

Transcriptional dynamics of the oligodendrocyte lineage and its regulation by the brain erythropoietin system

Corresponding Author: Professor Hannelore Ehrenreich

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 the manuscript entitled 'Transcriptional dynamics of the oligodendrocyte lineage and its regulation by the brain erythropoietin system', Ye et. al underline how OL lineage dynamics are affected by recombinant human erythropoietin (EPO), in the murine hippocampus. The authors show that acute rhEPO administration increases OPC numbers, while chronic rhEPO administration drives maturation of the entire OL lineage. Single nuclei RNA sequencing data reveal that chronic rhEPO administration results in downregulation of GABA-A signaling, suggesting that increased OPC migration (which was previously associated with decrease in GABA-A signaling) is the underlying mechanism for the increased cell numbers. Furthermore, paradigms that were previously shown to increase endogenous EPO levels show similar results of cell number increase along early OLC stages (until the NFOL stage). Finally, EPOR deletion from late stages of the OLC (NFOL stage onwards) was shown to result in subtle axon diameter changes and deficits to working memory in an extensive battery of behavioral tests.

Overall, the manuscript is well-written and informative. Specifically, the definition of oligodendrocyte lineage dynamics in figure 1a is thorough and will undoubtedly help many researchers in the field. The authors have done elegant and comprehensive work showing the 'flow' of increase in OLC cell numbers along rhEPO administration (figures 1+3).

However, I find the manuscript to have some flaws that should be addressed:

Major comments:

1. Figure 1 – is it possible to measure OPC migration in-vivo by additional methods? For example, stereotaxic injection of Brdu followed by IP injection of rhEPO and histological stainings after 24 hours?
2. While in-vivo results show unchanged proliferation OPC properties, the in-vitro results contradict this notion. More clarity is required in explaining these results. Specifically, does rhEPO increase OPC proliferation? Under what conditions? This should be better addressed in the text (possibly also in the discussion).
3. Figure 1 – Is it possible to measure transcript levels of the GABA-A and glutamate pathways in-vitro, from OPCs following rhEPO manipulation? Ideally, this can also be done in addition to pharmacology enhancing/blocking these pathways, to assess migration properties. This would help elucidate the exact mechanism, and the involvement of GABA-A/glutamate pathways in OPC proliferation/migration following rhEPO treatment.
4. The contrast between figures 1 and 2 is confusing, as in figure 1 the increased OPC numbers is observed 24 hours following the first IP injection of rhEPO (acute reaction of OPCs to rhEPO). However, in figure 2, the data are procured from mice that underwent chronic administration of rhEPO. I would be careful with associating these two datasets with one another. This is another reason why I feel an in-vitro experiment to assess the involvement of these pathways is required.
5. Overall, the mechanistic proof of increased migration of OPCs following rhEPO injection (derived mainly from figures 1 and 2) is thin and must be strengthened.
6. Figure 4 – To confirm that the mechanism underlying the result of the inspiratory hypoxia experiment is in fact the EPOR pathway, it would be ideal if the authors will repeat the CRW and inspiratory hypoxia (possibly only utilizing the CRW+hypoxia group and control group) experiment in mice lacking EPOR via *Cnp-Cre* deletion (hence, only assess number of NFOL/MOLs).
7. Taking into consideration data from figures 4h-k & figure 5h together – it seems the effects of EPOR deletion from MOLs on MOL numbers is only relevant when rhEPO is administrated. Combined with data from figure 5 there seems to be little-to-no myelin-related effect of EPOR deletion from MOLs, under normal physiological circumstances (i.e. – no rhEPO administration). Hence, the MOL cell-specific effect of EPO on myelin seems non-existent or negligible. However, the *Cnp-Cre* line is active at the NFOL stage onwards. Hence, there is an effect of EPO and EPOR on the OLC, that could be mainly attributed to earlier stages in OLC. I believe this should be highlighted in the text, possibly as one of the

takeaway messages of the manuscript.

8. The observed shift in axonal diameter (i.e. – same number of myelinated axons, myelinated axons with increased axonal diameters) is intriguing. This could mean that the lack of EPOR in MOLs somehow affects the interaction between axons and MOLs. However, the effect is unexplained. It would be ideal if the authors could also present the effect on myelin thickness, which I assume would also increase, in accordance with the increased axonal diameter. Should this be the case, it would seem again that EPOR deletion from MOL doesn't directly affect myelin, rather the interaction between MOL and axons.

9. Figure 6 – the results of the anxiety tests are rather confusing. More time spent in the open arms of the EZM is interpreted as a decrease in anxiety in KO mice. However, longer latency of entrance to the light on the light-dark box experiment is interpreted as increase in anxiety. Combined with the fear-conditioning results that suggest an increase in anxiety in KO mice, the results of the EZM test are rather confusing.

10. I'm uncertain what the main takeaway message of the paper is, and in what aspect exactly does the paper expand the knowledge on the EPO brain system and oligodendrocyte lineage. This doesn't diminish the importance of the findings presented in the manuscript, but rather underlines the importance of highlighting the main takeaway messages of the manuscript along the text.

11. In a previous study (<https://www.sciencedirect.com/science/article/pii/S1074742718300443>) the authors show that females, but not males, with EPOR KO from MOLs (same mice in the current manuscript) show cognitive deficits. This raises 2 questions – how does the current study expand the knowledge other than the resolution of behavioral tracking? And is the effect gender specific?

12. In general, it is unclear why in some aspects male mice were studied, and in some other aspects male mice were studied. This should be either well explained, or experimentally demonstrated.

Minor comments:

1. Abstract – line 58: 'life' instead of 'live'.

2. Abstract and Introduction – In the abstract it says that OL stands for “oligodendrocyte lineage”, while in the introduction it says that OLC stands for “oligodendrocyte lineage”. Later in the introduction it says that OL stands for oligodendrocytes. This is confusing, and a single abbreviation should be used along the whole text.

3. Introduction – I found the interplay between the main concepts introduced at the beginning quite challenging to grasp, as their integration wasn't clearly outlined. A more structured explanation providing an overview of the key processes and their interactions would be extremely helpful. This approach would allow for a better understanding before diving into more details.

4. Introduction – lines 86-89: The context of EPO physiological importance in neurons should be mentioned, to establish why studying EPO's role in oligodendrocytes lineage is important.

5. Introduction – lines 120-125: Please mention the main findings of the papers described, to help the reader better understand the current knowledge in the field and the gap in knowledge that the current study answers.

6. Introduction – Please include a thorough review of how rhEPO may affect the transcriptome, in the general cell population, and specifically in the OL.

7. The introduction lacks a segment in which the authors describe the main findings of the paper.

8. Results – line 164, 169, and other places in the text “oligodendrocyte” should be OL.

9. Results – Row 194, 12.043 should be 12,043

10. Figure 2b – is it NFOL instead of MFOL on the Y axis?

11. Figures 5i, 5k – please add representative images of EM from both experimental groups.

12. Figure 5N – There seems to be an outlier CnpCre::EPOR(f/f) group?

13. Figure 5o, 5p – Please include number of streamlines and FA scores.

14. Discussion – line 364-366 should be rephrased.

15. Discussion – please use caution when associating the cognitive deficits with the right-shift in axonal diameters in the fimbria, as this requires further experimental validation to be directly connected.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

In this paper, the authors evaluated the role of erythropoietin in oligodendrocyte differentiation and maturation in the adult mouse hippocampus by intraperitoneal application of recombinant human EPO in adult animals, as well as with stimulation of EPO brain expression with exercise (“functional hypoxia”) and with hypoxia (12% oxygen). In addition with single-nuclei-RNA-seq data, the paper provides differential gene expression analysis in oligodendrocytes revealing several EPO-regulated transcripts, including downregulation of GABA-A signaling, known to be associated with migration and OL progression.

By using a transgenic mouse lacking EPORs in mature oligodendrocytes (CNP:Cre;EPOR^{fx/fx}) the authors were able to determine a reduction in the number of mature myelinating oligodendrocytes, myelination impairment and alterations in working memory and executive performance.

This work adds to the already described role of EPO signaling in oligodendrocyte differentiation and myelination (Jatznie, 2013; Cervellini, 2013; Hassouna, 2016; Cho, 2012; Gyetvai, 2017; Muttathukunnel, 2023; Dewan, 2020), the relevance of oligodendroglial EPORs in maintaining myelination, adult neuroplasticity and cognition. The methodology meets the expected standards in the field, and the methods provide detail enough the work to be reproduced.

(Remarks on code availability)

The code does provide a READ me file with several package versions written in R: DEG_calculation, E_analysis, genie3_oligos, monocle3, regulon_annotation, trial_genie3

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)

This paper aims at further exploring the effects of EPO on the oligodendrocyte lineage. There are potentially some interesting observations contained within – albeit for the most part very subtle – but unfortunately the paper is written and put together in a manner which makes it very difficult to understand what has been done and why. Throughout, the results section is written in an overly colloquial fashion and the reference to the figures somewhat erratic. There also need to be a much clearer and logical rationale for why each part of the study have been undertaken. I fear the paper requires a major overhaul and re-write using a conventional scientific style. Below are just some examples illustrative of a more general problem.

The first section of the results is overlong and not really pertinent to the papers main (and new) findings. I suggest removing it.

What was the rationale for focusing on the hippocampus?

180-181 -it is not clear from the figures what enhancement (whatever that means) and 'remarkable decrease' is occurring at 72 hours (not a time point that appears in the figure).

If I have understood correctly, there are no changes in OPC proliferation or survival, but their numbers do changes (although the evidence for this is not obvious). Is this why migration becomes an issue? Of course, there are indeed no markers that prove migration but it's not clear how sequence data helps. Frankly, it's not clear what phenomenon is being investigated in the subsequent analysis of sequencing data.

Figure 1e – what are OPC changes? What does this mean exactly. Is this data from another study?

Personally, I would prefer that the OPC was defined as a progenitor rather than a precursor.

179 – these are not sketches – they are data figures

Abstract – perhaps be more specific about what EPO does rather than simply say 'stimulates all OL cells', which doesn't really convey what the specific effects are.

Abstract. Remove 'interestingly' – surely this is a decision for the reader to make. (And remove throughout).

Abstract. Explain what right shift in size distribution of myelinated axons actually means – I suspect this won't be obvious to the general reader.

Last line of the first paragraph of the introduction is something of a non sequitur – none of what comes before makes it clear why EPO is being studied.

Line 138 – Either there is clear evidence that EPO system is involved in adaptive myelination or there is not. For my reading the case has not been made and there are no hints that this might be so.

Line 94 – '.....that are haematopoiesis independent' perhaps?

Line 99 – what exactly is meant by 'overpopulating'?

Line 111 – perhaps find a less colloquial, more scientific term than 'brain hardware upgrade'

Line 124 – remove 'Altogether'

Explain cornu ammonis – or use the more widely understood term hippocampus

Line 145 – do the authors really mean 'immunological markers' – aren't they just markers, which ne can detect by immunocyto/histochemistry?

(Remarks on code availability)

n/a

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The revised manuscript addresses several key concerns, incorporating clarifications and adjustments to improve the presentation of data and conclusions. However, several major issues remain that require further attention to ensure the findings are well-supported.

My primary concern is that while the authors assert that "EPO stimulates all oligodendroglial cells and prompts their

differentiation and maturation" and that "our data support a working model in which brain EPO, induced by functional hypoxia through continual neuronal activity, drives adaptive myelination," the experiments conducted in this study do not seem sufficient to fully substantiate these claims. While the conclusions may ultimately be correct, additional data are needed to strengthen the evidence.

I acknowledge the limitations the authors are facing regarding the German regulation. However, without further experimental data to support the findings, it remains difficult to conclusively determine the in vivo effects of rhEPO on OPC migration (as also stated by reviewer 4). Relying on previous snRNAseq data does not necessarily help in explaining the migration-related phenotype, as this is in minimum correlative source of data that can help in choosing single targets to manipulate experimentally in order to mechanistically prove the underlying mechanism. At its current stage, the study presents only part of the story, limiting the strength of the conclusions. Also, some of the text should be moved to the Discussion section for better contextualization.

It is unclear which figure presents the results of in vitro EPO-stimulated OPCs. There appears to be a gap in the data that should be addressed. I understand the authors are currently working on this but have opted not to include these data in the manuscript. However, I believe this information is critical to support the study's conclusions. The observed reduction in GABA-A receptor mRNA is intriguing and may support altered migration properties following rhEPO stimulation, but on its own, it is not sufficient.

I appreciate the incorporation of snRNA-seq data, but as mentioned earlier, additional molecular and cellular validation is required to substantiate the findings, especially as this manuscript is being reviewed for a journal such as Nature Communications. Reviewer 4 also emphasizes that it is unclear how the snRNAseq data can mechanistically explain the discussed phenotypes. The text modifications made in response to previous comments are noted. However, additional experiments are needed to explore potential mechanisms underlying the observed effects.

While I understand the stated limitations, I strongly emphasize the necessity of the proposed experiment to validate the role of the EPOR pathway in mOLs.

There may have been a misunderstanding regarding my previous point (comment #8) — I meant that an increase in axonal diameter could suggest an increase in myelin thickness. While g-ratio values reflect the relationship between axonal diameter and myelin thickness, it would be beneficial to include absolute measurements of myelin thickness for additional clarity.

As previously mentioned, additional data are required to support the study's conclusions with greater confidence. Addressing these concerns through further data and clarifications would significantly enhance the strength and reliability of the manuscript.

Technical issues (as also mentioned by reviewer 4 about the manuscript design and readability): Please ensure the accuracy of references to figures throughout the main text. For example, in Figure 1 presented in the file, panels J,K,L are actually not presented in the figure, despite being cited in the main text. Similarly, references to Figures 2 and 3 seem to be incorrect in certain sections.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

The paper revises the impact of Erythropoietin (EPO) and its receptors (EPORs) on oligodendrocyte precursor cell (OPC) differentiation and myelination. It aligns with previous studies (Jatznie, 2013; Cervellini, 2013; Hassouna, 2016; Cho, 2012; Gyetvai, 2017; Muttathukunnel, 2023; Dewan, 2020). Although the paper does not introduce new concepts to the field, it reaffirms the critical role of EPO signaling in the oligodendrocyte lineage. Furthermore, it provides valuable insights into how EPO affects OPC differentiation during adulthood.

The methods and data analysis are presented clearly, with sufficient detail and a convincing interpretation of the results. The revision was thorough, and although some important experiments could not be included, the discussion was appropriately adjusted to reflect this.

(Remarks on code availability)

My questions have been satisfactorily addressed by the authors.

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)

This manuscript has certainly been improved from the earlier submitted version. I have a few comments that might improve it further.

Line 75 – Personally, I would remove ‘probably reflecting....’ – this is speculation and shouldn’t really appear in an abstract.

Line 77. Use ‘contributes to’ rather than ‘drives’. This would not only be more accurate but more in keeping with the appropriately circumspect conclusion drawn in line 145.

Line 80. Replace ‘on top of’ with ‘in addition to’

Line 91. Replace ‘highly likely’ with ‘a plausible hypothesis etc’

Lines 99 and 102. Delete commas

Line 187. Remove ‘remarkable’ – a judgement term not appropriate in a results section.

Line 183. Remove ‘First clues for potential involvement of migration’

Comments on responses

I am still of the view that mentioning that the cornu ammonis is part of the hippocampus would be helpful to many readers.

Citing a review that uses the term immunological markers does not make the term correct. I am sure the author’s see my point and I am sure they can come up with a more suitable term

I am very familiar with the current fashion to use the terms precursor and progenitor interchangeably. However, their meaning is subtly different – hence my suggestion. (No need for a response).

(Remarks on code availability)

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

While this is an area of ongoing interest, I believe the current version of the manuscript offers limited advancement in our understanding of these relationships. Much of the data on EPO and OPCs reiterates previously published findings, while the novel aspects related to mature oligodendrocytes and myelination, though potentially interesting, appear modest in scope.

That said, the authors have clearly invested effort in strengthening the manuscript, and I appreciate their responsiveness in addressing several of the prior concerns. My comments below are offered in the spirit of helping to clarify the novel contributions and to improve the overall clarity and impact of the manuscript. Below are my responses to the authors responses in the order they appear in the rebuttal letter:

1. I still find it somewhat challenging to clearly identify which conclusions stem from the current work and which are drawn from previous studies. This issue affects both the introduction and discussion sections. Greater distinction between background knowledge and new findings would significantly help the reader appreciate the manuscript’s contributions.

2. See comment #1 — clearer distinction between new and prior findings is needed.

3. I understand the limitations related to approval for in vivo experiments in Germany and appreciate the authors’ explanation. While the lack of in vivo OPC migration data remains a limitation, the additional in vitro data now provided, along with the revised discussion that acknowledges these constraints, is a reasonable compromise in my opinion.

4. Acknowledged.

5–6. The authors chose to highlight the involvement of GABA signaling, and while this may indeed be relevant, I felt that the mechanistic evidence provided is currently limited. My earlier comment was not intended as a critique of the inclusion of this pathway, but rather as a suggestion to either better support its role or to frame it more cautiously. As it stands, the data are quite general and not sufficient to support mechanistic conclusions about this pathway.

7. Acknowledged.

8. In my view, the key issue remains: the dataset, while of interest, supports only limited novel conclusions regarding the role of EPO/EPOR in oligodendrocyte lineage progression and myelination. The observed effects on mOLs are intriguing and worth pursuing further, but currently appear modest and somewhat preliminary.

9. Please see technical comments below.

Specific Comments:

Abstract:

Line 59: Consider rephrasing. Starting with “Here” and using the past tense (“thought”) is ambiguous — it’s unclear whether you’re referring to past work or current findings.

Line 60: “Using in the murine hippocampus” — please remove “in”.

Line 63: Clarify whether this is a novel finding. There is already substantial literature on EPO and OL lineage cells, including by the authors.

Line 73: The current data, while suggestive, may not yet fully support the closing claim regarding rhEPO as a clinically viable option. Consider softening the language. Also, please ensure font consistency here.

Introduction:

Line 100: It’s not clear whether “here” refers to this study or to previously cited findings. Please clarify, as this may confuse the reader.

Results:

Line 200–201: The differing effects of rhEPO in vitro and in vivo are well described. This distinction is important and should be emphasized in the discussion.

Line 261: Incorrect figure reference — it should be figure 4, not 3a–m. Please verify all figure references throughout the manuscript.

Line 271: Please indicate the specific figures (e.g., 5a–c for in vivo, and 5d for mOLs?).

Line 289: Please clarify what is meant by “absence of a response to rhEPO” — is this referring to decreased mOL numbers at week 4?

Ensure consistency in capitalization in graph and axis titles (e.g., Figure 6j).

Line 358: The sentence suggests a fully novel observation, but prior work has shown rhEPO-induced differentiation of OPCs. Consider framing this as an extension or refinement of previous findings, with emphasis on the mOL/myelination aspect as the primary novel contribution.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)

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(Remarks on code availability)

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February 08, 2025

POINT-BY-POINT RESPONSE to the REVIEWERS' COMMENTS

(Reviewers' comments in Calibri 11; **response in ARIAL 12 – blue**; reference to comment-relevant passages and changes in the manuscript labelled in yellow).

Reviewer #1:

In the manuscript entitled 'Transcriptional dynamics of the oligodendrocyte lineage and its regulation by the brain erythropoietin system', Ye et. al underline how OL lineage dynamics are affected by recombinant human erythropoietin (EPO), in the murine hippocampus. The authors show that acute rhEPO administration increases OPC numbers, while chronic rhEPO administration drives maturation of the entire OL lineage. Single nuclei RNA sequencing data reveal that chronic rhEPO administration results in downregulation of GABA-A signaling, suggesting that increased OPC migration (which was previously associated with decrease in GABA-A signaling) is the underlying mechanism for the increased cell numbers. Furthermore, paradigms that were previously shown to increase endogenous EPO levels show similar results of cell number increase along early OLC stages (until the NFOL stage). Finally, EPOR deletion from late stages of the OLC (NFOL stage onwards) was shown to result in subtle axon diameter changes and deficits to working memory in an extensive battery of behavioral tests.

We thank the reviewer for the time invested into reading our manuscript and for the insightful, stimulating and constructive feedback. With our response, we hope to fully correct and clarify any misunderstandings.

Overall, the manuscript is well-written and informative. Specifically, the definition of oligodendrocyte lineage dynamics in figure 1a is thorough and will undoubtedly help many researchers in the field. The authors have done elegant and comprehensive work showing the 'flow' of increase in OLC cell numbers along rhEPO administration (figures 1+3).

We thank the reviewer for the positive and inspiring evaluation of our work; we sincerely hope that this work will be of help to other researchers.

However, I find the manuscript to have some flaws that should be addressed:

Major comments:

1. Figure 1 – is it possible to measure OPC migration in-vivo by additional methods? For example, stereotaxic injection of Brdu followed by IP injection of rhEPO and histological stainings after 24 hours?

We do appreciate the idea to use BrdU for pulse labelling. Unfortunately, we do not have an animal ethics license in place that would cover this procedure. These days, writing such a proposal requests many weeks of full-time work of a qualified researcher. Even worse, approval by the regulatory bodies in Germany can easily take over 14-16 months (a politically intended impediment). We therefore have to postpone this interesting approach.

2. While in-vivo results show unchanged proliferation OPC properties, the in-vitro results contradict this notion. More clarity is required in explaining these results. Specifically, does rhEPO increase OPC proliferation? Under what conditions? This should be better addressed in the text (possibly also in the discussion).

We agree that this is an interesting *in vitro* / *in vivo* difference and have now revised and extended the respective paragraph on **page 6**:

“A sketch illustrates the course of numerical OPC changes, with early expansion, followed by a remarkable decrease at 72h and a steady-state thereafter (Figure 1c,d,e). In fact, at the 72h time point, OPC undergo prominent differentiation. But what about the early enhancement? Under pathological conditions such as traumatic brain injury (Shumilov et al 2023) or experimental autoimmune encephalomyelitis in mice (Zhang et al., 2005), or in a rat model of embolic focal cerebral ischemia (Cho et al., 2012), rhEPO has been found to increase OPC proliferation. During development (postnatal day 7), Muttathukunnel and colleagues (Muttathukunnel et al 2023) observed also enhanced OPC proliferation in the subventricular zone of Tg21 mice. However, using Ki67 as proliferation and cleaved-caspase 3 (CC3) as apoptosis markers, we can exclude cell division or death upon rhEPO under physiological conditions in adolescent or young adult mice as explanation of the early increase *in vivo* (Figure 1f-i). In contrast, stimulating OPC in cell culture, we find at 24h augmented BrdU incorporation upon rhEPO in a dose-dependent fashion, peaking at 1IU/ml [Figure 1j,k,l, with the bell-shaped curve showing a decrease after the peak as typical for many readouts after rhEPO application³⁸]. This somewhat contradictory proliferation response *in vitro* is best explained by the essentially pure OPC cultures, where regulatory interactions with other cell types that may restrict proliferation *in vivo* are excluded³⁹. There are several other possible explanations: (i) Differences in the proliferation markers used: In the *in vivo* experiments, we employed Ki67, a proliferative marker that labels cells in the G1, S, G2, and M phases, whereas in the *in vitro* experiments, we used BrdU, a proliferation marker, exclusively labelling S phase, hence providing perhaps a more precise snapshot. (ii) Delay in OPC proliferation *in vivo*: Although all experiments were conducted 24 h after rhEPO administration, *in vitro* experiments are straightforward, with cells being directly stimulated by rhEPO, whereas *in vivo*, circulation and subsequent cellular effects may take more time. As there are no established histological markers for migration of OPC, we explored this path next using our previously published single-nuclei-RNA-seq dataset¹⁵ as described in the following section.”

Shumilov, Kirill, et al. "Recombinant Erythropoietin Induces Oligodendrocyte Progenitor Cell Proliferation After Traumatic Brain Injury and Delayed Hypoxemia." *Neurotherapeutics* 20.6 (2023): 1859-1874.

Zhang, Jing, et al. "Erythropoietin treatment improves neurological functional recovery in EAE mice." *Brain research* 1034.1-2 (2005): 34-39.

Cho, Yun Kyung, et al. "Erythropoietin promotes oligodendrogenesis and myelin repair following lysolecithin-induced injury in spinal cord slice culture." *Biochemical and biophysical research communications* 417.2 (2012): 753-759.

Muttathukunnel, Paola, et al. "Erythropoietin regulates developmental myelination in the brain stimulating postnatal oligodendrocyte maturation." *Scientific Reports* 13.1 (2023): 19522.

3. Figure 1 – Is it possible to measure transcript levels of the GABA-A and glutamate pathways in-vitro, from OPCs following rhEPO manipulation? Ideally, this can also be done in addition to pharmacology enhancing/blocking these pathways, to assess migration properties. This would help elucidate the exact mechanism, and the involvement of GABA-A/glutamate pathways in OPC proliferation/migration following rhEPO treatment.

In our EPO-stimulated OPC, we observed a reduction of mRNA levels for 4 subunits of the GABA-A receptor: Gabrg3, Gabrb1, Gabrb2, Gabrb3). However, GABA-regulated (and glutamate regulated) OPC migration and differentiation is complex (PMID: 30957306; PMID: 33928492). While our data are compatible with GABA-regulated OPC inward migration and the numerical increase seen after 24h (Fig. 1d), we have intentionally refrained from exploring an isolated aspect of cell migration here. This is currently part of a separate study, which we do not want to spoil with premature conclusions from RT-PCR data or pharmacologically treated, cultured OPC. Moreover, valid mechanistic insight requires also *in vivo* data from EPO-treated mutant mice that remain to be generated first.

Habermacher C, Angulo MC, Benamer N. Glutamate versus GABA in neuron-oligodendroglia communication. *Glia*. 2019 Nov;67(11):2092-2106. doi: 10.1002/glia.23618. Epub 2019 Apr 7. PMID: 30957306.

Bai X, Kirchhoff F, Scheller A. Oligodendroglial GABAergic Signaling: More Than Inhibition! *Neurosci Bull*. 2021 Jul;37(7):1039-1050. doi: 10.1007/s12264-021-00693-w. Epub 2021 Apr 29. PMID: 33928492.

4. The contrast between figures 1 and 2 is confusing, as in figure 1 the increased OPC numbers is observed 24 hours following the first IP injection of rhEPO (acute reaction of OPCs to rhEPO). However, in figure 2, the data are procured from mice that underwent chronic administration of rhEPO. I would be careful with associating these two datasets with one another. This is another reason why I feel an in-vitro experiment to assess the involvement of these pathways is required.

There might be misunderstandings. We are not associating the two data sets with each other. We use our single-nuclei-RNA-seq dataset to search for transcriptional hints of OPC migration. These hints are not restricted to a particular time point as even pseudotime presentations are possible at each time point. Even though the sn-RNAseq dataset comes from ‘chronic’ rhEPO treatment, we still find undifferentiated OPC that respond to treatment comparable to the original OPC found at 24h.

5. Overall, the mechanistic proof of increased migration of OPCs following rhEPO injection (derived mainly from figures 1 and 2) is thin and must be strengthened.

We agree and have never talked about ‘mechanistic proof’ in the manuscript. Stimulated OPC migration was only discussed as a possibility, because it was

supported by certain gene regulatory findings. We make this point now clearer throughout the manuscript by dampening our respective wording.

6. Figure 4 – To confirm that the mechanism underlying the result of the inspiratory hypoxia experiment is in fact the EPOR pathway, it would be ideal if the authors will repeat the CRW and inspiratory hypoxia (possibly only utilizing the CRW+hypoxia group and control group) experiment in mice lacking EPOR via *Cnp-Cre* deletion (hence, only assess number of NFOL/MOLs).

We would gladly do this experiment, however, again, we would need a new animal ethics license since, unfortunately, we do not have an animal ethics license in place that would cover this experiment. Getting a permission would take 1-2 years before we can start the experiment (see #1 above).

7. Taking into consideration data from figures 4h-k & figure 5h together – it seems the effects of EPOR deletion from MOLs on MOL numbers is only relevant when rhEPO is administered. Combined with data from figure 5 there seems to be little-to-no myelin-related effect of EPOR deletion from MOLs, under normal physiological circumstances (i.e. – no rhEPO administration). Hence, the MOL cell-specific effect of EPO on myelin seems non-existent or negligible. However, the *Cnp-Cre* line is active at the NFOL stage onwards. Hence, there is an effect of EPO and EPOR on the OLC, that could be mainly attributed to earlier stages in OLC. I believe this should be highlighted in the text, possibly as one of the takeaway messages of the manuscript.

In Figure 5j we show that late OPC/COP/NFOL are not influenced by rhEPO upon EPOR deletion. However, we have now made changes, as requested, to introduction (page 3) and conclusions (page 12) that highlight our takeaway messages and the relevance of earlier stages in OLC in response to rhEPO treatment.

8. The observed shift in axonal diameter (i.e. – same number of myelinated axons, myelinated axons with increased axonal diameters) is intriguing. This could mean that the lack of EPOR in MOLs somehow affects the interaction between axons and MOLs. However, the effect is unexplained. It would be ideal if the authors could also present the effect on myelin thickness, which I assume would also increase, in accordance with the increased axonal diameter. Should this be the case, it would seem again that EPOR deletion from MOL doesn't directly affect myelin, rather the interaction between MOL and axons.

We agree and this is exactly why we determined the g-ratios, which were unaltered in fimbria and corpus callosum (Figure 6 i-l).

9. Figure 6 – the results of the anxiety tests are rather confusing. More time spent in the open arms of the EZM is interpreted as a decrease in anxiety in KO mice. However, longer latency of entrance to the light on the light-dark box experiment is interpreted as increase in anxiety. Combined with the fear-conditioning results that suggest an increase in anxiety in KO mice, the results of the EZM test are rather confusing.

We agree that the results of the anxiety tests are not all going in the same direction. This was unexpected but indicates a disturbed anxiety behavior. Since also in the open field test, the time spent in the center shows a tendency of an increase, we assume that our EPM results are valid. Please note that the latency to enter light in the LDB is based on another stimulus – namely light (LDB), versus wideness in

EPM/open field. We have added a sentence to this apparent contradiction on page 10.

10. I'm uncertain what the main takeaway message of the paper is, and in what aspect exactly does the paper expand the knowledge on the EPO brain system and oligodendrocyte lineage. This doesn't diminish the importance of the findings presented in the manuscript, but rather underlines the importance of highlighting the main takeaway messages of the manuscript along the text.

We have now summarized the main takeaway message in the discussion, page 11:

“Here, we uncover that all OLC respond to exogenously applied rhEPO with differentiation and maturation, as if the entire lineage, ready to differentiate towards MOL, remains 'in flow'. During lineage progression, increases in rhEPO-responsive cell stages occur in transitory waves, with individual markers and marker combinations rising at discrete time points. This pattern of pushed differentiation and maturation without DNA synthesis has been reported to occur very similarly in excitatory neurons and their precursors^{14, 15}. Importantly, stimulation of endogenous EPO by functional or inspiratory hypoxia or their combination²¹ leads to increases in OPC/COP/NFOL, analogous to rhEPO treatment. This finding suggests a physiological role of the EPO system in OLC that also contributes to adaptive myelination⁵⁷ which occurs in response to neuronal activity.”

11. In a previous study (<https://www.sciencedirect.com/science/article/pii/S1074742718300443>) the authors show that females, but not males, with EPOR KO from MOLS (same mice in the current manuscript) show cognitive deficits. This raises 2 questions – how does the current study expand the knowledge other than the resolution of behavioral tracking? And is the effect gender specific?

In the previous study, this finding was more of a ‘side observation’, which actually stimulated us to perform and extend the present work to see whether the cognitive deficits are an isolated finding. Regarding the results, there seems to be some misinterpretation of the previous paper, since both genders did show cognitive deficits. Only impaired place learning performance was present in male but not in female EPOR KO. Remarkably, the opposite was found for the reversal learning session (Dere et al 2018; Page 143, paragraph 3.2.2.1.), clearly indicating some gender specific effects.

12. In general, it is unclear why in some aspects male mice were studied, and in some other aspects female mice were studied. This should be either well explained, or experimentally demonstrated.

In order to reduce the number of unused ‘left-over’ mice and following many journals’ general request to investigate both genders, we included males and females and made thus full use of all mice born. Moreover, females have additional advantages, e.g. they do not require continued single housing after one test in which they had to be single housed (in contrast to males).

Minor comments:

1. Abstract – line 58: 'life' instead of 'live'.

Thank you for the correction!

2. Abstract and Introduction – In the abstract it says that OL stands for “oligodendrocyte lineage”, while in the introduction it says that OLC stands for “oligodendrocyte lineage”. Later in the introduction it says that OL stands for oligodendrocytes. This is confusing, and a single abbreviation should be used along the whole text.

We are sorry. We have now made it clearer that OLC stands for oligodendrocyte lineage cells (all cells) and OL for oligodendrocytes, page 3, first paragraph.

3. Introduction – I found the interplay between the main concepts introduced at the beginning quite challenging to grasp, as their integration wasn't clearly outlined. A more structured explanation providing an overview of the key processes and their interactions would be extremely helpful. This approach would allow for a better understanding before diving into more details.

Thanks for pointing this out. We have now added better explanatory sentences, page 3, first paragraph.

4. Introduction – lines 86-89: The context of EPO physiological importance in neurons should be mentioned, to establish why studying EPO's role in oligodendrocytes lineage is important.

Agreed. This has been done in connection with the point 3 above, page 3, first paragraph.

5. Introduction – lines 120-125: Please mention the main findings of the papers described, to help the reader better understand the current knowledge in the field and the gap in knowledge that the current study answers.

This has been added as requested, page 4, last paragraph.

6. Introduction – Please include a thorough review of how rhEPO may affect the transcriptome, in the general cell population, and specifically in the OL.

This has been added as requested on page 3, third paragraph.

7. The introduction lacks a segment in which the authors describe the main findings of the paper.

This has been done as requested, page 4, last paragraph (see also point 5 above)

8. Results – line 164, 169, and other places in the text “oligodendrocyte” should be OL.

Thank you! This has now been corrected throughout.

9. Results – Row 194, 12.043 should be 12,043

Thank you!

10. Figure 2b – is it NFOL instead of MFOL on the Y axis?

This is correct.

11. Figures 5i, 5k – please add representative images of EM from both experimental groups.

The EM images in Figure 5 (now Figure 6) would not show any differences between experimental groups, and were included only to demonstrate the quality of the EM data used for quantitation (@editor: please advise).

12. Figure 5N – There seems to be an outlier CnpCre::EPOR(fl/fl) group?

There was no outlier in Figure 5n. The error bars are SEM.

13. Figure 5o,5p – Please include number of streamlines and FA scores.

There seems to be a misunderstanding. The morphometric analyses were performed on anatomical images (magnetization transfer weighted). No diffusion-weighted images were acquired, so no fractional anisotropy (FA) map was calculated, nor was any tractography method applied.

As detailed in the methods section, the anatomical study template was generated using “twolevel_ants_dbm” script (<https://github.com/CoBrALab/>). This script uses the antsMultivariateTemplateConstruction2.sh function from ANTsX/ANTs (<https://github.com/ANTsX/ANTs>). It performs deformation-based morphometry and computes the Jacobian determinants. Figure 5o shows the result of the student’s t-test performed on the 3D Gaussian-smoothed maps of the Jacobian determinants (using the 3dttest++ function of AFNI). To visualize volume differences between mutants and controls, z-scores smaller than -2.57 (bluish) and larger than 2.57 (reddish) were overlaid on the study template.

14. Discussion – line 364-366 should be rephrased.

This has been done.

15. Discussion – please use caution when associating the cognitive deficits with the right-shift in axonal diameters in the fimbria, as this requires further experimental validation to be directly connected.

Agreed. This has now been more carefully formulated.

Reviewer #2:

In this paper, the authors evaluated the role of erythropoietin in oligodendrocyte differentiation and maturation in the adult mouse hippocampus by intraperitoneal application of recombinant human EPO in adult animals, as well as with stimulation of EPO brain expression with exercise (“functional hypoxia”) and with hypoxia (12% oxygen). In addition with single-nuclei-RNA-seq data, the paper provides differential gene expression analysis in oligodendrocytes revealing several EPO-regulated transcripts, including downregulation of GABA-A signaling, known to be associated with migration and OL progression.

By using a transgenic mouse lacking EPORs in mature oligodendrocytes (CNP:Cre;EPOR^{fx/fx}) the authors were able to determine a reduction in the number of mature myelinating oligodendrocytes, myelination impairment and alterations in working memory and executive performance. This work adds to the already described role of EPO signaling in oligodendrocyte differentiation and myelination (Jatznie, 2013; Cervellini, 2013; Hassouna, 2016; Cho, 2012; Gyetvai, 2017; Muttathukunnel, 2023; Dewan, 2020), the relevance of oligodendroglial EPORs in maintaining myelination, adult neuroplasticity and cognition. The methodology meets the expected standards in the field, and the methods provide detail enough the work to be reproduced.

We thank the reviewer for the careful reading of our manuscript and for providing insightful and constructive comments.

Reviewer #2 (Remarks on code availability):

The code does provide a README file with several package versions written in R: DEG_calculation, E_analysis, genie3_oligos, monocle3, regulon_annotation, trial_genie3

Thank you.

62-63: Here, we explored novel aspects of OL dynamics, namely effects of recombinant human erythropoietin (rhEPO) and its receptor (EPOR) on OL in murine hippocampus. Here, I am missing a link to why EPO is being studied in association with sustained neuronal activity in this work.

The link has now been provided.

-Where is EPO produced after functional hypoxia? Neuronal? Paracrine signaling?

Upon functional hypoxia, EPO is mainly produced by neurons which show the strongest hypoxia response, as assessed by snRNA-seq analysis.

(See Singh et al. Erythropoietin re-wires cognition-associated transcriptional networks. Nat Commun 14, 4777, 2023)

-Why did you remove EPORs from adult OLs? Justification missing.

We have added to page 4: In order to elucidate the effect of EPO on adult OL, we generated conditional EPOR KO mice.

-How comparable are the OPCs extracted from P7 mice brain in culture to adult oligodendrocytes in the hippocampus? EPORs expression varies according to age.

OPC extracted from P7 mouse brains for culture are not directly comparable to adult OL in the hippocampus. They are, however, comparable to OPC in the hippocampus. See Figures 1d and 2c.

-Other reports show an increase in GABA currents in the hippocampus and cortex with EPO (Khalid, 2021; Roseti, 2020). How do you discuss this point? Is a reduction in GABARs beneficial?

It is difficult to comment on the findings in slices by others. In our cultures, we find a downregulation of GABA-A signaling upon EPO, known to be associated with OPC migration and progression in oligodendrocyte differentiation.

-Figure 4 needs to add a supplementary figure in which comparisons between placebo and EPO can be visualized at specific time windows.

In Fig4a it is impossible to see the different oligodendrocyte types. Maybe arrows could help.

The requested comparison between placebo and EPO is shown in Figure 4e. In general, quantifications at specific time windows (e.g. Figure 4f) provide more solid information. We have added the arrows as requested.

In Figure 4e, NG2 staining is missing to confirm mature BCAS1+NG2-OLIG2+ cells specificity.

NG2 staining has now been added as suggested.

Figure 3g is not mentioned in the text, I guess should be written Figure 3g-j.

Done. Thank you.

-Figure 4 a-d could also be a supplementary figure.

We believe these data fit very well in Figure 5. An additional small supplementary figure would likely be confusing and may not be necessary. We have already 5 supplementary tables and a supplementary methods part.

-How is explained that chronic hypoxia causes hypomyelination and reduced motor coordination in adults (Chen, 2021) and neonates (e.g. Wang, 2018), with your data on positive impact of chronic hypoxia on oligodendrocyte numbers? Do you also expect myelination to be improved?

We can only guess, but our inspiratory hypoxia experiments were performed earlier and more mildly than those of Chen et al (2021). Our mice were treated at P28, i.e. in a juvenile time window of development, and gradually exposed to hypoxia (removing -3% O₂ per day) to a final concentration of 12% O₂, which was then left constant for 3 weeks.

Chen et al, in contrast, placed 3 months old (i.e. adult) mice abruptly into only 10% O₂ (for 12h) and repeated this abrupt exposure every day for an entire month. This is more likely to trigger a series of hypoxic stress moments and to diminish oligodendrogenesis, which is already low in 4 months old mouse brains. It is

possible that metabolic/energetic stress outweighs the effect of hypoxia-induced EPO signaling, but we refrained from commenting on this work in our paper.

Do you expect any difference between chronic hypoxia and functional hypoxia on myelination?

Again, we cannot say anything regarding the enhancement of myelination since this has not been investigated yet (only oligodendrocyte differentiation). However, chronic hypoxia and functional hypoxia would be expected to have similar effects – perhaps with a gradual difference. See for instance quantification of hypoxic cells in Butt et al. 2021, Figure 5.

Butt UJ, Steixner-Kumar AA, Depp C, Sun T, Hassouna I, Wüstefeld L, Arinrad S, Zillmann MR, Schopf N, Fernandez Garcia-Agudo L, Mohrmann L, Bode U, Ronnenberg A, Hindermann M, Goebbels S, Bonn S, Katschinski DM, Miskowiak KW, Nave KA, Ehrenreich H. Hippocampal neurons respond to brain activity with functional hypoxia. *Mol Psychiatry*. 2021 Jun;26(6):1790-1807. doi: 10.1038/s41380-020-00988-w. Epub 2021 Feb 9. PMID: 33564132; PMCID: PMC8440186.

-What does it mean thicker axons but reduced volumes in the striatum when EPORs are missing, please discuss.

Sorry for any misunderstanding. We did not mention thicker axons.

The word interestingly is too often repeated in the manuscript.

Agreed. The word ‘interestingly’ has now been eliminated throughout the manuscript.

-“Given that rhEPO is a clinically approved and safe drug, we assume that this knowledge will help developing novel therapeutic strategies combatting cognitive disability and decline in the increasing number of neuropsychiatric conditions that involve oligodendrocyte dysfunction”.

This is the last sentence of our conclusions, page 12. We hope you can agree.

If EPO is secreted by functional hypoxia (exercise), then a conclusion would also be to support exercise to prevent and treat neuropsychiatric conditions that also involve oligodendrocytes.

We agree that exercise should be considered in this context and have added a sentence in the conclusion section on page 12.

Reviewer #3:

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts

Thank you.

Reviewer #4:

This paper aims at further exploring the effects of EPO on the oligodendrocyte lineage. There are potentially some interesting observations contained within – albeit for the most part very subtle – but unfortunately the paper is written and put together in a manner which makes it very difficult to understand what has been done and why. Throughout, the results section is written in an overly colloquial fashion and the reference to the figures somewhat erratic. There also need to be a much clearer and logical rationale for why each part of the study have been undertaken. I fear the paper requires a major overhaul and re-write using a conventional scientific style. Below are just some examples illustrative of a more general problem.

We thank the reviewer for reading our paper, but do not comment on this.

The first section of the results is overlong and not really pertinent to the papers main (and new) findings. I suggest removing it.

We agree that the first section of results also serves an explanatory function to readers outside the oligodendrocyte field, which we feel is important. In response to this comment, we have shortened the first section though: [Page 5](#).

What was the rationale for focusing on the hippocampus?

Historically, we have studied the role of EPO/EPOR signaling in learning and memory focussing on hippocampal neurons. However, also for studying myelination, the hippocampus has certain advantages. Different from the optic nerve or corpus callosum, there is a lower density of oligodendrocytes, sparser myelination and later time course of developmental myelination, which makes it easier to capture subtle myelination differences in a white matter tract as a phenotype in mouse mutants. Moreover, we had invested in large snRNA-seq data sets of mouse hippocampi after rhEPO treatment which could be reutilized for this oligodendrogenesis project.

180-181 -it is not clear from the figures what enhancement (whatever that means) and ‘remarkable decrease’ is occurring at 72 hours (not a time point that appears in the figure).

This time point after rhEPO treatment was investigated in Hassouna et al 2016, as cited [directly in figure 1](#). We have clarified that now also in the [figure 1 legend](#).

Hassouna I, et al. Revisiting adult neurogenesis and the role of erythropoietin for neuronal and oligodendroglial differentiation in the hippocampus. *Mol Psychiatry* 21, 1752-1767 (2016).

If I have understood correctly, there are no changes in OPC proliferation or survival, but their numbers do changes (although the evidence for this is not obvious). Is this why migration becomes an issue? Of course, there are indeed no markers that prove migration but it’s not clear how sequence data helps. Frankly, it’s not clear what phenomenon is being investigated in the subsequent analysis of sequencing data.

A lower-level expression of GABA signaling pathway genes has been convincingly associated with the migration of OPC as well as the reduction of GABA-A receptors in these cells. In our EPO stimulated OPC, we likewise found a reduction in 4

subunits of GABA-A receptors (Gabra3, Gabrb1, Gabrb2, Gabrb3). Therefore, EPO-stimulated loss of GABA receptor signaling in OPC is compatible with OPC inward migration and thus their numerical increase after 24h (Figure 1d). However, we have refrained from exploring this isolated aspect of our current paper. GABA-regulated OPC migration and differentiation is complex and currently part of a separate study that we do not want to spoil with premature conclusions.

Figure 1e – what are OPC changes? What does this mean exactly. Is this data from another study? Personally, I would prefer that the OPC was defined as a progenitor rather than a precursor.

Yes, the 72h time point was investigated in Hassouna et al 2016, as cited directly in the figure.

Hassouna I, et al. Revisiting adult neurogenesis and the role of erythropoietin for neuronal and oligodendroglial differentiation in the hippocampus. *Mol Psychiatry* 21, 1752-1767 (2016).

In the literature, the term progenitor as well as the term precursor are often used interchangeably for OPC. As a short definition, a progenitor is more foundational and often denotes the direct ancestor or origin, while a precursor is something that comes before and prepares the way for something that follows. In this sense, both terms are valid for OPC.

179 – these are not sketches – they are data figures

This must be a misunderstanding. We refer to Figures 1 a, b, e as sketches and Figures 1 c, d, f-i as data figures. A sketch involves something quick, preliminary, and not yet fully developed. This is the case in all our sketches. They illustrate the state of our understanding.

Abstract – perhaps be more specific about what EPO does rather than simply say ‘stimulates all OL cells’, which doesn’t really convey what the specific effects are.

We have revised the abstract accordingly: *We found that EPO stimulates all oligodendroglial cells and prompts their differentiation and maturation. More about the specific effects is given extensively in text and figures.*

Abstract. Remove ‘interestingly’ – surely this is a decision for the reader to make. (And remove throughout).

The word ‘interestingly’ has now been eliminated in the whole manuscript.

Abstract. Explain what right shift in size distribution of myelinated axons actually means – I suspect this won’t be obvious to the general reader.

We have now explained this further in the text, page 11:

Lack of EPOR in MOL leads to volume reduction in both fimbria and striatum, and to a right shift in the size distribution of myelinated axons of fimbria (presented in the form of normal distributions).

Last line of the first paragraph of the introduction is something of a non sequitur – none of what comes before makes it clear why EPO is being studied.

This passage has now been reworded: Page 3.

Line 138 – Either there is clear evidence that EPO system is involved in adaptive myelination or there is not. For my reading the case has not been made and there are no hints that this might be so.

The sentence has been reworded: *We therefore asked whether in an integrated response, also OL and adaptive myelination would perhaps play a role, and whether this would mark a direct OLC response to EPO.* Page 4.

Line 94 – ‘.....that are haematopoiesis independent’ perhaps?

Thank you! This has now been added.

Line 99 – what exactly is meant by ‘overpopulating’?

We have rephrased the sentence: *We noticed novel populations of newly differentiating pyramidal neurons, which are about 2-fold more abundant after rhEPO treatment.* Page 3.

Line 111 – perhaps find a less colloquial, more scientific term than ‘brain hardware upgrade’

We had introduced this term in 2022 to denote a plethora of stable adaptive changes in the hippocampus after rhEPO treatment. We now cited the respective reference.

Ehrenreich H, Garcia-Agudo LF, Steixner-Kumar AA, Wilke JBH, Butt UJ. Introducing the brain erythropoietin circle to explain adaptive brain hardware upgrade and improved performance. *Mol Psychiatry*. 2022 May;27(5):2372-2379. doi: 10.1038/s41380-022-01551-5. Epub 2022 Apr 12. PMID: 35414656; PMCID: PMC9004453.

Line 124 – remove ‘Altogether’

Has been done.

Explain cornu ammonis – or use the more widely understood term hippocampus

Cornu ammonis (CA) is only one part of the hippocampus, but in all textbooks well defined.

Line 145 – do the authors really mean ‘immunological markers’ – aren’t they just markers, which ne can detect by immunocyto/histochemistry?

Yes, ‘immunological markers’ is an established term, referring to any molecular feature that can be identified using immunological methods, typically antibodies. See for instance the paper below.

Huang H, He W, Tang T, Qiu M. Immunological Markers for Central Nervous System Glia. *Neurosci Bull* 39, 379-392 (2023).
Reviewer #4 (Remarks on code availability): n/a

Thank you.

POINT-TO-POINT RESPONSE

Refers to changes made in the manuscript

Reviewer #1:

1) The revised manuscript addresses several key concerns, incorporating clarifications and adjustments to improve the presentation of data and conclusions.

Response: We thank this reviewer for the help in further improving our manuscript. With our response below, we hope to convince both reviewer and editor of our properly revised and resubmitted manuscript. We have added experiments, changed the discussion where necessary, and most importantly, **have more clearly distinguished between our conclusions and resulting working hypotheses**, which appears to have caused some confusion.

2) However, several major issues remain that require further attention to ensure the findings are well-supported. My primary concern is that while the **authors assert that "EPO stimulates all oligodendroglial cells and prompts their differentiation and maturation"** and that **"our data support a working model in which brain EPO, induced by functional hypoxia through continual neuronal activity, drives adaptive myelination,"** the experiments conducted in this study do not seem sufficient to fully substantiate these claims. While the conclusions may ultimately be correct, additional data are needed to strengthen the evidence.

Response: We apologize, if our two quoted statements (one on EPO as a driver of oligodendrocyte differentiation, the other on EPO as a potential mediator of adaptive myelination) have been misunderstood as equally robust conclusions. This is certainly not the case and it was never intended.

Our primary data and gene expression profiles were used to **conclude** that all oligodendrocyte lineage cells are responsive to rhEPO stimulation and driven towards a mature phenotype. These findings were also supported by the analysis of conditional mouse mutants lacking EPO receptors.

However, our **resulting working hypothesis** of hypoxia-induced and EPO-stimulated adaptive myelination was clearly no more than intriguing points of discussion that we felt are of interest to investigators in the field of myelin biology, where adult myelin plasticity is a major focus of interest. To make that distinction clearer, **we completely shifted all reference to this working hypothesis to the discussion section, further emphasizing its speculative character, and removed this idea from the abstract.**

3) I acknowledge the limitations the authors are facing regarding the German regulation. However, without further experimental data to support the findings, **it remains difficult to conclusively determine the in vivo effects of rhEPO on OPC migration** (as also stated by reviewer 4). Relying on previous snRNAseq data does not necessarily help in explaining the migration-related phenotype, as this is in minimum correlative source of data that can help in choosing single targets to manipulate experimentally in order to mechanistically prove the underlying mechanism. At its current stage, the study presents only part of the story, limiting the strength of the conclusions.

Response: We agree in principle and would have liked to establish a technology to visualize EPO-stimulated OPC migration *in vivo*. However, the latter is impossible in the frame of a paper revision and the fact that invasive animal experiments in Germany can take years to be approved should not be held against the scientists.

More importantly, “migration” of OPC needs to be better discussed. In nervous system development, OPC (similar neural crest cells) migrate over long distances, but that is presumably independent of EPO signaling, as evidenced here by the rather normal development of our mutant mice. For adaptive myelination and local myelin repair, that is different and OPC build largely on their normal repulsive behavior and the fact that they have already “tiled” the brain. This means “migration” of adult OPC to close-by (unmyelinated) axons involves only short distances (1-2 cell diameters), which is difficult to quantify *in vivo* in time and space, as many other local clues contribute to OPC dynamics at the same time (vascular cues, electrical activity, metabolic signals, inflammatory signals, etc.). Dissecting out selectively the EPO/EPOR signaling as a “variable” will require such a large number of *in vivo* observations that it is in our view doubtful to constitute a realistic experimental aim. Note also that the underlying biological trigger of adult OPC migration (brain injury, acute demyelination, or acute neuronal stimulation) are very difficult to standardize *in vivo*, which further adds to a statistical problem. We therefore think that a clean and simplified *in vitro* experiment is much better suited to show the “migratory” behavior of OPC in response to EPO stimulation, knowing that these are not adult OPC and the results can only provide some *proof-of-principle*. We have performed these experiments and have added new Figure 2d and 2e, showing EPO-induced increased OPC motility over short distances, as it is also realistic in remyelination/adaptive myelination situations *in vivo*.

We have also modified our text accordingly in the results section, discussion part and abstract.

4) Also, some of the text should be moved to the Discussion section for better contextualization.

Response: As acknowledged above, the strict separation of conclusions from resulting working hypotheses made these text moves mandatory.

5) It is unclear which figure presents the results of *in vitro* EPO-stimulated OPCs. There appears to be a gap in the data that should be addressed. I understand the authors are currently working on this but have opted not to include these data in the manuscript. However, I believe this information is critical to support the study’s conclusions. The observed reduction in GABA-A receptor mRNA is intriguing and may support altered migration properties following rhEPO stimulation, but on its own, it is not sufficient.

Response: The entire figure 2 is now devoted to results with *in vitro* EPO-stimulated OPC.

With respect to GABA signaling, we observed indeed the reduction of mRNAs for 4 different receptor subunits (Gabrg3, Gabrb1, Gabrb2, Gabrb3). While this is compatible with GABA-regulated OPC migration (Fig. 1d), we had to refrain from exploring this isolated aspect of cell migration much further. Please note that in EPO-stimulated OPC, we cannot judge the relative contribution of GABA signaling to the overall response to numerous (other) transcriptional changes. This has to await the time-consuming *in vivo* analysis of OPC from novel conditional mouse mutants. As this reviewer will appreciate, it would be in no one’s interest to add premature “conclusions” from RT-PCR data or pharmacologically treated, cultured OPC. On the other hand, we may agree it is reasonable to alert the

interested reader to these findings. We therefore restrict any speculation about GABA signaling in OPC migration to the discussion section.

6) I appreciate the incorporation of snRNA-seq data, but as mentioned earlier, additional molecular and cellular validation is required to substantiate the findings, especially as this manuscript is being reviewed for a journal such as Nature Communications. Reviewer 4 also emphasizes that it is unclear how the snRNAseq data can mechanistically explain the discussed phenotypes. The text modifications made in response to previous comments are noted. **However, additional experiments are needed to explore potential mechanisms underlying the observed effects. While I understand the stated limitations,** I strongly emphasize the necessity of the proposed experiment to validate the role of the EPOR pathway in mOLs.

Response: The central question of our paper was whether or not erythropoietin, already known to be expressed within the CNS, has a direct effect on oligodendrocyte lineage cells. This was comprehensively studied *in vitro* and *in vivo*. We could even generate downstream gene expression data of the affected cell types that are complex but compatible with the observed OPC behavior and the mouse mutant phenotype. However, we do not think it is appropriate to reset the bar at that stage and ask completely different questions now at the level of EPO receptor downstream mechanisms. Obviously, we share the interest in these questions, but this is much more than a new layer of analysis. By all likelihood, it would constitute a severalfold larger project package, as we know already that several candidate mechanisms must be explored. Picking just GABA_R signaling for a start ("*additional experiments are needed to explore potential mechanisms.*"), would only pay "lip service" to the truly important question about further mechanistic insight, which will have to test a range of candidate genes. Lip service experiments are more likely than not to leave the reader of our paper with the wrong take home message. **We have added a paragraph (on page 11) to explain this caveat in the discussion.**

Moreover, we had pointed out before, there is a huge administrative problem associated with planning, applying and performing all these genetic *in vivo* experiments in Germany, which would result in a further delay of this publication by about two years. We therefore ask you to acknowledge that these requests constitute different projects for the next generation of students and postdocs.

7) There may have been a misunderstanding regarding my previous point (comment #8) — I meant that an increase in axonal diameter could suggest an increase in myelin thickness. While g-ratio values reflect the relationship between axonal diameter and myelin thickness, it would be beneficial to include absolute measurements of myelin thickness for additional clarity.

Response: in Figures 6i and 6j, we had shown highly significant right-shifts of myelinated axon calibers in hippocampal fimbria (i.e. increased axon thickness), but no significant change of g-ratios, as demonstrated in the scatter plot, and an unaltered density of myelinated axons per μm^2 . Since the g-ratio expresses the ratio of myelinated fiber diameter divided by the axon diameter, some increased "myelin thickness" is automatically captured in this formula. However, the very fact that "*absolute measurements of myelin thickness*" are usually not helpful (if one assesses an entire spectrum of axon calibers), is the reason that the myelin research community has agreed on g-ratios as the best measure. Of course, this requires the graphical depiction of the g-ratio scatter plot to be included (in addition to regression lines or calculated numerical averages), which he have provided in Fig 6 as well. We acknowledge that maintaining g-ratios may involve increased myelin synthesis.

8) As previously mentioned, **additional data are required to support the study's conclusions** with greater confidence. Addressing these concerns through further data and clarifications would significantly enhance the strength and reliability of the manuscript.

Response: This is the same, somewhat general point made before that we respond again to in two ways. For one, we have now sharpened our “conclusions” and have separated them clearly from any “resulting working hypotheses”, that require additional experiments in order to lead to conclusions in the future. The latter points thus move entirely into the discussion section. Secondly, we must emphasise that picking single candidate mechanisms for more detailed mechanistic insight experiments would be a mere “lip service”, given the number of transcriptional changes observed (each being a candidate to be tested for functional relevance). The required time and effort goes far beyond what is reasonable for a single paper revision and should be given to the next generation of students. Some have already started their thesis projects on that.

9) Technical issues (as also mentioned by reviewer 4 about the manuscript design and readability): Please ensure the accuracy of references to figures throughout the main text. For example, in Figure 1 presented in the file, panels J,K,L are actually not presented in the figure, despite being cited in the main text. Similarly, references to Figures 2 and 3 seem to be incorrect in certain sections.

Response: we apologize for these oversights and **have corrected** the references to figures.

Reviewer #2:

The paper revises the impact of Erythropoietin (EPO) and its receptors (EPORs) on oligodendrocyte precursor cell (OPC) differentiation and myelination. It aligns with previous studies (Jatznie, 2013; Cervellini, 2013; Hassouna, 2016; Cho, 2012; Gyetvai, 2017; Muttathukunnel, 2023; Dewan, 2020). Although the paper does not introduce new concepts to the field, it reaffirms the critical role of EPO signaling in the oligodendrocyte lineage. Furthermore, it provides valuable insights into how EPO affects OPC differentiation during adulthood.

The methods and data analysis are presented clearly, with sufficient detail and a convincing interpretation of the results. The revision was thorough, and although some important experiments could not be included, the discussion was appropriately adjusted to reflect this. My questions have been satisfactorily addressed by the authors.

Response: We thank the reviewer for the sympathetic and understanding feedback.

Reviewer #3:

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Thank you!

Reviewer #4:

This manuscript has certainly been improved from the earlier submitted version. I have a few comments that might improve it further.

Response: We thank the reviewer for the suggestions that further improved our manuscript.

1) Line 75 – Personally, I would remove ‘probably reflecting....’ – this is speculation and shouldn’t really appear in an abstract.

Done.

2) Line 77. Use ‘contributes to’ rather than ‘drives’. This would not only be more accurate but more in keeping with the appropriately circumspect conclusion drawn in line 145.

Done.

3) Line 80. Replace ‘on top of’ with ‘in addition to’

Done.

4) Line 91. Replace ‘highly likely’ with ‘a plausible hypothesis etc’

Done.

5) Lines 99 and 102. Delete commas

Done.

6) Line 187. Remove ‘remarkable’ – a judgement term not appropriate in a results section.

Done.

7) Line 183. Remove ‘First clues for potential involvement of migration’

Done.

8) Comments on responses

I am still of the view that mentioning that the cornu ammonis is part of the hippocampus would be helpful to many readers.

Response: This useful explanation **has now been integrated.**

9) Citing a review that uses the term immunological markers does not make the term correct. I am sure the author’s see my point and I am sure they can come up with a more suitable term

Response: we have **removed the term ‘immunological’ markers.**

10) I am very familiar with the current fashion to use the terms precursor and progenitor interchangeably. However, their meaning is subtly different – hence my suggestion. (No need for a response).

Response: We have now **replaced the term *precursor* by *progenitor* throughout the manuscript.**

REVIEWER COMMENTS (our answers inserted in blue)

Reviewer #1 (Remarks to the Author):

While this is an area of ongoing interest, I believe the current version of the manuscript offers limited advancement in our understanding of these relationships. Much of the data on EPO and OPCs reiterates previously published findings, while the novel aspects related to mature oligodendrocytes and myelination, though potentially interesting, appear modest in scope.

We obviously do not fully agree with this standpoint. Nevertheless, we respect this Reviewer's position and thank him/her again for the time invested into reading and constructively commenting on the paper.

That said, the authors have clearly invested effort in strengthening the manuscript, and I appreciate their responsiveness in addressing several of the prior concerns.

Thank you very much indeed!

My comments below are offered in the spirit of helping to clarify the novel contributions and to improve the overall clarity and impact of the manuscript. Below are my responses to the authors responses in the order they appear in the rebuttal letter:

1. I still find it somewhat challenging to clearly identify which conclusions stem from the current work and which are drawn from previous studies. This issue affects both the introduction and discussion sections. Greater distinction between background knowledge and new findings would significantly help the reader appreciate the manuscript's contributions.

We have now in both introduction and discussion tried to provide greater distinction between background knowledge and new findings. See yellow labeled sentences.

2. See comment #1 — clearer distinction between new and prior findings is needed.

We have now provided greater distinction between background knowledge and new findings. See yellow labeled sentences.

3. I understand the limitations related to approval for in vivo experiments in Germany and appreciate the authors' explanation. While the lack of in vivo OPC migration data remains a limitation, the additional in vitro data now provided, along with the revised discussion that acknowledges these constraints, is a reasonable compromise in my opinion.

Thank you very much for your understanding and support.

4. Acknowledged.

Thank you!

5–6. The authors chose to highlight the involvement of GABA signaling, and while this may indeed be relevant, I felt that the mechanistic evidence provided is currently limited. My earlier comment was not intended as a critique of the inclusion of this pathway, but rather as a

suggestion to either better support its role or to frame it more cautiously. As it stands, the data are quite general and not sufficient to support mechanistic conclusions about this pathway.

Agreed. We therefore restricted any speculation about GABA signaling in OPC migration to the discussion section.

7. Acknowledged.

Thank you!

8. In my view, the key issue remains: the dataset, while of interest, supports only limited novel conclusions regarding the role of EPO/EPOR in oligodendrocyte lineage progression and myelination. The observed effects on mOLs are intriguing and worth pursuing further, but currently appear modest and somewhat preliminary.

We obviously do not fully agree with this standpoint. Nevertheless, we respect this Reviewer's position and thank him/her again for the time invested into reading and constructively commenting on the paper.

9. Please see technical comments below.

Specific Comments:

Abstract:

Line 59: Consider rephrasing. Starting with “Here” and using the past tense (“thought”) is ambiguous — it's unclear whether you're referring to past work or current findings.

Line 60: “Using in the murine hippocampus” — please remove “in”.

Line 63: Clarify whether this is a novel finding. There is already substantial literature on EPO and OL lineage cells, including by the authors.

Line 73: The current data, while suggestive, may not yet fully support the closing claim regarding rhEPO as a clinically viable option. Consider softening the language. Also, please ensure font consistency here.

The Reviewer will notice that we have now shortened the abstract to 150 words. The necessary re-writing has now solved all the here mentioned issues.

Introduction:

Line 100: It's not clear whether “here” refers to this study or to previously cited findings. Please clarify, as this may confuse the reader.

Here has now been replaced by ‘In this region...’

Results:

Line 200–201: The differing effects of rhEPO in vitro and in vivo are well described. This distinction is important and should be emphasized in the discussion.

We agree that the distinction is important. Thank you for the positive feedback! We decided, however, not to repeat this topic in the discussion to avoid lengthy redundancy.

Line 261: Incorrect figure reference — it should be figure 4, not 3a–m. Please verify all figure references throughout the manuscript.

We thank this Reviewer for making us aware of the figure references. This has indeed been very important to correct. They have now all been properly adjusted.

Line 271: Please indicate the specific figures (e.g., 5a–c for in vivo, and 5d for mOLs?).
Line 289: Please clarify what is meant by “absence of a response to rhEPO” — is this referring to decreased mOL numbers at week 4?

We have now reworded: *Importantly, after 4 weeks, only CNP-expressing MOL (lacking EPOR expression) failed to respond to rhEPO (Figure 5g-k).*

Ensure consistency in capitalization in graph and axis titles (e.g., Figure 6j).

Thank you! We did.

Line 358: The sentence suggests a fully novel observation, but prior work has shown rhEPO-induced differentiation of OPCs. Consider framing this as an extension or refinement of previous findings, with emphasis on the mOL/myelination aspect as the primary novel contribution.

We have tried to make this now very clear in the manuscript.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Thank you!