Dihydrofolate reductase activity controls neurogenic transitions in the developing neocortex
Sulov Saha, Thomas T. Jungas, David Ohayon, Christophe Audouard, Tao Ye, Mohamad-Ali Fawal and Alice Davy
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Original submission

First decision letter

MS ID#: DEVELOP/2023/201696

MS TITLE: Dihydrofolate reductase activity controls neurogenic transitions in the developing neocortex

AUTHORS: Sulov Saha, Thomas TJ Jungas, David Ohayon, Christophe Audouard, Tao Ye, Mohamad-Ali Fawal, and Alice Davy

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some comments that in our view should be addressed as completely as possible before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.
Reviewer 1

Advance summary and potential significance to field

Review for “Dihydrofolate reductase activity controls neurogenic transitions in the developing neocortex”

Saha et al. address the biological question "whether DHFR plays a role in specific steps of neocortex development"? and clearly showed that this is the case. The authors used genetic and pharmacologic perturbation of DHFR in mouse embryos, and pharmacologic inhibition of the enzyme in human neural organoids to show shifts in neural populations in the developing neocortex. The data supports the conclusion that DHFR plays a role in normal neocortex development, and that perturbation of DHFR results in changes in histone-methylation patterns, gene expression profile and cell identity. Causality of each of these components is not assessed but the axis is a well-established one and each of these results of DHFR perturbation is interesting on its own. Reporting these findings is of interest to the community.

The paper reads well and is easy to follow.

I have a few addressable comments:

Comments for the author

I have a few addressable comments:
1. Figure 3A contains some points that can be better represented.
   A. Folate is not the relevant form to be imported into brain cells in the embryo. It is 5-methyl THF that is the abundant form of folate in the plasma. Although neurons in culture (such as in the NEO system) do import folate, because that is the form provided with commercial media, the scheme is misleading and should reflect the in vivo scenario as well.
   B. In order to convey that folate is utilized in a recyclable manner (that likely explains why DHFR+/Δ cells don’t die), two adjustments can be done: in the TYMS reaction - add the reaction product DHF; in the MTR reaction - add the reaction product THF.
   C. “Methenyl THF” - please change to 5,10-methenyl THF.
   D. “Formyl THF” - please change to 10-formyl THF (the relevant form for purine synthesis).
2. Better description of some of the methods will be helpful. Specifically - the assays used to assess DHFR activity and THF levels should be mentioned in the legend (not only in the methods).
3. Please add the dose used for MTX treatment in relevant panels in Figure 3 or at least to the legend (2&61549; M is mentioned only in figure 4).
4. Figure 4B - the authors mentioned in the correction that they increased the n number for this experiment. Currently n equals 2-3. Is this the updated number?
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6. The last sentence in the results section feels like an overstatement. Although it is better explained in the discussion, in its current form and place it reads as an overstatement. Reviewer 2 commented on this in the first round, and although I read the response and understand the rationale behind the statement because the focus of the data up to this point is on ratios between populations and not total increase in cell numbers, I recommend rethinking the wording of this last sentence in the results: either build up the rationale better before this sentence, or just keep it for the discussion.
7. Please adjust this sentence in the discussion: “..., this is not true for rate limiting enzymes” to “... this is not true for rate limiting some enzymes.”
Reviewer 2

Advance summary and potential significance to field

Saha et al. investigated the function of dihydrofolate reductase (DHFR) in cortical neurogenesis. First, they made the interesting observation that DHFR was strikingly expressed only at very early stages of corticogenesis. Then they generated DHFR mutant mice and examined cortical neurogenesis at several stages throughout embryogenesis. At early stages (E12.5), they found that DHFR haploinsufficiency leads to a reduction in the number of apical progenitors and deep layer neurons (Ctip2+) while at mid-corticogenesis stages (E14.5) DHFR mutant mice display increased number of Tbr2+ basal progenitors and upper layer neurons (Satb2+), and at latest stages analyzed (E16.5) they find a decrease in intermediate progenitors while the number of Satb2+ neurons remains much increased, seemingly at the expense of CTIP2+ deep layer neurons. This altered neuronal composition is also found to be maintained at P21. At the molecular level, DHFR mutant mice show decreased levels of H3K4me3 levels at E12.5, while treatment with MTX, which leads to DHFR inhibition, on mouse cortical cells cultures affected H3K4me3 levels at the level of some cortical neuron fate marker genes. MTX treatment in utero also led to changes in some neuronal fate marker expression. Finally, treatment of human cerebral organoids with MTX led to increased proportion of intermediate progenitor-like cells and neurons expressing TBR1, CTIP2 and SATB2. The authors conclude that the balance of direct vs indirect neurogenesis is impaired following DHFR inhibition. This is a potentially interesting study on a timely topic, combing mouse in vivo and ex vivo and human in vitro analyses on the impact of an important metabolic pathway on cortical neurogenesis. However, the data presented to do not support strongly the conclusion of an imbalance of direct vs. indirect neurogenesis. Indeed the only parameter that goes in line with this conclusion is the change in proportion of Tbr2 cells, but it depends on the stage considered (first unchanged, then increased, then decreased). Phenotypes could be equally linked, for instance, to changes in proliferation, differentiation, or temporal patterning, which have not been examined sufficiently. The molecular analyses are presented in a somewhat superficial way, making it difficult to judge them in full. There are also some important informations missing on the methods of quantification of mouse embryos and organoids. Major points are summarized below.

Comments for the author

1. The method of quantification of fate markers on DHFR control vs het mice should be explained in much more detail, as this is a delicate process prone to sampling artifacts. Which level of the brain was chosen and how? How many cells / levels / animals were used? This is very important given the well-known differences in cell fate proportions depending on the area and stage considered. Moreover the numbers should be provided in detail in every legend not just in the suppl table where it is difficult to find the relevant information.

2. Similarly, how the organoids were quantified should be explained in much more detail. Firstly, the authors should provide more evidence that they are indeed analyzing ‘cortical-like’ tissue, for instance by focusing on FoxG1 or Emx1-positive parts of the organoids. This is crucial given the known variability in the regional identity of cells generated with these systems. Numbers of experiments, organoids, regions of interest and cells should be provided in much more detail to give a better idea of variability in their experiments - including in the legends. Without appropriate validation of the regional identity of the ROI examined the analysis focusing on Tbr2 and CTIP2/Satb2 could be very misleading.

3. The data shown are not sufficient to support the conclusion that the balance between direct vs indirect neurogenesis is altered in the mutant mice. The authors should perform additional analyses to understand better the origin of the fate changes and explore equally interesting and important alternatives. BrdU short-term (1hr-24hrs) labeling should be performed to determine the levels of proliferation and cell cycle exit, which could equally explain differences in the proportion of neurons of different fate/layer identity. BrdU pulse-chase labelings should also be determined to determine the timing of birthdate of the neurons, as alterations of temporal patterning could be another important mechanism that is altered here.

4. The differences observed between human vs mouse models are intriguing (CTIP2 vs Satb2 neurons) but difficult to interpret given that the stages examined are not all the same, and whether they are linked to intermediate progenitors remains quite unclear from the data shown.
Unless more analysis as in point 3 are performed in the human system, this should be discussed in a much more conservative fashion.

5. In the MTX treatment in vivo: what happens to Tbr2 and Satb2? These should be shown and quantified to be compared with the genetic model and the organoid data.

6. In the ChiPseq data, how do the authors explain that some genes display higher levels and others lower levels of methylation? How does this fit with the data that global levels of Histone methylation are decreased? In any case it would be much more convincing to show all the ChiPseq data in order to interpret them optimally, instead of focusing on 4 genes only.

7. The authors show changes of number of CtIP2+ and Satb2+ and conclude that deep and upper layer composition is changed in mutant mice. It would be important to test other layer markers (e.g. Foxp2, Cux1, Brn2,…) to support further the interpretation of a genuine fate change.

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**First revision**

**Author response to reviewers' comments**

We thank the reviewers for their constructive comments which we addressed either by modifying the text (in red) and/or the figures, or by providing additional data.

Reviewer 1 Advance Summary and Potential Significance to Field:
Saha et al. address the biological question "whether DHFR plays a role in specific steps of neocortex development"? and clearly showed that this is the case. The authors used genetic and pharmacologic perturbation of DHFR in mouse embryos, and pharmacologic inhibition of the enzyme in human neural organoids to show shifts in neural populations in the developing neocortex. The data supports the conclusion that DHFR plays a role in normal neocortex development, and that perturbation of DHFR results in changes in histone-methylation patterns, gene expression profile, and cell identity. Causality of each of these components is not assessed but the axis is a well-established one and each of these results of DHFR perturbation is interesting on its own. Reporting these findings is of interest to the community.

The paper reads well and is easy to follow. I have a few addressable comments:

Reviewer 1 Comments for the Author: I have a few addressable comments:

1. Figure 3A contains some points that can be better represented. A. Folate is not the relevant form to be imported into brain cells in the embryo. It is 5-methyl THF that is the abundant form of folate in the plasma. Although neurons in culture (such as in the NEO system) do import folate, because that is the form provided with commercial media, the scheme is misleading and should reflect the in vivo scenario as well.

B. In order to convey that folate is utilized in a recyclable manner (that likely explains why DHFR+/Δ cells don’t die), two adjustments can be done: in the TYMS reaction - add the reaction product DHF; in the MTR reaction - add the reaction product THF.

C. “Methenyl THF” - please change to 5,10-methenyl THF.

D. “Formyl THF” - please change to 10-formyl THF (the relevant form for purine synthesis).

We modified the schematic presented in Fig4A (Fig3A in the previous version) as proposed by the reviewer.

2. Better description of some of the methods will be helpful. Specifically - the assays used to assess DHFR activity and THF levels should be mentioned in the legend (not only in the methods).

We modified the text accordingly, we included the Material and Methods section in the main document and added the assays used for DHFR activity, THF and SAM levels in the Figure legends.

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3. Please add the dose used for MTX treatment in relevant panels in Figure 3 or at least to the legend (2µM is mentioned only in figure 4).

We modified the figures accordingly.

4. Figure 4B - the authors mentioned in the correction that they increased the n number for this experiment. Currently n equals 2-3. Is this the updated number?

These are the updated numbers. Each biological replicate is composed of 6-8 organoids. For one condition we quantified 2 biological replicates while for all other conditions we quantified 3 biological replicates.

5. Please add n numbers to the legends of
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   • Figure 2: A, B, C, D, E-G, H-M
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We will added n numbers in the graphs.

6. The last sentence in the results section feels like an overstatement. Although it is better explained in the discussion, in its current form and place it reads as an overstatement. Reviewer 2 commented on this in the first round, and although I read the response and understand the rationale behind the statement, because the focus of the data up to this point is on ratios between populations and not total increase in cell numbers, I recommend rethinking the wording of this last sentence in the results: either build up the rationale better before this sentence, or just keep it for the discussion.

We changed the last sentence of the Result section to « Altogether, these findings indicate that inhibition of DHFR in human neural organoids leads to an early developmental delay followed by an increased and accelerated neuronal production. »

7. Please adjust this sentence in the discussion: “... this is not true for rate limiting enzymes” to “this is not true for rate limiting some enzymes”.

We adjusted the sentence in the discussion.

Reviewer 2 Advance Summary and Potential Significance to Field:

Saha et al. investigated the function of dihydrofolate reductase (DHFR) in cortical neurogenesis. First, they made the interesting observation that DHFR was strikingly expressed only at very early stages of corticogenesis. Then they generated DHFR mutant mice and examined cortical neurogenesis at several stages throughout embryogenesis. At early stages (E12.5), they found that DHFR haploinsufficiency leads to a reduction in the number of apical progenitors and deep layer neurons (Ctip2+) while at mid-corticogenesis stages (E14.5) DHFR mutant mice display increased number of Tbr2+ basal progenitors and upper layer neurons (Satb2+), and at latest stages analyzed (E16.5) they find a decrease in intermediate progenitors while the number of Satb2+ neurons remains much increased, seemingly at the expense of Ctip2+ deep layer neurons. This altered neuronal composition is also found to be maintained at P21. At the molecular level, DHFR mutant mice show decreased levels of H3K4me3 levels at E12.5, while treatment with MTX, which leads to DHFR inhibition, on mouse cortical cells cultures affected H3K4me3 levels at the level of some cortical neuron fate marker genes. MTX treatment in utero also led to changes in some neuronal fate marker expression. Finally, treatment of human cerebral organoids with MTX led to increased proportion of intermediate progenitor-like cells and neurons expressing TBR1, CTIP2 and SATB2. The authors conclude that the balance of direct vs indirect neurogenesis is impaired following DHFR inhibition.

This is a potentially interesting study on a timely topic, combing mouse in vivo and ex vivo and human in vitro analyses on the impact of an important metabolic pathway on cortical neurogenesis.
However, the data presented to do not support strongly the conclusion of an imbalance of direct vs. indirect neurogenesis. Indeed the only parameter that goes in line with this conclusion is the change in proportion of Tbr2 cells, but it depends on the stage considered (first unchanged, then increased, then decreased). Phenotypes could be equally linked, for instance, to changes in proliferation, differentiation, or temporal patterning, which have not been examined sufficiently. The molecular analyses are presented in a somewhat superficial way, making it difficult to judge them in full. There are also some important informations missing on the methods of quantification of mouse embryos and organoids. Major points are summarized below.

Reviewer 2 Comments for the Author:

1. The method of quantification of fate markers on DHFR control vs het mice should be explained in much more detail, as this is a delicate process prone to sampling artifacts. Which level of the brain was chosen and how? How many cells / levels / animals were used? This is very important given the well-known differences in cell fate proportions depending on the area and stage considered. Moreover the numbers should be provided in detail in every legend not just in the suppl table where it is difficult to find the relevant information.

We included images to show ROI in the various samples. We provided the n numbers in the graphs.

2. Similarly, how the organoids were quantified should be explained in much more detail. Firstly, the authors should provide more evidence that they are indeed analyzing ‘cortical-like’ tissue, for instance by focusing on FoxG1 or Emx1-positive parts of the organoids. This is crucial given the known variability in the regional identity of cells generated with these systems. Without appropriate validation of the regional identity of the ROI examined the analysis focusing on Tbr2 and CTIP2/Satb2 could be very misleading.

We stained organoid cryosections with Emx1 to show that the structures we consider « cortical-like » are indeed similar to dorsal telecephalon. This data is provided in Fig. S5C.

Numbers of experiments, organoids, regions of interest and cells should be provided in much more detail to give a better idea of variability in their experiments - including in the legends.

We modified the Material and methods section to provide more details on quantifications and included the n numbers in the graphs.

3. The data shown are not sufficient to support the conclusion that the balance between direct vs indirect neurogenesis is altered in the mutant mice. The authors should perform additional analyses to understand better the origin of the fate changes and explore equally interesting and important alternatives. BrdU short-term (1hr-24hrs) labeling should be performed to determine the levels of proliferation and cell cycle exit, which could equally explain differences in the proportion of neurons of different fate/layer identity. BrdU pulse-chase labelings should also be determined to determine the timing of birthdate of the neurons, as alterations of temporal patterning could be another important mechanism that is altered here.

This reviewer has two distinct comments : 1) provide additional support to the conclusion that direct vs indirect neurogenesis is modified in DHFR mutant, and 2) explore additional explanations for the phenotypes.

The complete set of experiments proposed by this reviewer is extremely interesting, however, we would like to point out that they represent a significant amount of work (and animals). We thus kindly asked the editor whether it would be sufficient to focus on the first point, which she agreed on (email 06/04/2023).

To do this, we performed birthdating EdU pulse chase experiments by injecting EdU at E12.5 and analyzed embryonic brains at E14.5. We co-stained sections with Tbr2, Ctip2 and Satb2. As expected, we observed a decrease in the number of neurons produced (Ctip2+/EdU+ cells) and an increase in the number of intermediate progenitors (EdU+/Tbr2+) in DHFR heterozygotes. This data indicated that cycling cells at E12.5 produced more Tbr2+ progenitors at the expense of Ctip2+ neurons. Further, quantification of EdU+/Satb2+ cells suggested a potential alteration in
specification of these early born neurons. This data is presented in Fig. 3H, I.

In addition to these experimental data, we modified the manuscript to tone down our interpretation of the phenotypes.

4. The differences observed between human vs mouse models are intriguing (CTIP2 vs Satb2 neurons) but difficult to interpret given that the stages examined are not all the same, and whether they are linked to intermediate progenitors remains quite unclear from the data shown. Unless more analysis as in point 3 are performed in the human system, this should be discussed in a much more conservative fashion.

We modified the text accordingly and tone down this point in the discussion.

5. In the MTX treatment in vivo: what happens to Tbr2 and Satb2? These should be shown and quantified to be compared with the genetic model and the organoid data.

The MTX experiment was originally designed to support the ChIP-Seq data on Bcl11b (Ctip2). However, we repeated these experiments and quantified Satb2+ cells. We observed a modest increase in Satb2+ neurons in the intermediate zone (shown in Fig. S4B, C).

6. In the ChIPseq data, how do the authors explain that some genes display higher levels and others lower levels of methylation? How does this fit with the data that global levels of Histone methylation are decreased? In any case it would be much more convincing to show all the ChIPseq data in order to interpret them optimally, instead of focusing on 4 genes only.

Several published studies, including our own, have shown that decreased SAM levels affects different histones differently (i.e. H3K4me3 is more sensitive to SAM levels than other H3 methylation marks) and alters gene expression in a gene-specific manner (i.e. Mentch et al. Cell Metabolism 2015, Fawal et al. Cell Reports 2018). To the best of our knowledge, the reasons for these specificities is not known. While a decreased H3K4me3 level is expected following inhibition of 1C metabolism, we can not exclude that increased levels of H3K4me3 at some genes are secondary consequences of changes in gene expression.

Nevertheless, to address this reviewer’s concern that the ChIP-Seq data, we provide additional more global analyses of the ChIP-Seq data (Fig. 4E and Fig. S4A).

7. The authors show changes of number of Ctip2+ and Satb2+ and conclude that deep and upper layer composition is changed in mutant mice. It would be important to test other layer markers (e.g. Foxp2, Cux1, Brn2,…) to support further the interpretation of a genuine fate change.

We provide quantifications of the upper layer marker Cux1 (Fig 3G, H).
appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the final referee comment is satisfactorily addressed. Please attend to all of the reviewers’ comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referees’ comments, and we will look over this and provide further guidance.

Reviewer 2

Advance summary and potential significance to field

The authors have adequately revised the manuscript. There is still one pending issue though to make sure the organoid analysis is state of the art.

Comments for the author

Regarding the organoid identity the authors provide convincing pics for Emx1 staining, but how representative is this of the organoids that they analyzed for other fate markers? The authors should provide some quantification of the proportion of Emx1-positive VZ-like structures in their organoid batches, and if this proportion is not high enough, the analysis of the fate markers should be focused on Emx1-positive structures. This additional analysis/information is mandatory to make sure that the reported changes reflect genuine changes in cortical neurogenesis, and not other more global changes in regional identity.

Second revision

Author response to reviewers’ comments

Reviewer 2’s comments

Regarding the organoid identity the authors provide convincing pics for Emx1 staining, but how representative is this of the organoids that they analyzed for other fate markers? The authors should provide some quantification of the proportion of Emx1-positive VZ-like structures in their organoid batches, and if this proportion is not high enough, the analysis of the fate markers should be focused on Emx1-positive structures. This additional analysis/information is mandatory to make sure that the reported changes reflect genuine changes in cortical neurogenesis, and not other more global changes in regional identity.

We understand this reviewer’s concern about HNO analyses and we adhere to their objective to promote state of art analyses. With this objective in mind, the engineer in the team who is in charge of HNO cultures and analyses undertook in 2018 the Hands-On training provided by STEMCELL Technologies (one of the leading company in this field). Since then, we have been using STEMCELL’s optimized organoids media kits and protocols to generate cerebral organoids (https://cdn.stemcell.com/media/files/brochure/BR27069-STEMdiff_Cerebral_Organoid_Kit.pdf). These reagents and protocols are based on the original formulation published by Madeline Lancaster et al. (Lancaster MA et al. Nature, 2013 and Lancaster MA et al. Science, 2014) and they are still used by her team to generate cerebral organoids (Pellegrini L, et al. Science, 2020). We would like to stress that in the manuscript, we use the term ‘human neural organoids’ instead of ‘human cerebral organoids’ in order to follow recent nomenclature guidelines (Pasca et al. Nature 2022).
To select cortical-like structures, we used morphometric parameters (structures located at the periphery of the organoids, presence of a lumen, elongated shape of pseudostratified nuclei, densely packed ventricular zone at least 5 nuclei thick, all criteria which are now listed in the Materials and Methods section) as well as markers listed in the STEMCELL brochure and commonly used in the field, namely PAX6, TBR2, TBR1, CTIP2 and SATB2. We would like to emphasize that the combination of these markers, and their spatial organization in superposed layers, ensures that the structures studied are indeed cortical-like, as shown by leading groups in the field (Velasco S et al. Nature 2019; Rosebrock D et al. Nature Cell Biology 2022; Quadrato G et al. Nature 2017). In response to this reviewer’s initial comments we performed immunostaining with EMX1 as an independent validation, to show that the morphometric parameters we used to select cortical-like structures were indeed sufficient to select EMX1+ structures within our control and MTX-treated HNO (100% of the structures selected on morphometric criteria were EMX1+ ; n=10). Now, we provide a low magnification picture of an HNO to show several representative examples of structures that were selected for analyses (matching EMX1 staining) and others that were excluded (EMX1-) (Fig S5C).

Altogether, this information confirms that our analyses were focused on genuine EMX1+ cortical-like structures. We agree that a systematic quantification of the proportion of EMX1+ structures in control and MTX conditions would assess potential changes of regional identities in response to MTX treatment, an interesting question, but outside the scope of this manuscript.

Third decision letter
MS ID#: DEVELOP/2023/201696
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AUTHORS: Sulov Saha, Thomas TJ Jungas, David Ohayon, Christophe Audouard, Tao Ye, Mohamad-Ali Fawal, and Alice Davy
ARTICLE TYPE: Research Article

Thank you for revising your manuscript to address the remaining reviewer question. I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks. Thank you for sharing your interesting work with us.