulk brain RNA-seq of these mice that exons 6 and 7 are skipped in a large portion of transcripts, which are not completely degraded through NMD (**Revision Figure 3c**). Perhaps due to this incomplete NMD and significant intron retention, total estimated *Syngap1* RNA expression (which includes intron-including pre-mRNA) is not significantly lower in these mice compared to wild-type littermates (**Revision Figure 3e**). The difference with qPCR and Northern blot quantification could be due to transcripts with intron retention and immature polyA tails, which affect these measures differently. Given the halved Syngap1 protein expression and absence of substantial truncated protein species (**Revision Figure 3a**), this suggests that the transcripts from the knockout allele may be protected from NMD to an extent but are nevertheless translationally inactive. Substantial intron retention of intron 7 in *Syngap1*$^{+/-}$ mice (Araki et al. PNAS 2023 *SI Appendix*, Figure S3) further suggests that nuclear retention of unspliced pre-mRNA may contribute to this resistance to NMD, which occurs in the cytosol only after mRNA maturation. Alternative splicing events, including exon 14 skipping, exon 17 5′ extension (VK), exon 18 5′ extension (β vs. non-β), exon 18 3′ extensions, and exon 19 skipping (α1, α2, γ), did not show significant changes in ratio (Araki et al. PNAS 2023 *SI Appendix*, Figure S2 *B*–*E*). As the reviewer suggested, whereas an N-terminal splice junction unique to full-length *Syngap1* (A1/2/4; exon 3-4) was significantly reduced in both *Syngap1*$^{+/c.3583\text{-}9G>A}$ (P = 0.0210, two-way ANOVA and Šídák's post hoc test) and *Syngap1*$^{+/-}$ mice (P = 0.0437), other isoforms, including the second-most abundant D isoform, did not show a significant RNA reduction (**Revision Figure 3f**). This suggests that these N-terminal isoforms may be subject to less efficient NMD. The less pronounced downregulation of these N-terminal isoforms might contribute to the <50% decrease observed in *Syngap1* mRNA levels (**Revision Figure 3c**). These transcripts nevertheless are minor isoforms and likely contribute a small amount of Syngap1 protein (**Revision Figure 3a and b**).



Revision Figure 3. mRNA and protein measurements in *Syngap1*$^{+/-}$ mice. a Western blot of *Syngap1*$^{+/-}$ and *Syngap1*$^{+/L813fsX}$ mice brain samples. **b** Northern blot of *Syngap1*$^{+/-}$ mouse brains with a *Syngap1* probe targeting exons 8-11 and a control S26 RNA probe. **c** qPCR of *Syngap1*$^{+/-}$ mice brain samples using exon-exon junction assays. **d** Exon junction-spanning read abundance in bulk brain RNA-seq of *Syngap1*$^{+/-}$ mice. Exons 6 and 7 are skipped in exon 5-8 junction reads. **e** RNA-seq measurement of total *Syngap1* expression (which includes intron-including pre-mRNA) is not significantly lower in these mice compared to wild-type littermates. **f** Exon junction-spanning read abundance of N-terminal splice isoforms of *Syngap1* (A1/2/4; A3; B; D; E isoforms).

The other question is whether this is a true species difference or is an unrelated effect of the techniques used in the other studies. The authors might be able to analyze the trajectory of SYNGAP1 in human and mouse neuroprogenitors from available expression data to determine whether there might be a difference that accounts for this discrepancy. To address this question, we have analyzed previously published single-cell RNA sequencing datasets from mouse[31] and human[32] neocortex and included this analysis in **Supplementary Figure 1**. In support of our findings, the expression levels of *Syngap1* are very low in all cell types analyzed during embryonic development in both human and mouse neocortex.

Third, there appears to be some differences between the mouse background strains used in this manuscript vs those studied in Birtele et al paper. It is possible, and in fact common, that some phenotypes of a pathogenic variant (in this case in Syngap1) can only be detected in a certain mouse strain background but not in others. Adding more details of the mixed background used in this paper to provide a more detailed way to compare the differences between the two papers and adding this possibility to the discussion would be helpful. The *Syngap1*KO mice used in this manuscript are the same mice and under the same genetic background as the mice used by Birtele et al.[22] in their study. We have added details and made this clear in the revised manuscript.

Finally, the literature on the brain/head size of patients with SYNGAP1-related intellectual disability (SYNGAP1-ID) is mixed. There appears to be a subset of patients with microcephaly while this is not a uniform manifestation of this condition. It would be helpful to add some comments related to the significance of these mouse findings in the setting of the clinical picture. Microcephaly and structural brain anomalies are very rare in SYNGAP1 patients, which aligns with our findings showing no brain size abnormalities in *Syngap1* haploinsufficient mice that model key aspects of SYNGAP1-related intellectual disability. Similarly, despite observing changes in the ratio of cell types, Birtele et al.[22] reported no overall size differences in the analyzed human organoids. We have revised the manuscript to clarify this point and emphasize that the absence of structural phenotypes in our model is consistent with observations in SYNGAP1 patients.

NCOMMS-25-05776_Response to reviewer comments

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1) One of the other reviewers mentioned the possibility of another isoform of Syngap1 being expressed in the mouse models that could account for the lack of effect on early development. The authors provided a thorough response in the rebuttal but I think it would be worth discussing this more in the manuscript itself. The discussion was changed to incorporate the reviewer's suggestion.

2) There may be a word missing in the sentence in lines 199-200: do the authors mean "undergoing differentiative division"? The text was changed to correct it.

3) In line 200 – perhaps remove this part of the statement "…properties of their neocortical progenitor cells" since the properties of the cells were not directly addressed here. The text was changed to incorporate the reviewer's suggestion.

4) In lines 217-218 the authors state that "only a reduced subset of human SYNGAP1 haploinsufficiency patients are diagnosed with microcephaly or structural brain abnormalities". It would be helpful to be more specific here, i.e. approximately what percentage of SYNGAP1 patients have microcephaly? The text was changed to incorporate the reviewer's suggestion.

5) The authors sometimes use "SYNGAP1" when referring to the mouse gene (instead of "Syngap1") The text was changed to correct this along the manuscript.

6) In lines 219-220, the authors state that the phenotypes likely arise due to SynGAP's synaptic funcitons. Perhaps this could be broadened to say SynGAP's roles in neuronal physiology (including synaptic and intrinsic excitability changes) The text was changed to incorporate the reviewer's suggestion.

Reviewer #3 (Remarks to the Author)

The authors have addressed all my comments. N/A.