nature portfolio

Peer Review File

Incomplete remyelination via therapeutically enhanced oligodendrogenesis is sufficient to recover visual cortical function

Corresponding Author: Professor Ethan Hughes

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 work by Nunes and colleagues they take on the important question of how rapidly and efficiently oligodendrocytes are replaced after demyelination in the cortex. They use two-photon imaging to find that the best predictor of new oligodendrocyte replacement is oligodendrocyte loss (peaking about 7 days before the replacement). This is fascinating as it likely has biological implications, such as new signals that could be causing this effect. Another important finding is that in the cortex the gain of oligodendrocytes, where there is robust loss, is insufficient. They go on to find that when using a new CNS honing thryroid hormone mimic they can enhance the rate of oligodendrocyte replacement and improve certain electrophysiological and visual system outcomes. Interestingly, the target medication shown by many groups clementine to improve remyelination does not enhance the max oligodendrocyte gain rate, where the this thyroid hormone derivative does. I think overall this is a rigourous study that will have impacts on the field of remyelination and myelin biology. The live imaging adds new, novel, information to the field. Overall, I commend the authors for their outstanding work
My only more major concern is that the discussion leans more towards a rehash of the results instead of an integration of this work in the literature. Specifically, this work is all on grey matter remyelination and so I hoped to find a discussion point on how grey matter and white matter may be different (or the same). I recognize due to technical limitations that a direct comparison is not possible (yet), but at a minimum it should be clear that some of the biology could be specific to oligodendogenesis in the grey matter. Also, it would be interesting to have some speculation as to what signals stimulate oligodendrocyte production due to oligodendrocyte loss.
Minor points:
Line 132: important to mention somewhere here that these gains also coincide with the removal of cuprizone diet.
Line 134: It is worth stating that point a and point b are not mutually exclusive
Line 146: I found the line “does not underlie” too firm for a correlational finding
Line 155: You mention here that you are measuring cumulative loss. At times you are probably measuring cumulative gain. I found throughout the paper that this was not stated explicitly enough. I would like the work cumulative to be embedded in the figure where relevant. For measurement up to a given date, is it always cumulative or is this at times the amount of oligodendrocytes gained between imaging sessions? Either way, make it more explicit in the legends and the figures.
Line 212: I found this line unclear

Reviewer #2

(Remarks to the Author)
In their study, Nunes et al. have investigated aspects of oligodendrocyte (OL) dynamics, neuronal network dysfunctions and thyromimetic therapy-based recovery after experimentally-induced demyelination. In particular, they applied longitudinal in vivo two-photon imaging of visual cortical area V1 to investigate OL loss and gain in a transgenic Mobp-EGFP mouse model of subacute cuprizone exposure-induced de- and remyelination.
Their main findings show that (I) OL regeneration fails under conditions of high OL death rates, and that (II) the best predictor of OL gain rate at a given timepoint was OL loss rate seven days prior, suggesting that an OL death-derived acute signal induces new OL formation. Subsequently, by comparing the therapeutic potential of clemastine fumarate with a new thyromimetic compound (LL-341070), they found that LL-341070 transiently (during the first half of treatment) accelerates OL

gain rate and that LL-341070 is more effective than clemastine fumarate at augmenting OL restoration. Finally, they performed in vivo-electrophysiology in V1 to assess visually-evoked neural activity following LL-341070 treatment. Their data suggest that LL-341070 treatment promotes the recovery of both single-neuron response latencies as well as V1 neuronal population responses to visual stimuli by restoring the myelinating OL population after cuprizone-induced demyelination.
Overall, this is a well performed study that provides detailed insight into cortical OL dynamics in the context of cuprizone-induced de- and remyelination with relevance for demyelinating disorders. These data suggest a therapeutic potential of a novel thyromimetic compound (LL-341070) by promoting OL differentiation following cuprizone-induced OL loss. Moreover, authors found that LL-341070 treatment accelerates the recovery of visual neuronal network functions after demyelination; however, untreated controls reached a similar recovery of neuronal network functions at later stages. Nevertheless, these are important findings that provide a framework for additional preclinical studies, especially when considering a critical time window for endogenous remyelination, as suggested by the data provided. Ultimately, thyromimetic treatment might become highly relevant in human patients, particularly under chronic conditions of de/remyelination, with a much more limited capacity for OPC differentiation into myelinating OL.
While the data presented in the manuscript demonstrates high quality and supports the overarching conclusions, there are instances where the authors infer causation from correlation alone. To strengthen their argumentation, the authors should include additional data and engage in further discussion addressing the following comments.

1) The interpretation of study outcomes (including the transient nature of LL-341070 effects) would benefit from providing some additional histological data, at least for selective timepoints, e.g. 1 week versus 7 weeks post cuprizone treatment. This should include OPCs (considering their discussed possible depletion by LL-341070 treatment), microglia and astrocytes (there are extensive microglial and astrocytic responses in the cuprizone model that might interact with/are modified by LL-341070 treatment).
2) In addition, the study uses elevated numbers of Mobp+ OL as a proxy for (increased) remyelination, but there are no direct data that support increased (intracortical) remyelination as a driver of functional recovery. Also, as two-photon imaging mainly detects OL in cortical layers I-III, whereas in vivo electrophysiology preferentially concerns deeper layers, authors should at least discuss implications and possible limitations of their study (including possible involvement of effects throughout the visual path).
3) Finally, to provide more challenging conditions (and to possibly identify distinct longer-term recovery plateaus), it would be informative to combine LL-341070 treatment with (a more common) 6 weeks cuprizone exposure protocol.
4) While oligodendrocyte gain during remyelination is induced by recent oligodendrocyte loss, the number of new oligodendrocytes formed per lost oligodendrocyte depends on the rate at which the oligodendrocytes were lost. According to Fig. 2H this only applies to low/moderate oligodendrocyte loss rates.
5) In Fig. 3H there seems to be no significant differences between groups in OL gain at 7 weeks. This seems to be at variance with data in Fig. 5F
6) In Fig. 6/7 presentation of data as ‘super plots’ with distinct color label of data points from individual mice would provide more information to the reader.
7) In Fig. 7D) the emergence of shape distortion in high LL-341070 group at 7 weeks (especially when
8) Chaudhary et al. Thyroid hormone and thyromimetics inhibit myelin and axonal degeneration and oligodendrocyte loss in EAE. J. Neuroimmunol. (2021) should be referenced and discussed.
9) The paper would profit from a discussion on the distinct side effects/tolerability of clemastine versus LL-341070, considering similar long-term outcomes.

Reviewer #3

(Remarks to the Author)
The manuscript titled " Incomplete remyelination via endogenous or therapeutically enhanced oligodendrogenesis is sufficient to recover visual cortical function" authored by Nunes et al., is well written, topical and a technically rigorous study.
What are the noteworthy results?
Given that myelin loss causes brain dysfunction and is linked to neurodegenerative, understanding endogenous remyelination and developing treatments to restore neuronal function are critical. Although visual pathway abnormalities are among the initial symptoms experienced by individuals with MS, research on the impact of myelin loss, a key characteristic of MS, on visual function remains limited. Using in vivo two-photon microscopy and electrophysiology, the authors show that endogenous remyelination is triggered by recent oligodendrocyte loss and is very successful following mild demyelination, but less efficient after more prolonged exposure to cuprizone resulting in more severe demyelination. In comparison to clemastine fumarate, a new thyromimetic LL-341070 increases oligodendrocyte gain during remyelination and speeds up neural function recovery in the early phase, where clemastine exhibited slower but more prolonged effects, even after discontinuation. The therapeutic efficacy of LL-341070 only occurs after moderate to severe demyelination. Finally, the data shows that partial oligodendrocyte regeneration is enough to restore visual neuronal function as measured electrophysiologically.
Will the work be of significance to the field and related fields? How does it compare to the established literature? If the work is not original, please provide relevant references.
Yes. This study presents in vivo findings to describe a correlation between myelin loss and visual function. They authors propose that treatments promoting remyelination, such as the thyromimetic LL-341070, may facilitate functional recovery by boosting oligodendrocyte regeneration in conditions associated with myelin loss. Among the agents currently under investigation, LL-341070, a thyromimetic prodrug, stands out for its ability to enhance oligodendrocyte regeneration in experimental models. The design of this agent optimizes central versus peripheral exposure through selective activation upon contact with FAAH, expressed in the brain. LL-341070 also exhibits selectivity by targeting the thyroid hormone

receptor beta. These features position LL-341070 as a promising candidate for advancing our understanding and potential treatment of myelin-related disorders, particularly in the context of multiple sclerosis. The findings of this paper add new insights into ongoing research on remyelinating therapies for multiple sclerosis, emphasizing the rationale for promoting myelin sheath repair for functional recovery.
Does the work support your conclusions and claims, or is additional evidence needed?
The author's work substantiates the presented conclusions and claims. However, some issues should be experimentally addressed and further discussion is needed to clarify the obstacles inhibiting remyelination in MS.

Critique:
- This study did not characterize how LL-341070 increases the generation of new mature oligodendrocyte. The author should discuss the potential source of the remyelinating cells. It has been demonstrated that OPCs first begin to accumulate in the demyelinating region 2–3 weeks after initiating the cuprizone intoxication. The applied drug LL-341070 might influence the differentiation rather than the proliferation of OPCs (or eventually both). Perhaps this question can be answered by imaging NG2-mEGFP line, track OPC migration, proliferation, differentiation, and death?
- Is the increased Oligo numbers following treatments due to the effect of treatment on the number of cortical OPC or recruited from nearby brain region?
- Does the remyelination modulate myelin sheath number per newborn oligos? It is unclear whether myelin sheath underwent remodeling during remyelination.
- The study showed that the magnitude of oligo loss and gain is not one to one. Although remyelination failed to restore oligodendrocyte number but seems to restore neuronal function. It is unclear whether the restoration of neuronal function can be attributed to an increase in sheath numbers generated by new oligodendrocytes? Alternatively, it raises the possibility that existing mature oligodendrocytes might play a role in generating myelin, aligning with current evidence indicating the potential participation of mature oligodendrocytes in remyelination in rodent models (PMID: 37071991). This nuanced relationship between oligodendrocyte numbers, myelin generation, and neuronal function adds complexity to our understanding of the mechanisms underlying remyelination and warrants further investigation.
- Axons become to become affected with longer exposures to cuprizone and I wonder if the authors can comment on drop out of axons affecting their measurements both at the tissue level and by EP, where pseudonormalization can occur by recording only remaining healthy axons. While less than optimal there are now some improved methods of measuring vision in mice.
- The study shows that only mice with low level of oligo loss regenerate their original oligo population after remyelination but mice with high level oligo loss exhibited a regeneration deficit. It is unclear why there is regeneration gap. Possible factors contributing to this gap could include a shortage in the pool of OPCs and the presence of reactive gliosis, and axonal pathology, as mentioned above. The authors should discuss these potential confounding factors. Addressing these uncertainties will be crucial for advancing our understanding of the mechanisms involved in oligodendrocyte regeneration after remyelination, ultimately informing potential therapeutic interventions.
- Please discuss that cuprizone may not accurately model all of the inhibitory cues present in an MS plaque.
- The study shows that the rate of oligo gain during remyelination is driven by recent loss. The authors mention the possibility of acute signaling that might occur around the death of oligodendrocytes, thereby inducing the formation of new oligos approximately one week later, but this section could be exapnded.
Are there any flaws in the data analysis, interpretation and conclusions? Do these prohibit publication or require revision?
- In figure 1 B and C, the optic nerve is only assesses at -3.5 and 0. A trend towards reduced myelination is seen and it would be important to examine at the later time point to settle this point of contention in the recent literature. In Figure 3, panel 3E, although there is no significant difference in the mean lost oligodendrocytes between groups, the observed variability is notably high. Considering the established correlation that more oligo loss to lead to more gain, the one should be careful of this substantial variability in the extent of oligodendrocyte loss within the visual cortex after cuprizone intoxication. The discussion should address the factors contributing to the wide range of oligodendrocyte loss observed, ranging from 14% to 80% demyelination (line 110) at the 3-week cuprizone. similarly, high variability is shown in Fig 5C, where oligo loss is shown after 3 weeks cuprizone intoxication between treatment groups. Details regarding the experimental design should be added, such as the number of times the experiments were repeated independently to overcome the high variability in a small sample size (n).

- Additionally, the impact of sex on LL-341070 therapy should be addressed. Understanding whether and how sex influences the response to treatment is crucial for the applicability and generalizability of the findings. Any observed differences in treatment outcomes based on sex should be discussed.

- In Supplementary Figure 4F and G, the remyelinating treatment with low dose LL-341070 (0.1 mg/kg) appears to worsen oligodendrocyte gain as compared to the vehicle. Please comment.
Is the methodology sound? Does the work meet the expected standards in your field?
The methodology is sound and meets the standards.
Is there enough detail provided in the methods for the work to be reproduced?
- In the method section, it is written that cortical layer MOBP–EGFP image stacks were acquired in a volume of 425 μm × 425 μm × 336 μm; corresponding to layers I-III, 0–336 μm from the cortical surface (lines 566-568). In Fig6A Neuropixel probes was inserted in the primary visual cortex. Please add an illustration to show where exactly the Neuropixel probes record single neuron activity. While two-photon microscopy records layer I-III. Please provide more details how these data are correlated if captured from different layers?
Data arrangement and presentation in the manuscript:
- The organization and presentation of data in certain panels create confusion and can be challenging to follow. The manuscript benefit for clearly explaining these terms in a separated section, e.g. in methods and provide details (equation) how they were calculated. All the values below are presented as percentage and could be very confusing.

Lost or new OL (%), OL number (%), OL gain rate (%), Oligodendrocyte replacement (line 88) (%), Cumulative oligodendrogenesis (%).

- The text discusses some panels out of order, causing confusion for readers. For instance, between lines 212 to 221, the explanation of panel F occurs after the discussions on panels G and H. Similarly, at line 112, Fig 1G is explained after Fig 1H. This arrangement makes it challenging for readers to follow the data logically. It is recommended to reorganize the text to ensure that the discussion aligns with the order of the panels, enhancing the overall clarity and facilitating a smoother comprehension of the presented data.

- In Figure 3, Panel 3F, it would be more effective to present the data in a video format since each image was taken from a mouse in a separate group, making direct comparisons challenging. The statement "We found that mice treated with the high dose of LL-341070 gained substantially more new oligodendrocytes by 1.5 weeks as compared to mice treated with the low dose or vehicle" would benefit from visual representation using a video file, allowing for a dynamic presentation of the observed differences among the treatment groups on the generation of new oligodendrocytes over time.

Reviewer #4

(Remarks to the Author)
Nunes et al. have submitted a paper that addresses the question of how remyelination after toxic demyelination occurs in the murine visual cortex, how this can be boosted by myelin-promoting drug candidates, and how this impacts some aspects of visual cortex physiology.
While the question addressed is important, and combining a remyelination therapy with a functional readout could be exciting, I have substantial concerns about some of the data and arguments that, in my view, preclude publication in this form.

General comments:

1) Throughout the paper, the authors seem to equate the restoration of OL cell body number with “remyelination”, including in the title. Would this claim not require analyzing internodes, not OL numbers?

2) The authors’ arguments in the first part of their paper are largely based on correlations resulting from the intrinsic variability of the initial demyelination in the area of observation (visual cortex). The sole reliance on curpizone as a demyelination tool is a limitation here. Cuprizone affects myelin in many parts of the CNS (and in very variable ways). Cuprizone can also be neurotoxic, e.g. Höflich et al., Brain Res 2016; Nyamoya et al., 2017 and references therein) and hepatotoxic, and its mechanism of action is somewhat cryptic. This is relevant for variance analysis of remyelination triggers (as there might be many hidden covariant factors) and for functional analysis (as there might be remote and off-target effects). Without an understanding of what causes the variability of demyelination (even whether this is a local or a global phenomenon for a given mouse), it is hard to ascertain that the drawn conclusions are the only possible ones. Even if one accepts the description that “OL loss rate a week prior” predicts current OL gain, in my view, this could still be compatible with a short and transient “homeostatic” signal that originates, e.g., from other cells that are supported by the dying cells and monitor one of their functions. My reading of the text seems to suggest, however, that the authors favor a view where the death of OLs (or cells in general) in itself somehow triggers a regenerative signal. This could be tested, e.g., by additionally killing additional cells, perhaps with other kinetics (OLs vs. other cells, e.g. astrocytes) and seeing how the signals add up (I assume that just killing some non-Ols would not induce any de novo myelination - which, if true, to me would suggest that at least some homeostatic signal is needed that determines the type, if not the extent, of the repair reaction). Overall, I think without finding the actual signals that trigger remyelination, I am not sure this part rises beyond a (albeit in my view valuable) description of some of the characteristics of the remyelination-inducing signal: It seems to be scaled to loss of OLs and relatively short-lived; but whether it is induced by the death of cells, or by demyelination of axons (or any other function/change in Ols that the system monitors) seems to me hard to conclusively answer from the data provided – and I would suggest a revision that tones down the more specific mechanistic claims if no additional evidence can be provided.

3) For remyelination-supporting therapy, the authors compare two established drugs: a thyromimetic (LL-341070, short “LL”) and clemastine fumarate (the current “gold standard” even though its effects are not fully understood). Both have in prior work been shown to support remyelination, but LL is the much less explored drug – in part because it is a company development by “autobahn therapeutics”, a company that is also listed among some of the authors’ affiliations (two co-authors hold/held company stocks according to a 2022 disclosure) and which funded the research. So, considering that the experiments amount to a “benchmarking” against a best-in-class drug, revealing conflicts of interest and ensuring transparency are important here (the company link is mentioned in the “competing interest” section, but the extent of the current financial involvement should be made clear). In this context, it is unfortunate that the relevant key reference (Ref 54) is incomplete and – at least for me - hard to find (I assume it is https://doi.org/10.1212/WNL.98.18_supplement.3865, although the drug there has another name and it is really only an abstract/meeting supplement). Also, the methods list that the experimenters were blinded “during drug administration” (L. 526) – I assume this to mean that the study was conducted as a completely blinded and randomized (at “0w”) study from animal assignment to end of analysis? I would ask the authors to clarify. Then, the number of data points that were included also needs to be made transparent. For instance, in Fig 4A – how many samples are there with 75% loss in the high dose group that supports the claim that LL “more than doubles” the OL gain. If I correctly read Fig. 3G, the sample size might actually be n=2 for this subgroup (1.5w, 75% loss, 0.3mg/kg), which supports a key conclusion. This would raise serious doubts, and I assume I am wrong (how could an S.E.M. be meaningful here?), but I cannot simply find the answer as to why this reading is not correct. In any case, it seems a bit concerning that

the authors in this part of the study (Fig. 3 and 4) chose not to plot the data points, but rather resort to a measure of mean and S.E.M.; while with careful referencing some of this information can be deduced (or extracted from the legends), the casual reader might miss the actual sample size underlying some claims - after all only 6 mice were measured I this part of the study, not much for an analysis that in part is variance based. I find the study underpowered in this key experiment. While the main Figs 3 and 4 suggest a dose-dependent effect of LL, in Supp Fig 4, the picture is different – the low-dose group is all over the place, neither resembling the vehicle nor the high-dose (see, e.g., Supp Fig 4E), suggesting that here the analysis exceeds what the data can tell. In addition, some data are replotted and combined in a way that is not always transparent or even correct (see below).

4) While I find the Neuropixel recordings an interesting addition, I am not sure if this justifies the title that claims “recovery of visual function”. What is measured here is not visual function but parameters of visual system electrophysiology. Claiming the extent of visual function recovery would need a more comprehensive analysis, including of the information content of the population response and behavioral read-outs. In any case, the extent of demyelination (and other pathology), spontaneous or induced repair along the visual pathway, are unknown. Similarly, which pathology actually explains the altered recordingsis also unclear. Thus a direct linking of the extent of remyelination in the observed small part of visual cortex and the degree of recovery in the recording parameters, which probe a large part of the visual system, including the retina, optic nerves and tracts, thalamus and subcortical white matter, seems not very meaningful.

Specific comments:

Across all figures: There is replotting and reanalysis – this needs to be made transparent. For instance, Fig. 3D/E and 5B/C are in parts identical (LL high dose in 3E is LL in 5C and I suspect the controls are pooled, where some “vehicle” data points from 3E are also in the “vehicle” group from 5C). Same for 4D and 5G – and this probably then also applies to 3I, J and 5D, E – but here it is additionally confusing that the error bars (e.g., but not only, in the 1.5-3 w groups of 3J and 5D) are different. Overall, I recommend carefulyl checking the data and avoiding replots – Figures 3, 4 and 5 largely describe the same experiment and should be treated and plotted as such – with the two vehicle treatments clearly separated and all data points individually plotted. In the same spirit, identical subanalyses for clemastine should be included that were done for the LL high dose.

Fig 1A: It is my understanding that the schematic is not correct. Most mice were gavaged during early remyelination with “1% 1-methyl-2-pyrrolidone (Sigma 522 270458) and 1% Kolliphor HS 15 (Sigma 42966)” or a “10% DMSO solution” – see Supp Fig 5A. While the authors show that they cannot detect differences between these groups, this should still be indicated here.

Fig 1B/C: I am not convinced these data suffice to claim that the “myelin in the anterior visual pathway” is unaffected. For that claim, I would expect comprehensive structural data from all the myelinated parts of the prethalamic visual projections and higher resolution imaging not only of para-/nodal structures but also of myelin, including ultrastructure.

Fig. 1D/E: I suspect the authors have shown this before, but I want to be sure this also applies to this specific cuprizone dose and treatment length – are the oligodendrocytes that cannot be seen anymore really dead? Is Mobp-GFP expression insensitive to cuprizone? I appreciate that in Fig. 1E most cells that appear are in positions distinct from the original cells, but I want to make sure this is systematically true.

Fig 1K: It might be worth considering that another interpretation of the results is that there are really two groups of mice – in which cuprizone has different effects. The segment of 30-60% loss is conspicuously empty. Could there be two groups of mice (males/females; weight, age, etc.) that differ biologically in terms of loss of oligodendrocytes and regeneration capacity?

Supp Fig. 2F: While obviously the significance for the correlation between loss and gain gets lost at 7w, how certain are the authors that this is not just a question of power? This is only relevant because the authors cite this result as evidence that the gap between the low and high loss groups will not close later – which, in my view, it still well could. If the authors want to claim that eventually remyelination remains incomplete, they should simply image longer.

Line 371: I am not sure how the authors arrive at “87%” as the threshold for functional recovery, but as I said before, I think it is not sound to correlate the recovery in layers I-III of a part of the visual cortex to an electrophysiological recording that probes the entire visual system. I do not see how such a claim could be proved without chronic recordings and then selectively re-ablating the observed “new” cells that supposedly mediate this recovery.

Line 454: The third option could simply be assessed by OPC stainings.

Reviewer #5

(Remarks to the Author)

Reviewer #6

(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.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed my concerns

Reviewer #2

(Remarks to the Author)
The authors have adequately addressed all my previous remarks and provided substantial additional data, which significantly strengthen the manuscript. This particularly refers to the quantification of remyelination and the characterization of oligodendrocyte dynamics along the visual pathway which substantially add to the manuscript. However, the newly included histological quantification of microgliosis and astrogliosis does not, in my view, sufficiently support the statement: “Neither astrocytes nor microglia were grossly affected by LL-341070, suggesting that a direct effect of LL-341070 on other glial cells is unlikely (Supplementary Fig. 9).” This conclusion is hampered by limitations in the analysis, which relies on a single homeostatic microglial marker and seems underpowered, with only n = 4 per group and a high variability (wide SD). Notably, the mean GFAP level is reduced by half in the treatment group, and the lack of statistical significance appears to result from the combination of high variability and low sample size. While the data on remyelination are now more convincing, the possibility of an immunomodulatory contribution cannot be excluded based on the evidence presented and this aspect should be adequately addressed and discussed.

Reviewer #3

(Remarks to the Author)
The revisions are thorough and have addressed our concerns.
Marjan Gharagozloo and Peter Calabresi

REVIEWER COMMENTS

We thank all the reviewers for their comments; they have greatly improved our manuscript. We have performed numerous new experiments and analyses to rigorously address all reviewer feedback by:

1. Adding new mice to support our finding that a novel remyelination therapeutic, the thyromimetic LL-341070, improves regenerative oligodendrogenesis during remyelination.
2. Directly measuring myelin sheaths during remyelination to demonstrate that LL-341070 increases myelin levels during remyelination.
3. Imaging oligodendrogenesis in healthy mice to compare recovery following demyelination to healthy levels.
4. Characterizing demyelination throughout the visual pathway to demonstrate selective posterior demyelination.
5. Characterizing glial activation and neuronal degeneration in response to LL-341070 and demyelination.
6. Examined the mechanism of action of the novel thyromimetic LL-341070.
7. Investigating the factors contributing to variability in demyelination and establishing that demyelination and remyelination therapy do not exhibit sexual dimorphism.

These findings have been added to Figs. 3, 4, and 7 and Supplementary Figs. 8 and 10 and have generated entirely new content: new Supplementary Figs. 1, 2, 6, 9, and 16, new Supplementary Tables 1 and 2. Furthermore, we have revised the text in numerous places to incorporate reviewer feedback.

Please see our responses to specific feedback below.

Reviewer #1 (Remarks to the Author):

In the work by Nunes and colleagues they take on the important question of how rapidly and efficiently oligodendrocytes are replaced after demyelination in the cortex. They use two-photon imaging to find that the best predictor of new oligodendrocyte replacement is oligodendrocyte loss (peaking about 7 days before the replacement). This is fascinating as it likely has biological implications, such as new signals that could be causing this effect. Another important finding is that in the cortex the gain of oligodendrocytes, where there is robust loss, is insufficient. They go on to find that when using a new CNS honing thryroid hormone mimic they can enhance the rate of oligodendrocyte replacement and improve certain electrophysiological and visual system outcomes. Interestingly, the target medication shown by many groups clementine to improve remyelination does not enhance the max oligodendrocyte gain rate, where the this thyroid hormone derivative does. I think overall this is a rigourous study that will have impacts on the field of remyelination and myelin biology. The live imaging adds new, novel, information to the field. Overall, I commend the authors for their outstanding work

We thank the reviewer for their review of our manuscript and their thoughtful and supportive comments.

My only more major concern is that the discussion leans more towards a rehash of the results instead of an integration of this work in the literature. Specifically, this work is all on grey matter remyelination and so I hoped to find a discussion point on how grey matter and white matter may be different (or the same). I recognize due to technical limitations that a direct comparison is not possible (yet), but at a minimum it should be clear that some of the biology could be specific to oligodendogenesis in the grey matter.

We have now added several points to the Discussion to integrate this work into the literature, including a new paragraph in the Discussion addressing gray vs. white matter differences (Lines 533-540).

Also, it would be interesting to have some speculation as to what signals stimulate oligodendrocyte production due to oligodendrocyte loss.

We added this point to the Discussion (Lines 459-470).

Minor points:

Line 132: important to mention somewhere here that these gains also coincide with the removal of cuprizone diet.

Indeed, oligodendrocyte gain is suppressed during the delivery of cuprizone and increases following removal of cuprizone diet. Previously, we determined that this suppression during cuprizone is due to an almost 100% death rate of newly generated oligodendrocytes during cuprizone (Bacmeister et al., 2020). We have added new text describing in the Results section (Lines 111-114).

Line 134: It is worth stating that point a and point b are not mutually exclusive

We changed "or" to "and/or" to acknowledge that these options may not be mutually exclusive (Lines 146-148).

Line 146: I found the line "does not underlie" too firm for a correlational finding

This has been changed to now read: "… is not a primary factor…" (Line 160).

Line 155: You mention here that you are measuring cumulative loss. At times you are probably measuring cumulative gain. I found throughout the paper that this was not stated explicitly enough. I would like the work cumulative to be embedded in the figure where relevant. For measurement up to a given date, is it always cumulative or is this at times the amount of oligodendrocytes gained between imaging sessions? Either way, make it more explicit in the legends and the figures.

This has been made more explicit in the text, figures, and legends throughout. We thank the reviewer for helping to make these measurements clearer to the reader. Gain and loss % measurements are all cumulative measurements and have been changed to "Cumul. lost OLs (%)" or "Cumul. new OLs (%)".

Line 212: I found this line unclear

The line in question was referring to the analysis of covariance with unequal slopes described in the previous paragraph. We have now combined these paragraphs in order to make it clearer that they refer to the same analysis (Lines 222-230).

Reviewer #2 (Remarks to the Author):

In their study, Nunes et al. have investigated aspects of oligodendrocyte (OL) dynamics, neuronal network dysfunctions and thyromimetic therapy-based recovery after experimentally-induced demyelination. In particular, they applied longitudinal in vivo two-photon imaging of visual cortical area V1 to investigate OL loss and gain in a transgenic Mobp-EGFP mouse model of subacute cuprizone exposure-induced de- and remyelination.
Their main findings show that (I) OL regeneration fails under conditions of high OL death rates, and that (II) the best predictor of OL gain rate at a given timepoint was OL loss rate seven days prior, suggesting that an OL death-derived acute signal induces new OL formation. Subsequently, by comparing the therapeutic potential of clemastine fumarate with a new thyromimetic compound (LL-341070), they found that LL-341070 transiently (during the first half of treatment) accelerates OL gain rate and that LL-341070 is more effective than clemastine fumarate at augmenting OL restoration. Finally, they performed in vivo-electrophysiology in V1 to assess visually-evoked neural activity following LL-341070 treatment. Their data suggest that LL-341070 treatment promotes the recovery of both single-neuron response latencies as well as V1 neuronal population responses to visual stimuli by restoring the myelinating OL population after cuprizone-induced demyelination.
Overall, this is a well performed study that provides detailed insight into cortical OL dynamics in the context of cuprizone-induced de- and remyelination with relevance for demyelinating disorders. These data suggest a therapeutic potential of a novel thyromimetic compound (LL-341070) by promoting OL differentiation following cuprizone-induced OL loss. Moreover, authors found that LL-341070 treatment accelerates the recovery of visual neuronal network functions after demyelination; however, untreated controls reached a similar recovery of neuronal network functions at later stages. Nevertheless, these are important findings that provide a framework for additional preclinical studies, especially when considering a critical time window for endogenous remyelination, as suggested by the data provided. Ultimately, thyromimetic treatment might become highly relevant in human patients, particularly under chronic conditions of de/remyelination, with a much more limited

capacity for OPC differentiation into myelinating OL.
While the data presented in the manuscript demonstrates high quality and supports the overarching conclusions, there are instances where the authors infer causation from correlation alone. To strengthen their argumentation, the authors should include additional data and engage in further discussion addressing the following comments.

We thank the reviewer for their insightful review of our manuscript.

1) The interpretation of study outcomes (including the transient nature of LL-341070 effects) would benefit from providing some additional histological data, at least for selective timepoints, e.g. 1 week versus 7 weeks post cuprizone treatment. This should include OPCs (considering their discussed possible depletion by LL-341070 treatment), microglia and astrocytes (there are extensive microglial and astrocytic responses in the cuprizone model that might interact with/are modified by LL-341070 treatment).

We have now performed histological analyses and evaluated glial responses in animals treated with 0.3 mg/kg LL-341070 or vehicle for 3 weeks post-cuprizone. We did not identify any gross changes in glial reactivity in the primary visual cortex (V1). Vehicle or treatment groups had equivalent area coverage by homeostatic microglia (labeled with P2RY12) and similar density of reactive astrocytes (labelled with GFAP) and total astrocytes (labelled with S100β). These data suggest that gliosis is unlikely to underlie the effects of LL-341070 on remyelination or neuronal function observed at this timepoint. These new data are reported in **Supplementary Fig. 9** and discussed in the Results section (Lines 239-240).

Since there is a strong drive to maintain a constant OPC population (Hughes et al., 2013), fluctuations in the OPC population would be best measured using a method with high sensitivity and temporal resolution like longitudinal *in vivo* imaging. This is the focus of ongoing studies in the laboratory. We revised our Discussion to include the additional possibility of a varied OPC response to demyelination via subpopulations (Lines 519-524).

2) In addition, the study uses elevated numbers of Mobp+ OL as a proxy for (increased) remyelination, but there are no direct data that support increased (intracortical) remyelination as a driver of functional recovery.

We thank the reviewer for raising this important point. also raised by **Reviewer #3, point 4**. We have included new data directly quantifying remyelination, presented in **Fig. 4G-J** and **Supplementary Fig. 10C, I**. We quantified the number of sheaths, length of sheaths, and total length of myelin made by new oligodendrocytes in vehicle-treated and LL-341070-treated mice during remyelination as well as in healthy controls. We did not observe statistical differences in any metric of myelin levels between conditions. This new data is reported is reported in the Results section (Lines 305-314).

Using these measurements, we estimated total myelin levels in healthy and remyelinating mice and compared these levels to restoration of neuronal function. We find that mice treated with 0.3 mg/kg LL-341070 restore function at three weeks with myelin still well below healthy levels. These results are presented in new **Fig. 4J**, **Supplementary Fig. 10E**, and **Fig. 7E** and presented in the Results section (Lines 314-322 and Lines 420-435) and Discussion (Lines 570-614).

Also, as two-photon imaging mainly detects OL in cortical layers I-III, whereas in vivo electrophysiology preferentially concerns deeper layers, authors should at least discuss implications and possible limitations of their study (including possible involvement of effects throughout the visual path).

Thank you for raising this point also raised by **Reviewer #3, point 12**.. We have added a graph indicating the depth of units recorded from the Neuropixel probes (**Supplementary Fig. 12D**).

We expect demyelination in layers I-III to affect neuronal function in deeper cortical layers. In the visual cortex, as in all areas of mammalian neocortex, neurons in the deep cortex extend dendrites into the superficial layers of the cortex (Peng et al., 2021; Thomson & Lamy, 2007). Moreover, layer I of V1 serves as an important site of integration of feedback and local signals (Burkhalter et al., 2024), and neurons in superficial and deep layers of V1 are highly interconnected (Yao et al., 2023). Our study captures the degree of demyelination and remyelination of axons in layers I-III, which includes axons that are transmitting signals to dendrites of neurons in deeper cortical layers.

Furthermore, it is known that demyelination levels in deeper cortical layers are similar (Thornton et al., 2024) or even higher (Orthmann-Murphy et al., 2020) than that in superficial layers following cuprizone, while remyelination levels tend to be lower in deeper cortical layers (Orthmann-Murphy et al., 2020; Thornton et al., 2024). Overall, these measurements - which are a good proxy for myelin changes throughout the cortex - are likely to be an overestimation of myelin repair and have profound impacts neurons throughout cortical layers.

To expand our analysis beyond the visual cortex, we have now completed an extensive histological characterization of demyelination throughout the visual pathway, which is reported in our new **Supplementary Fig. 1**. We identified clear demyelination of the primary visual cortex and the underlying optic radiation, but we were unable to detect significant demyelination in the dorsal lateral geniculate nucleus of the thalamus or the optic nerve. Therefore, our acute cuprizone protocol triggers preferential demyelination of the posterior visual pathway, as previously suggested (Araújo et al., 2017). Our histological approach also confirmed that the visual cortex is affected across all cortical layers. We expanded our Discussion to include these points and discuss limitations of our study (Lines 542-557).

3) Finally, to provide more challenging conditions (and to possibly identify distinct longer-term recovery plateaus), it would be informative to combine LL-341070 treatment with (a more common) 6 weeks cuprizone exposure protocol.

We have elected to use a 3.5-week cuprizone protocol in order to focus our analysis on cortical de/remyelination while minimizing other damage to the CNS. In our new data, presented in new **Supplementary Fig. 1**, we find that our cuprizone protocol causes demyelination in the visual cortex and optic radiation but does not cause significant demyelination in more anterior parts of the visual pathway, including visual thalamus and optic nerve. Other studies show that 6 weeks of cuprizone induces substantially greater signs of axonal degeneration and glial activation than 3 weeks of cuprizone without inducing comparable increases in demyelination (Crawford et al., 2009), making longer cuprizone administration a less selective myelin manipulation. In future pre-clinical work, it will certainly be essential to test the role of LL-341070 in numerous demyelination models, but we feel that this is beyond the scope of this manuscript. We are optimistic of the generalizability of our findings across models given that other thyromimetics have be shown to augment remyelination in lysolecithin, 12-week cuprizone, and a genetic model of demyelination (Hartley et al., 2019) . We have now included a Discussion point highlighting the importance of assessing our findings in other demyelination models (Lines 533-540).

4) While oligodendrocyte gain during remyelination is induced by recent oligodendrocyte loss, the number of new oligodendrocytes formed per lost oligodendrocyte depends on the rate at which the oligodendrocytes were lost. According to Fig. 2H this only applies to low/moderate oligodendrocyte loss rates.

The reviewer is correct in observing that the **number** of new OLs formed per lost OL depends on the rate at which the oligodendrocytes were lost (**Fig. 2H**). At loss rates under approximately 1.5%/day, mice gain more than 1 new cell per lost cell (approx. 1.25 new cells/lost cell). However, at loss rates over 1.5%/day, mice gain less than 1 new cell per lost cell (approx. 0.75 new cells/lost cell at 2% loss/day, and approx. 0.5 new cells/lost cell at 4% loss/day). Importantly, even at these higher OL loss rates, OL gain rates still correlate with OL loss rates approximately one week prior. The OL gain rate is highly correlated with the OL loss rate approximately one week prior across all values of OL loss (**Fig. 2E** for example plot, all plots summarized in **Fig. 2F**).

What we are describing by observing that the “the **number** of new OLs formed per lost OL depends on the rate at which the oligodendrocytes were lost” is that the slope of the regression line of the between OL gain rate and OL loss rate is less than 1. We added text clarifying this point in the Results section (Line 180-181). A slope of 1 (aka the line of equality) is indicated by the black dotted line in **Fig. 2H**, and is visually helpful to demonstrate that at low rates of OL loss, as many new OLs are generated as are lost, but at high rates of OL loss, many fewer OLs are generated than are lost. This line is not a line of best fit for these data points. We added a label to this line on **Fig. 2H** and clarified it in the legend.

5) In Fig. 3H there seems to be no significant differences between groups in OL gain at 7 weeks. This seems to be at variance with data in Fig. 5F

This is correct as the datasets analyzed in each figure are different, resulting in the discrepancy in statistical results. To allow for a fair comparison of LL-341070 to clemastine-treated mice, we had previously combined the clemastine and the LL-341070 vehicles. The combined vehicle group had more mice than the LL-341070 vehicle mice alone, making statistical differences easier to detect, which is why some differences could be detected in **Fig. 5** but not in **Fig. 3**. Since this was also mentioned by **Reviewer #4**, we have now analyzed the LL-341070 and the clemastine datasets separately and adjusted the text accordingly. The clemastine data are presented in our new **Fig. 5** and reported in the Results (Lines 324-352) and Discussion (Lines 507-531).

6) In Fig. 6/7 presentation of data as 'super plots' with distinct color label of data points from individual mice would provide more information to the reader.

We have changed the presentation of the data to superplots in these figures.

7) In Fig. 7D) the emergence of shape distortion in high LL-341070 group at 7 weeks (especially when

This sentence was cut short in the reviewer's comments, but we believe the reviewer is asking about the emergence of significant shape distortion in the 0.3 mg/kg group (compared to healthy) in **Fig. 7D** at 7 weeks, which was not present at 3 weeks of remyelination (**Fig. 6J**). This is a great point and we think may have two explanations: 1) the shape distortion metric is calculated based on the VEP shape of healthy animals at the baseline timepoint (-3.5w, as indicated in **Fig. 6B**); thus, it is possible that VEP shape slowly changes with age and this is what we are detecting; 2) visual inspection of the VEP traces indicate that, at the 7 weeks timepoint, some animals demonstrate alterations to its shape in the form of sequential ripples (similar shape but lower magnitude as the first VEP wave) following the initial wave, which may indicate that the population activity is not quite the same as the pre-cuprizone activity. We now commented on this result in Lines 415-418.

8) Chaudhary et al. Thyroid hormone and thyromimetics inhibit myelin and axonal degeneration and oligodendrocyte loss in EAE. J. Neuroimmunol. (2021) should be referenced and discussed.

We have now compared our results to those of the cited manuscript in the Results section (Lines 217-220).

9) The paper would profit from a discussion on the distinct side effects/tolerability of clemastine versus LL-341070, considering similar long-term outcomes.

Given that LL-341070 remains in the pre-clinical stages, there is unfortunately no safety data available to share at this time. Of course, such considerations will be important as remyelination therapeutics move forward through FDA-approval processes. We modified our Discussion to comment about the safety of LL-341070 and other thyromimetics (Lines 510-514).

Reviewer #3 (Remarks to the Author):

The manuscript titled " Incomplete remyelination via endogenous or therapeutically enhanced oligodendrogenesis is sufficient to recover visual cortical function" authored by Nunes et al., is well written, topical and a technically rigorous study.
What are the noteworthy results?
Given that myelin loss causes brain dysfunction and is linked to neurodegenerative, understanding endogenous remyelination and developing treatments to restore neuronal function are critical. Although visual pathway abnormalities are among the initial symptoms experienced by individuals with MS, research on the impact of myelin loss, a key characteristic of MS, on visual function remains limited. Using in vivo two-photon microscopy and electrophysiology, the authors show that endogenous remyelination is triggered by recent oligodendrocyte loss and is very successful following mild demyelination, but less efficient after more prolonged exposure to cuprizone resulting in more severe demyelination. In comparison to clemastine fumarate, a new thyromimetic LL-341070 increases oligodendrocyte gain during remyelination and speeds up neural function recovery in the early phase, where clemastine exhibited slower but more prolonged effects, even after discontinuation. The therapeutic efficacy of LL-341070 only occurs after moderate to severe demyelination. Finally, the data shows that partial oligodendrocyte regeneration is enough to restore visual neuronal function as measured electrophysiologically.

Will the work be of significance to the field and related fields? How does it compare to the established literature? If the work is not original, please provide relevant references.
Yes. This study presents in vivo findings to describe a correlation between myelin loss and visual function. They authors propose that treatments promoting remyelination, such as the thyromimetic LL-341070, may facilitate functional recovery by boosting oligodendrocyte regeneration in conditions associated with myelin loss. Among the agents currently under investigation, LL-341070, a thyromimetic prodrug, stands out for its ability to enhance oligodendrocyte regeneration in experimental models. The design of this agent optimizes central versus peripheral exposure through selective activation upon contact with FAAH, expressed in the brain. LL-341070 also exhibits selectivity by targeting the thyroid hormone receptor beta. These features position LL-341070 as a promising candidate for advancing our understanding and potential treatment of myelin-related disorders, particularly in the context of multiple sclerosis. The findings of this paper add new insights into ongoing research on remyelinating therapies for multiple sclerosis, emphasizing the rationale for promoting myelin sheath repair for functional recovery.
Does the work support your conclusions and claims, or is additional evidence needed?
The author's work substantiates the presented conclusions and claims. However, some issues should be experimentally addressed and further discussion is needed to clarify the obstacles inhibiting remyelination in MS.

We thank the reviewer for their thorough review of our manuscript.

Critique:
- This study did not characterize how LL-341070 increases the generation of new mature oligodendrocyte. The author should discuss the potential source of the remyelinating cells. It has been demonstrated that OPCs first begin to accumulate in the demyelinating region 2–3 weeks after initiating the cuprizone intoxication. The applied drug LL-341070 might influence the differentiation rather than the proliferation of OPCs (or eventually both). Perhaps this question can be answered by imaging NG2-mEGFP line, track OPC migration, proliferation, differentiation, and death?

We thank the reviewer for this question. Given the well-documented role of thyroid hormone in promoting OPC differentiation through thyroid hormone receptor beta (TRβ) on OPCs (Baxi et al., 2014; Hartley et al., 2019), we expect the thyroid hormone receptor beta-targeting thyromimetic LL-341070 acts through a similar mechanism. We have now added new data to address this point. We confirmed the selectivity of LL-341070 for TRβ, which is presented in new **Supplementary Fig. 6A**. To test the ability of LL-341070 to induce OPC differentiation, we assayed OPC differentiation in cultured primary OPCs. We find that LL-341070 induces OPC differentiation in a dose-dependent manner. These new data are included as a new **Supplementary Fig. 6B-D.** This new figure is discussed in the Results section (Lines 209-212).

- Is the increased Oligo numbers following treatments due to the effect of treatment on the number of cortical OPC or recruited from nearby brain region?

There are two potential sources for new oligodendrocytes: 1) parenchymal OPCs dispersed throughout the brain that differentiate locally, 2) neural precursor cells in the SVZ that migrate to nearby regions and differentiate into OPCs that then generate oligodendrocytes. The contribution of neural precursors to remyelination is modest and most evident in region of the brain closest to the SVZ (Brousse et al., 2015; Butti et al., 2019; Moyon et al., 2023; Serwanski et al., 2018; Xing et al., 2014). Therefore, it is well-accepted that cortical oligodendrogenesis is mediated locally by parenchymal OPCs, with limited recruitment from nearby regions or from neural precursors from the SVZ. Importantly, the population of parenchymal OPCs in the cortex is not reduced by cuprizone (Baxi et al., 2017; Thornton et al., 2024) and, thus, there is no need for recruitment of OPCs from other brain regions.

- Does the remyelination modulate myelin sheath number per newborn oligos? It is unclear whether myelin sheath underwent remodeling during remyelination.
- The study showed that the magnitude of oligo loss and gain is not one to one. Although remyelination failed to restore oligodendrocyte number but seems to restore neuronal function. It is unclear whether the restoration of neuronal function can be attributed to an increase in sheath numbers generated by new oligodendrocytes?

We thank the reviewer for raising this important point also raised by **Reviewer #2, point 2**. We have included new data directly quantifying remyelination, presented in **Fig. 4G-J** and **Supplementary Fig. 10C, I**. We quantified the number of sheaths, length of sheaths, and total length of myelin made by new oligodendrocytes in vehicle-treated and LL-341070-treated mice during remyelination as well as in healthy

controls. We did not observe statistical differences in any metric of myelin levels between conditions. This new data is reported is reported in the Results section (Lines 305-314).

Using these measurements, we estimated total myelin levels in healthy and remyelinating mice and compared these levels to restoration of neuronal function. We find that mice treated with 0.3 mg/kg LL-341070 restore function at three weeks with myelin still well below healthy levels. These results are presented in new **Fig. 4J**, **Supplementary Fig. 10E**, and **Fig. 7E** and presented in the Results section (Lines 420-435) and Discussion (Lines 570-614).

Alternatively, it raises the possibility that existing mature oligodendrocytes might play a role in generating myelin, aligning with current evidence indicating the potential participation of mature oligodendrocytes in remyelination in rodent models (PMID: 37071991). This nuanced relationship between oligodendrocyte numbers, myelin generation, and neuronal function adds complexity to our understanding of the mechanisms underlying remyelination and warrants further investigation.

Mature oligodendrocytes that survive a demyelinating insult can form a few new myelin sheaths (Bacmeister et al., 2020; Mezydlo et al., 2023; Neely et al., 2022), but our laboratory has previously found that this does not occur after cuprizone alone (Bacmeister et al., 2020). Moreover, remyelination therapies (clemastine/metformin) were shown to be ineffective at promoting surviving oligodendrocyte remyelination (Mezydlo et al., 2023). Thus, although this is a relevant question, it is not to be expected that remyelination by surviving oligodendrocytes occurs in this model after endogenous or therapeutic-induced remyelination. Indeed, a cursory analysis did not find any examples. We did quantify the number of sheaths, length of sheaths, and total length of myelin made by new oligodendrocytes in vehicle-treated and LL-341070-treated mice during remyelination as well as in healthy controls. We did not observe statistical differences in any metric of myelin levels between conditions. This new data is reported is reported in the Results section (Lines 305-314).

- Axons become to become affected with longer exposures to cuprizone and I wonder if the authors can comment on drop out of axons affecting their measurements both at the tissue level and by EP, where pseudonormalization can occur by recording only remaining healthy axons. While less than optimal there are now some improved methods of measuring vision in mice.

We have now performed multiple assays to assess neuronal/axonal degeneration following our acute 0.2% cuprizone protocol. We used a new antibody that detects cleaved neurofilament (Degenotag^{TM}) (Shaw et al., 2023) and confirmed its utility in assessing axonal damage. We demonstrate that our protocol does not lead to evident axon degeneration in the primary visual cortex and in the optic radiation, the regions where we observed demyelination. We also did not detect any effect of cuprizone on the density of retinal ganglion cells. This new data is reported in **Supplementary Figure 2** and discussed in the Results and Discussion (Lines 94-96, and Lines 597-599). These results indicate that the health of neurons and axons is overall preserved in the visual pathway using our experimental cuprizone protocol.

- The study shows that only mice with low level of oligo loss regenerate their original oligo population after remyelination but mice with high level oligo loss exhibited a regeneration deficit. It is unclear why there is regeneration gap. Possible factors contributing to this gap could include a shortage in the pool of OPCs and the presence of reactive gliosis, and axonal pathology, as mentioned above. The authors should discuss these potential confounding factors. Addressing these uncertainties will be crucial for advancing our understanding of the mechanisms involved in oligodendrocyte regeneration after remyelination, ultimately informing potential therapeutic interventions.

We agree that the regeneration deficit specific to mice with high levels of OL loss is a fascinating finding and important for informing therapeutic interventions. We have expanded our discussion of the factors that may inhibit oligodendrogenesis and added a discussion of gliosis (Lines 487-491). Furthermore, we address that it is unlikely a shortage in the pool of OPCs, given that enhancing OPC differentiation using LL-341070 eliminated the recovery deficit at high loss levels (Lines 491-493). Similarly, this would suggest that this deficit is not due to any structural issues with axons, as they are able to be remyelinated when OPC differentiation is augmented. We edited the Discussion to clarify these points (Lines 472-495).

- Please discuss that cuprizone may not accurately model all of the inhibitory cues present in an MS plaque.

We have now added a new discussion paragraph discussing the importance of validating these findings in other brain regions and demyelination models, which may exhibit distinct biology (Lines 533-540).

- The study shows that the rate of oligo gain during remyelination is driven by recent loss. The authors mention the possibility of acute signaling that might occur around the death of oligodendrocytes, thereby inducing the formation of new oligos approximately one week later, but this section could be exapnded.

We thank the reviewer for raising this important point also raised by **Reviewer #4, point 2**. We are also very interested in which signals from oligodendrocyte loss stimulate oligodendrocyte production. Please see our revised discussion speculating as to the potential source of such a signal (Lines 459-470).

Are there any flaws in the data analysis, interpretation and conclusions? Do these prohibit publication or require revision?
- In figure 1 B and C, the optic nerve is only assesses at -3.5 and 0. A trend towards reduced myelination is seen and it would be important to examine at the later time point to settle this point of contention in the recent literature.

We have now performed an extensive analysis of demyelination throughout the visual pathway following our demyelination model (3.5 weeks of 0.2% cuprizone), including additional analyses in the optic nerve. These findings are summarized in a new **Supplementary Fig. 1** and discussed in Lines 91-94. We do not observe any evidence of demyelination in the optic nerve or visual thalamus, but confirmed that the visual cortex is demyelinated, as well as the optic radiation just underneath it. This points to a preferential demyelination of the posterior visual pathway following cuprizone, a finding that supports previous results from the literature (Araújo et al., 2017).

In Figure 3, panel 3E, although there is no significant difference in the mean lost oligodendrocytes between groups, the observed variability is notably high. Considering the established correlation that more oligo loss to lead to more gain, the one should be careful of this substantial variability in the extent of oligodendrocyte loss within the visual cortex after cuprizone intoxication. The discussion should address the factors contributing to the wide range of oligodendrocyte loss observed, ranging from 14% to 80% demyelination (line 110) at the 3-week cuprizone. similarly, high variability is shown in Fig 5C, where oligo loss is shown after 3 weeks cuprizone intoxication between treatment groups. Details regarding the experimental design should be added, such as the number of times the experiments were repeated independently to overcome the high variability in a small sample size (n).

Given the variability in loss between mice, we have corrected our data by the level of loss each mouse incurred. These methods have been clarified in the Results section (Lines 220-230) and Methods section “Statistics and modeling” (Lines 901-914). To summarize, we used an analysis of covariance with unequal slopes, wherein:

Dependent variable: oligodendrocyte gain
Independent variable: drug treatment
Covariate: oligodendrocyte loss
Interaction term: treatment x loss

Including loss as a covariate allows us to control for the high levels of variability in loss and still be able to test the effect of treatment on gain.

The reason for the high variability in oligodendrocyte loss in V1 between mice is unclear. In fact, even the mechanism by which cuprizone induces oligodendrocyte death has remained elusive (Morgan et al., 2022; Zirngibl et al., 2022). Although not a subject of investigation in this manuscript, we tested possible contributions to variability in loss, which are reported in our new **Supplementary Fig. 16**. We did not observe effects of sex on cumulative oligodendrocyte loss levels at 3 weeks following cuprizone (**Supplementary Fig. 16A**). Next, we hypothesized that the amount of cuprizone chow consumed by the mice may determine their loss levels. As a proxy for consumption of cuprizone chow (the mice’s only food source during the cuprizone period), we used the change in mouse body weight over the cuprizone period, with a decrease in body weight indicating less consumption of cuprizone chow. We did not observe any correlation between weight change during cuprizone and cumulative oligodendrocyte loss 3 weeks following cuprizone (**Supplementary Fig. 16D**). Finally, we assayed whether we saw differences in oligodendrocyte loss between cohorts, and found no changes (**Supplementary Fig. 16E**). A detailed

description of the number of replicate cohorts for the drug studies have now been added to the Methods section “Statistics and modeling” (Lines 888-893).

Finally, to increase our sample size, we have added an additional cohort to our *in vivo* imaging data, including one more animal from each group: vehicle, 0.1 mg/kg LL-341070, and 0.3 mg/kg LL-341070.

- Additionally, the impact of sex on LL-341070 therapy should be addressed. Understanding whether and how sex influences the response to treatment is crucial for the applicability and generalizability of the findings. Any observed differences in treatment outcomes based on sex should be discussed.

We agree that sex as a biological factor is a crucial factor to be considered and thank the reviewer for raising this point. To address this point, we analyzed our data using sex as an independent variable. We did not observe any effects of sex on the level of oligodendrocyte loss in mice from all treatment groups. Neither did we observe effects of sex on oligodendrocyte gain during endogenous remyelination or with 0.3 mg/kg LL-341070 treatment. These results are summarized in our new **Supplementary Fig. 16**. As the study was not designed to determine differences between sex, we are underpowered to statistically assess the role of sex in every condition and timepoint. However, sex was distributed between treatment groups, and we did not observe any evidence of sexual dimorphism that warranted deeper investigation. We edited the Methods to include these findings (Lines 623-625).

- In Supplementary Figure 4F and G, the remyelinating treatment with low dose LL-341070 (0.1 mg/kg) appears to worsen oligodendrocyte gain as compared to the vehicle. Please comment.

Supplementary Fig. 4F and **4G** (now **Supplementary Fig. 8F** and **8G**) represent the analysis of covariance with unequal slopes to compare the oligodendrocyte gain rates between treatment groups while correcting for oligodendrocyte loss and the interaction between oligodendrocyte loss and treatment. This involves performing a linear regression for each treatment group of gain rate versus loss, which is displayed in **Supplementary Fig. 4F** and **4G** (now **Supplementary Fig. 8F** and **8G**). The visual representation of this analysis is useful as we can see that across levels of oligodendrocyte loss (x axis), low dose LL-341070 actually appears to have similar or higher rates of oligodendrocyte gain than does the vehicle. To statistically compare the treatment groups (**Fig. 3J**), we must use Tukey’s HSD to compare the least square means (oligodendrocyte gain rate corrected by cumulative oligodendrocyte loss) derived from the linear regressions at the average level of oligodendrocyte loss. **Fig. 3J** shows that there is no statistical difference between the gain rates of the low dose and vehicle groups.

Is the methodology sound? Does the work meet the expected standards in your field?
The methodology is sound and meets the standards.

We thank the reviewer for this feedback.

Is there enough detail provided in the methods for the work to be reproduced?
- In the method section, it is written that cortical layer MOBP–EGFP image stacks were acquired in a volume of 425 µm × 425 µm × 336 µm; corresponding to layers I-III, 0–336 µm from the cortical surface (LINES 566-568). In Fig6A Neuropixel probes was inserted in the primary visual cortex. Please add an illustration to show where exactly the Neuropixel probes record single neuron activity. While two-photon microscopy records layer I-III. Please provide more details how these data are correlated if captured from different layers?

Thank you for raising this point also raised by **Reviewer #2, point 2**. Neuropixel probes span the entire visual cortex, capturing neuronal activity of all cortical layers, as described in our Methods section. We have added a graph indicating the depth of units recorded from the Neuropixel probes (**Supplementary Fig. 12D**).

We expect demyelination in layers I-III to affect neuronal function in deeper cortical layers. In the visual cortex, as in all areas of mammalian neocortex, neurons in the deep cortex extend dendrites into the superficial layers of the cortex (Peng et al., 2021; Thomson & Lamy, 2007). Moreover, layer I of V1 serves as an important site of integration of feedback and local signals (Burkhalter et al., 2024), and neurons in superficial and deep layers of V1 are highly interconnected (Yao et al., 2023). Our study captures the degree of demyelination and remyelination of axons in layers I-III, which includes axons that are transmitting signals to dendrites of neurons in deeper cortical layers.

Furthermore, it is known that demyelination levels in deeper cortical layers are similar (Thornton et al., 2024) or even higher (Orthmann-Murphy et al., 2020) than that in superficial layers following cuprizone, while remyelination levels tend to be lower in deeper cortical layers (Orthmann-Murphy et al., 2020; Thornton et al., 2024). Overall, these measurements - which are a good proxy for myelin changes throughout the cortex - are likely to be an overestimation of myelin repair and have profound impacts neurons throughout cortical layers.

To expand our analysis beyond the visual cortex, we have now completed an extensive histological characterization of demyelination throughout the visual pathway, which is reported in our new **Supplementary Fig. 1**. We identified clear demyelination of the primary visual cortex and the underlying optic radiation, but we were unable to detect significant demyelination in the dorsal lateral geniculate nucleus of the thalamus or the optic nerve. Therefore, our acute cuprizone protocol triggers preferential demyelination of the posterior visual pathway, as previously suggested (Araújo et al., 2017). Our histological approach also confirmed that the visual cortex is affected across all cortical layers. We expanded our Discussion to include these points and discuss limitations of our study (Lines 542-557).

Data arrangement and presentation in the manuscript:
- The organization and presentation of data in certain panels create confusion and can be challenging to follow. The manuscript benefit for clearly explaining these terms in a separated section, e.g. in methods and provide details (equation) how they were calculated. All the values below are presented as percentage and could be very confusing.
Lost or new OL (%), OL number (%), OL gain rate (%), Oligodendrocyte replacement (line 88) (%), Cumulative oligodendrogenesis (%).

We have now standardized our references in the figures and text and expanded on our description of these terms in the **Methods section** “Statistics and modeling” (Lines 916-923). We also removed any reference to “oligodendrocyte replacement”.

“To account for variations in baseline cell number, all oligodendrocyte metrics are reported as a percentage of baseline oligodendrocytes. The following metrics were derived from the raw oligodendrocyte counts:

$$Cumul.\,lost\ or\ new\ OLs\ (\%) = \frac{Cumul.\ no.\ of\ lost\ or\ new\ OLs}{No.\ of\ baseline\ OLs}$$

$$OL\ no.\ (\%) = \frac{No.\ of\ OLs}{No.\ of\ baseline\ OLs}$$

$$OL\ loss\ or\ gain\ rate\ (\%/day) = \frac{\left(\frac{No.\ of\ lost\ or\ new\ OLs\ from\ t1\ to\ t2}{No.\ of\ baseline\ OLs}\right)}{t2 - t1\ days}$$

“

- The text discusses some panels out of order, causing confusion for readers. For instance, between LINES 212 to 221, the explanation of panel F occurs after the discussions on panels G and H. Similarly, at line 112, Fig 1G is explained after Fig 1H. This arrangement makes it challenging for readers to follow the data logically. It is recommended to reorganize the text to ensure that the discussion aligns with the order of the panels, enhancing the overall clarity and facilitating a smoother comprehension of the presented data.

We have now reordered panels in the figures to ensure all panels are discussed in order. **Fig. 1F** and **G** (previously Fig. **1G** and **H**) have been swapped. **Fig. 3 F** is now first discussed with panels **Fig. 3D-F** (Lines 213-215).

- In Figure 3, Panel 3F, it would be more effective to present the data in a video format since each image was taken from a mouse in a separate group, making direct comparisons challenging. The statement "We found that mice treated with the high dose of LL-341070 gained substantially more new oligodendrocytes by 1.5 weeks as compared to mice treated with the low dose or vehicle" would benefit from visual representation using a video file, allowing for a dynamic presentation of the observed differences among the treatment groups on the generation of new oligodendrocytes over time.

We find that video format is actually more difficult to present this type of comparison than static figures. Dynamic representation of loss and gain in these mice can be observed in static images in **Supplementary Fig. 7.**

Reviewer #4 (Remarks to the Author):

Nunes et al. have submitted a paper that addresses the question of how remyelination after toxic demyelination occurs in the murine visual cortex, how this can be boosted by myelin-promoting drug candidates, and how this impacts some aspects of visual cortex physiology.
While the question addressed is important, and combining a remyelination therapy with a functional readout could be exciting, I have substantial concerns about some of the data and arguments that, in my view, preclude publication in this form.

We thank the reviewer for their considered review that has strengthened our manuscript.

General comments:

1) Throughout the paper, the authors seem to equate the restoration of OL cell body number with "remyelination", including in the title. Would this claim not require analyzing internodes, not OL numbers?

We thank the reviewer for raising this important point also raised by **Reviewer #2, point 2**, and **Reviewer #3, point 4**. We have included new data directly quantifying remyelination, presented in **Fig. 4G-J** and **Supplementary Fig. 10C, I**. We quantified the number of sheaths, length of sheaths, and total length of myelin made by new oligodendrocytes in vehicle-treated and LL-341070-treated mice during remyelination as well as in healthy controls. We did not observe statistical differences in any metric of myelin levels between conditions. This new data is reported is reported in the Results section (Lines 305-314).

Using these measurements, we estimated total myelin levels in healthy and remyelinating mice and compared these levels to restoration of neuronal function. We find that mice treated with 0.3 mg/kg LL-341070 restore function at three weeks with myelin still well below healthy levels. These results are presented in new **Fig. 4J**, **Supplementary Fig. 10E**, and **Fig. 7E** and presented in the Results section (Lines 420-435) and Discussion (Lines 570-614).

2) The authors' arguments in the first part of their paper are largely based on correlations resulting from the intrinsic variability of the initial demyelination in the area of observation (visual cortex). The sole reliance on curpizone as a demyelination tool is a limitation here. Cuprizone affects myelin in many parts of the CNS (and in very variable ways). Cuprizone can also be neurotoxic, e.g. Höflich et al., Brain Res 2016; Nyamoya et al., 2017 and references therein) and hepatotoxic, and its mechanism of action is somewhat cryptic. This is relevant for variance analysis of remyelination triggers (as there might be many hidden covariant factors) and for functional analysis (as there might be remote and off-target effects). Without an understanding of what causes the variability of demyelination (even whether this is a local or a global phenomenon for a given mouse), it is hard to ascertain that the drawn conclusions are the only possible ones. Even if one accepts the description that "OL loss rate a week prior" predicts current OL gain, in my view, this could still be compatible with a short and transient "homeostatic" signal that originates, e.g., from other cells that are supported by the dying cells and monitor one of their functions. My reading of the text seems to suggest, however, that the authors favor a view where the death of OLs (or cells in general) in itself somehow triggers a regenerative signal. This could be tested, e.g., by additionally killing additional cells, perhaps with other kinetics (OLs vs. other cells, e.g. astrocytes) and seeing how the signals add up (I assume that just killing some non-OIs would not induce any de novo myelination - which, if true, to me would suggest that at least some homeostatic signal is needed that determines the type, if not the extent, of the repair reaction). Overall, I think without finding the actual signals that trigger remyelination, I am not sure this part rises beyond a (albeit in my view valuable) description of some of the characteristics of the remyelination-inducing signal: It seems to be scaled to loss of OLs and relatively short-lived; but whether it is induced by the death of cells, or by demyelination of axons (or any other function/change in OIs that the system monitors) seems to me hard to conclusively answer from the data provided – and I would suggest a revision that tones down the more specific mechanistic claims if no additional evidence can be provided.

We thank the reviewer for raising this important point also raised by **Reviewer #3, point 8**. We agree that our data offer a valuable contribution to understanding important characteristics of the remyelination-inducing signal: it is time-locked to the loss of oligodendrocytes and proportional to the magnitude of that loss. We do not intend to project more insights than our data allows, and do not interpret our data to

indicate that the signal is the “death” of oligodendrocytes. We have now ensured to describe this signal as “signaling around the time of loss of myelinating oligodendrocytes” to include all possibilities the reviewer has described that may make up this signal. Specifically, in the Results section Lines 181-183, “death” has been changed to “loss”.

3) For remyelination-supporting therapy, the authors compare two established drugs: a thyromimetic (LL-341070, short “LL”) and clemastine fumarate (the current “gold standard” even though its effects are not fully understood). Both have in prior work been shown to support remyelination, but LL is the much less explored drug – in part because it is a company development by “autobahn therapeutics”, a company that is also listed among some of the authors’ affiliations (two co-authors hold/held company stocks according to a 2022 disclosure) and which funded the research. So, considering that the experiments amount to a “benchmarking” against a best-in-class drug, revealing conflicts of interest and ensuring transparency are important here (the company link is mentioned in the “competing interest” section, but the extent of the current financial involvement should be made clear).

We agree this is of the upmost importance. We have now clarified the role of Autobahn Therapeutics in the study under the “Author contributions” (Lines 1198-1213) and “Competing interests” (Lines 1215-1222).

In this context, it is unfortunate that the relevant key reference (Ref 54) is incomplete and – at least for me - hard to find (I assume it is https://doi.org/10.1212/WNL.98.18_supplement.3865, although the drug there has another name and it is really only an abstract/meeting supplement).

We apologize that this reference (a meeting abstract) was hard to find. The reference has now been removed and the relevant data, produced by Autobahn Therapeutics, has been included as new **Supplementary Fig. 6**. That these data came from Autobahn Therapeutics has been made transparent in the “Author contributions” (Lines 1198-1213) and “Competing interests” (Lines 1215-1222).

Also, the methods list that the experimenters were blinded “during drug administration” (L. 526) – I assume this to mean that the study was conducted as a completely blinded and randomized (at “0w”) study from animal assignment to end of analysis? I would ask the authors to clarify.

We have rewritten this section to clarify the blinding and randomizing protocol (Lines 646-657). To summarize, drugs were provided blinded to experimenters. Mice were allocated to blinded groups either prior to or during cuprizone treatment. In some instances, the level of oligodendrocyte loss during cuprizone was assessed prior to mouse allocation to attempt to ensure an even distribution of oligodendrocyte loss levels between blinded groups. Mice were unblinded following image or electrophysiological analysis.

Then, the number of data points that were included also needs to be made transparent. For instance, in Fig 4A – how many samples are there with 75% loss in the high dose group that supports the claim that LL “more than doubles” the OL gain. If I correctly read Fig. 3G, the sample size might actually be n=2 for this subgroup (1.5w, 75% loss, 0.3mg/kg), which supports a key conclusion. This would raise serious doubts, and I assume I am wrong (how could an S.E.M. be meaningful here?), but I cannot simply find the answer as to why this reading is not correct. In any case, it seems a bit concerning that the authors in this part of the study (Fig. 3 and 4) chose not to plot the data points, but rather resort to a measure of mean and S.E.M.; while with careful referencing some of this information can be deduced (or extracted from the legends), the casual reader might miss the actual sample size underlying some claims - after all only 6 mice were measured I this part of the study, not much for an analysis that in part is variance based. I find the study underpowered in this key experiment. While the main Figs 3 and 4 suggest a dose-dependent effect of LL, in Supp Fig 4, the picture is different – the low-dose group is all over the place, neither resembling the vehicle nor the high-dose (see, e.g., Supp Fig 4E), suggesting that here the analysis exceeds what the data can tell. In addition, some data are replotted and combined in a way that is not always transparent or even correct (see below).

For every analysis of covariance with unequal slopes, we have provided two graphical representations. First, all data points are plotted during the linear regression step, where gain vs. loss is plotted for each mouse. The graphical representation of this step can mostly be found in the supplementary figures (ex: **Fig. 1G**, **Supplementary Fig. 8A, B**). Second, the analysis of covariance calculates for each group their least square mean of gain (gain corrected by loss at the average loss value of all groups) and SEM, which is used then used to compare groups with post-hoc tests. The graphical representation of this step is typically

found in the main figures (ex: **Fig. 1H**). It is not possible to plot data points on these graphs as the group is corrected by loss as a whole – data points are not corrected individually. Importantly, both graphs are always referenced in the same location in the text, so the raw data points can easily be found. Additionally, every panel is referenced in the legend with the number of mice for each group. We have now rewritten the Methods section for "Statistics and modeling" to clarify our statistical analyses and graphical representations (Lines 901-914).

To increase our sample size, we have added an additional cohort to our *in vivo* imaging data, including one more animal from each group: vehicle, 0.1 mg/kg LL-341070, and 0.3 mg/kg LL-341070.

In **Fig. 4A**, to ensure our data analysis and the mice included therein is clearer, we have now sub-divided our data into mice that had < 50% loss and those with ≥ 50% loss by 1.5 weeks. We used a one-way ANOVA with post-hoc Tukey's HSD to test whether there are differences between vehicle, 0.1 mg/kg LL-341070, and 0.3 mg/kg LL-341070 treated mice in oligodendrocyte gain by 1.5 weeks. Consistent with our previous analysis, we found that 0.3 mg/kg LL-341070 induces an increase in oligodendrocyte gain but only in mice with high levels of loss (**Fig. 4A**).

As indicated below, we have revised our data analysis so that we are no longer replotting data to compare to clemastine (see revised **Fig. 5**).

4) While I find the Neuropixel recordings an interesting addition, I am not sure if this justifies the title that claims "recovery of visual function". What is measured here is not visual function but parameters of visual system electrophysiology. Claiming the extent of visual function recovery would need a more comprehensive analysis, including of the information content of the population response and behavioral read-outs.

While we agree with the reviewer that visual function likely includes visual information at the population level, and can include behavioral outputs, the measurements we present of the neural activity in the primary visual cortex are measures of visual function. Without this V1 activity humans report no visual experience as well as behavioral deficits. This brain electrical activity, which we show is altered and recovers, underlies visual function. We agree that a follow up study can examine information quantification and behavioral measurements inspired by the demonstration of visual functional recovery we show here. To make this topic more clear to the reader, we altered the text throughout to clarify that we are assessing neuronal parameters of visual function; for example:

Lines 453-454:
"Moreover, our data showed that even partial remyelination can restore metrics of visual neuronal function."

Lines 543-545:
"Thus, we utilized high-density extracellular in vivo recordings in a model with preferential posterior visual demyelination (**Supplementary Fig. 1**) to investigate the impact of demyelination on cortical visual neuronal responses."

Lines 570-571:
"After seven weeks of remyelination, animals treated with LL-341070 reached a plateau of functional recovery of visual response metrics similar to healthy levels (**Fig. 7A-D**)."

In any case, the extent of demyelination (and other pathology), spontaneous or induced repair along the visual pathway, are unknown. Similarly, which pathology actually explains the altered recordingsis also unclear. Thus a direct linking of the extent of remyelination in the observed small part of visual cortex and the degree of recovery in the recording parameters, which probe a large part of the visual system, including the retina, optic nerves and tracts, thalamus and subcortical white matter, seems not very meaningful.

Thank you for raising this point also raised by **Reviewer #2, point 2** and **Reviewer #3, point 12**. To expand our analysis beyond the visual cortex, we have now completed an extensive histological characterization of demyelination throughout the visual pathway, which is reported in our new **Supplementary Fig. 1**. We identified clear demyelination of the primary visual cortex and the underlying optic radiation, but did not detect demyelination in the dorsal lateral geniculate nucleus of the thalamus or the optic nerve. Therefore, our acute cuprizone protocol triggers preferential demyelination of the posterior visual pathway, as previously suggested (Araújo et al., 2017). Our histological approach also confirmed

that the visual cortex is affected across all cortical layers. We expanded our Discussion to include these points and discuss limitations of our study (Lines 542-557).

Furthermore, we have assessed other potential pathological changes in our mice. We have performed multiple assays to assess neuronal/axonal degeneration following our acute 0.2% cuprizone protocol. We used a new antibody that detects cleaved neurofilament (Degenotag^{TM}) (Shaw et al., 2023) and confirmed its utility in assessing axonal damage. We demonstrate that our protocol does not lead to evident axon degeneration in the primary visual cortex and in the optic radiation, the regions where we observed demyelination. We also did not detect any effect of cuprizone on the density of retinal ganglion cells. This new data is reported in **Supplementary Figure 2** and discussed in the Results and Discussion (Lines 94-96, and Lines 597-599). These results indicate that the health of neurons and axons is overall preserved in the visual pathway using our experimental cuprizone protocol.

We have now performed histological analyses and evaluated glial responses in animals treated with 0.3 mg/kg LL-341070 or vehicle for 3 weeks post-cuprizone. We did not identify any gross changes in glial reactivity in the primary visual cortex (V1). We found that area coverage by homeostatic microglia (labeled with P2RY12) and similar density of reactive astrocytes (labelled with GFAP) and total astrocytes (labelled with S100β) were found in vehicle or treatment groups. These data suggest that gliosis is unlikely to underly the effects of LL-341070 on neuronal function observed at this timepoint. This new data is reported in **Supplementary Fig. 9**, reported in the results section (Lines 239-240).

Specific comments:

Across all figures: There is replotting and reanalysis – this needs to be made transparent. For instance, Fig. 3D/E and 5B/C are in parts identical (LL high dose in 3E is LL in 5C and I suspect the controls are pooled, where some "vehicle" data points from 3E are also in the "vehicle" group from 5C). Same for 4D and 5G – and this probably then also applies to 3I, J and 5D, E – but here it is additionally confusing that the error bars (e.g., but not only, in the 1.5-3 w groups of 3J and 5D) are different. Overall, I recommend carefulyl checking the data and avoiding replots – Figures 3, 4 and 5 largely describe the same experiment and should be treated and plotted as such – with the two vehicle treatments clearly separated and all data points individually plotted. In the same spirit, identical subanalyses for clemastine should be included that were done for the LL high dose.

Thank you for this feedback. We have now analyzed the LL-341070 and the clemastine datasets separately and adjusted the text accordingly (see revised **Fig. 5**).

Fig 1A: It is my understanding that the schematic is not correct. Most mice were gavaged during early remyelination with "1% 1-methyl-2-pyrrolidone (Sigma 522 270458) and 1% Kolliphor HS 15 (Sigma 42966)" or a "10% DMSO solution" – see Supp Fig 5A. While the authors show that they cannot detect differences between these groups, this should still be indicated here.

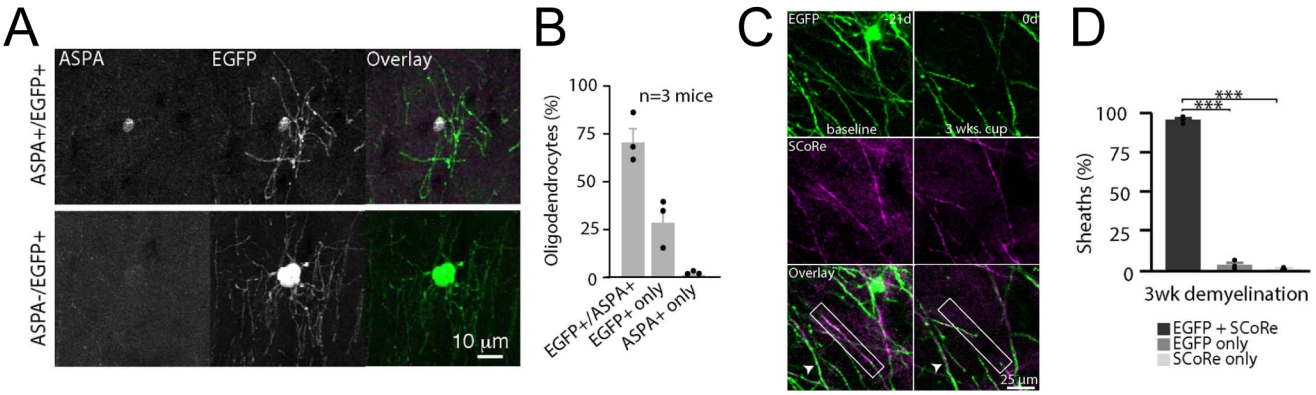
We adjusted in several places to reflect that many animals were treated with vehicle: the schematic in **Fig. 1A**; the legend ("Untreated and vehicle-treated mice were used for analysis of endogenous remyelination, as we did not observe differences between groups."); the Results (Lines 102-104); and the Methods (Lines 658-659).

Fig 1B/C: I am not convinced these data suffice to claim that the "myelin in the anterior visual pathway" is unaffected. For that claim, I would expect comprehensive structural data from all the myelinated parts of the prethalamic visual projections and higher resolution imaging not only of para-/nodal structures but also of myelin, including ultrastructure.

As described above in **Reviewer 4, point 4**, we expanded our analysis beyond the visual cortex to more of the visual path, we have now completed an extensive histological characterization of demyelination throughout the visual pathway, which is reported in our new **Supplementary Fig. 1**. We identified clear demyelination of the primary visual cortex and the underlying optic radiation, but we were unable to detect significant demyelination in the dorsal lateral geniculate nucleus of the thalamus or the optic nerve. Therefore, our acute cuprizone protocol triggers preferential demyelination of the posterior visual pathway, as previously suggested (Araújo et al., 2017). Our histological approach also confirmed that the visual cortex is affected across all cortical layers. We expanded our discussion to include these points (Lines 542-557). Our histological approach also confirmed that the visual cortex is affected across all cortical layers.

Fig. 1D/E: I suspect the authors have shown this before, but I want to be sure this also applies to this specific cuprizone dose and treatment length – are the oligodendrocytes that cannot be seen anymore really dead? Is Mobp-GFP expression insensitive to cuprizone? I appreciate that in Fig. 1E most cells that appear are in positions distinct from the original cells, but I want to make sure this is systematically true.

We have previously extensively characterized this (Bacmeister et al., 2020), and have included it here for ease of viewing in **Reviewer Fig. 1**. In summary, after 3-week-cuprizone demyelination, we found virtually no ASPA+ cells that were EGFP- (**Reviewer Fig. 1A, B**) and virtually no myelin visible by SCoRe imaging that was EGFP- (**Reviewer Fig. 1C, D**), indicating that EGFP expression is a reliable indicator of oligodendrocyte and myelin presence. The reviewer is correct in observing that new cells appear in different positions than lost cells – we observe this almost every time, similar to other groups (Orthmann-Murphy et al., 2020). Furthermore, we reliably observe that oligodendrocyte cell bodies shrink in size prior to loss, and newly generated oligodendrocytes cell bodies are very large and bright upon appearance. Thus, we are confident in our assessment of oligodendrocyte loss and gain in this model.



Reviewer Fig. 1: MOBP-EGFP faithfully captures oligodendrocytes and myelin. (A) Maximum projection of an EGFP$^+$/ASPA$^+$ oligodendrocyte (top) and an EGFP$^+$/ASPA$^-$ oligodendrocyte (bottom). Note the large size of the ASPA$^-$/EGFP$^+$ cell soma suggesting it is a recently born oligodendrocyte in the early stages of the maturation process. **(B)** After three weeks of cuprizone treatment, 70.73 ± 12.78% of oligodendrocytes are EGFP$^+$/ASPA$^+$, 0.97 ± 0.84% of cells are ASPA$^+$/EGFP$^-$, while the remainder are EGFP$^+$ only (n_{mice}=3, n_{cells} = 185), which are likely newly-generated oligodendrocytes in the early stages of the maturation process. **(C)** Maximum projections of oligodendrocytes showing colocalization of in vivo MOBP-EGFP and SCoRe imaging for myelin both before cuprizone administration (−21 days) and immediately following its removal (0 days). **(D)** Following 3 weeks of cuprizone diet, most myelin sheaths are MOBP-EGFP$^+$/SCoRe$^+$ (95.71 ± 1.16; ANOVA, $F_{2,6}$ = 2012.94, $p < 0.0001$) and almost no sheaths are SCoRe$^+$ and MOBP-EGFP$^-$.

Fig 1K: It might be worth considering that another interpretation of the results is that there are really two groups of mice – in which cuprizone has different effects. The segment of 30-60% loss is conspicuously empty. Could there be two groups of mice (males/females; weight, age, etc.) that differ biologically in terms of loss of oligodendrocytes and regeneration capacity?

We noticed this gap in loss levels as well in the untreated and vehicle-treated mice. However, we do find some mice with loss between 30% and 50% in the low dose LL-341070, high dose LL-341070 (Fig. 3E), and clemastine (Fig. 5C) groups, suggesting that loss levels are continuous not bimodal and suggesting the lack of mice in this range in the untreated and vehicle-treated groups may be due to random variability.

The reason for the high variability in oligodendrocyte loss in V1 between mice is unclear and this important point also raised **Reviewer #3, point 9**. In fact, even the mechanism by which cuprizone induces oligodendrocyte death has remained elusive (Morgan et al., 2022; Zirngibl et al., 2022). Although not a subject of investigation in this manuscript, we tested possible contributions to variability in loss, which are reported in our new **Supplementary Fig. 16**. We did not observe effects of sex on cumulative oligodendrocyte loss levels at 3 weeks following cuprizone (**Supplementary Fig. 16A**). Next, we hypothesized that the amount of cuprizone chow consumed by the mice may determine their loss levels. As a proxy for consumption of cuprizone chow (the mice's only food source during the cuprizone period),

we used the change in mouse body weight over the cuprizone period, with a decrease in body weight indicating less consumption of cuprizone chow. We did not observe any correlation between weight change during cuprizone and cumulative oligodendrocyte loss 3 weeks following cuprizone (**Supplementary Fig. 16D**). Finally, we assayed whether we saw differences in oligodendrocyte loss between cohorts, and found no changes (**Supplementary Fig. 16E**).

Supp Fig. 2F: While obviously the significance for the correlation between loss and gain gets lost at 7w, how certain are the authors that this is not just a question of power? This is only relevant because the authors cite this result as evidence that the gap between the low and high loss groups will not close later – which, in my view, it still well could. If the authors want to claim that eventually remyelination remains incomplete, they should simply image longer.

We have now also compared the rate of oligodendrocyte gain between remyelinating mice and healthy mice and found that the gain rate from 5-7 weeks post-cuprizone in all groups is not significantly elevated above healthy levels. These new data can be found in new **Fig. 4F**. These data also support the finding that the remyelination response has grossly ended by 7 weeks post-cuprizone, and mice have returned to healthy oligodendrogenesis rates.

The reviewer is correct that we are underpowered to detect small correlations in cumulative oligodendrocyte loss and oligodendrocyte gain from 5-7 weeks with n = 11 mice. With n = 11 mice, we are powered (α=0.05, power = 0.80) to detect a correlation with an $R2 \geq 0.47$, in range of what we have detected at other timepoints (**Supplementary Fig. 5A-E**). Thus, by not detecting a significant correlation in **Supplementary Fig. 5F**, we are confident in concluding that we lose the clear relationship between cumulative oligodendrocyte loss and oligodendrocyte gain rate that exists at earlier timepoints during remyelination. However, there may be a small correlation between these parameters that persists to 7 weeks of remyelination that is below our ability to statistically detect with our sample size. Thus, we have now performed calculations to estimate the period of time required for mice with high loss to recover their oligodendrocyte levels to those with low loss. Using the equation of the line of best fit in **Supplementary Fig. 5F**, we calculated the rate of oligodendrocyte gain for 75% loss and 25% loss (**Supplementary Table 1**). We assumed mice would continue adding cells at the same rate as 5-7 weeks of remyelination. Using these parameters, it would take 19.6 additional weeks for a mouse with 75% loss to have the same number of oligodendrocytes as a mouse with 25% loss (**Supplementary Table 1**). This estimate assumes that mice maintain the same levels of oligodendrogenesis as 5-7 weeks post-remyelination until they are approximately 9-months-old. However, given the radical decrease in oligodendrogenesis rates with aging (Wang et al., 2020), it is highly unlikely that mice will maintain these levels, realistically causing it to take even longer than 19.6 weeks – if ever – for mice with high loss to catch up. We have now updated the Results section reporting these findings (Lines 130-139).

Line 371: I am not sure how the authors arrive at "87%" as the threshold for functional recovery, but as I said before, I think it is not sound to correlate the recovery in layers I-III of a part of the visual cortex to an electrophysiological recording that probes the entire visual system. I do not see how such a claim could be proved without chronic recordings and then selectively re-ablating the observed "new" cells that supposedly mediate this recovery.

We restructured our Results and Discussion based on this and other comments and we have removed this threshold. We hope our new text offers a more balanced interpretation of our findings. We also provide additional analyses of demyelination in other areas of the visual pathway, and glial reactivity and axon degeneration in V1, which we hope complement our discussion about how the parameters of the of neuronal visual processing could be modulated in our study. Most critically, changes to superficial visual cortex neuronal signaling are expected to alter the parameters of visual function probed in this study. Rather than assigning a threshold of remyelination for functional recovery, our restructured manuscript makes the important observation the remyelination to healthy levels in superficial V1 is not required to restore visual responses.

Line 454: The third option could simply be assessed by OPC stainings.

Since there is a strong drive to maintain a constant OPC population (Hughes et al., 2013), fluctuations in the OPC population would be best measured using a method with high sensitivity and temporal resolution like longitudinal *in vivo* imaging. This is the focus of ongoing studies in the laboratory. We revised our

Discussion to include the additional possibility of a varied OPC response to demyelination via subpopulations (Line 526-527).

Reviewer #6 (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.

We thank this reviewer for their contribution to our manuscript.

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Response to Reviewer Comments: Della-Flora Nunes, Osso et al.

Reviewer #1 (Remarks to the Author):

The authors have addressed my concerns.

We thank the reviewer for their contribution to our manuscript.

Reviewer #2 (Remarks to the Author):

The authors have adequately addressed all my previous remarks and provided substantial additional data, which significantly strengthen the manuscript. This particularly refers to the quantification of remyelination and the characterization of oligodendrocyte dynamics along the visual pathway which substantially add to the manuscript. However, the newly included histological quantification of microgliosis and astrogliosis does not, in my view, sufficiently support the statement: “Neither astrocytes nor microglia were grossly affected by LL-341070, suggesting that a direct effect of LL-341070 on other glial cells is unlikely (Supplementary Fig. 9).” This conclusion is hampered by limitations in the analysis, which relies on a single homeostatic microglial marker and seems underpowered, with only n = 4 per group and a high variability (wide SD). Notably, the mean GFAP level is reduced by half in the treatment group, and the lack of statistical significance appears to result from the combination of high variability and low sample size. While the data on remyelination are now more convincing, the possibility of an immunomodulatory contribution cannot be excluded based on the evidence presented and this aspect should be adequately addressed and discussed.

We thank the reviewer for their contribution to our manuscript. We agree that some changes may occur in astrocytes and microglia that were not captured by our analysis. We have altered the statement in question to now read: “Gross changes in astrocytes or microglia were not apparent after LL-341070 treatment (Supplementary Fig. 9), though effects not captured by our analyses are possible.”

Reviewer #3 (Remarks to the Author):

The revisions are thorough and have addressed our concerns.
Marjan Gharagozloo and Peter Calabresi

We thank the reviewers for their contribution to our manuscript.