JCB

# Completion of neuronal remodeling prompts myelination along developing motor axon branches

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## Review Timeline:

| | |
|---|---|
| Submission Date: | 2019-12-05 |
| Editorial Decision: | 2020-01-21 |
| Revision Received: | 2020-11-20 |
| Editorial Decision: | 2020-12-18 |
| Revision Received: | 2020-12-25 |

*Monitoring Editor: Elior Peles*

*Scientific Editor: Tim Spencer*

## Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

**DOI: https://doi.org/10.1083/jcb.201911114**

January 21, 2020

Re: JCB manuscript #201911114

Prof. Thomas Misgeld
Institute of Neuronal Cell Biology
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Germany

Dear Prof. Misgeld,

Thank you for submitting your manuscript entitled "Completion of neuronal remodeling prompts myelination along developing motor axon branches". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that although all three reviewers find the study to be interesting and timely, they each raise a number of substantive issues that will need to be resolved before the paper would be suitable for publication in JCB. We hope that you will be able to address each of the reviewers' concerns in full in a revised manuscript.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

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The typical timeframe for revisions is three months; if submitted within this timeframe, novelty will not be reassessed at the final decision. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Elior Peles, PhD
Monitoring Editor
Journal of Cell Biology

Tim Spencer, PhD
Executive Editor
Journal of Cell Biology

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Reviewer #1 (Comments to the Authors (Required)):

In this paper, Wang and colleagues examine the relationship between myelination and the completion of activity-dependent competition for synaptic innervation of the neuromuscular junction (NMJ). The NMJ has been used as a model to study the reciprocal signaling between neurons and muscle cells as they form the precise molecular specialization of the NMJ. While much is known about the neuron-dependent cues that control NMJ and muscle differentiation, much less is known about the cues that drive presynaptic specializations. Among these presynaptic specializations are myelination. Since myelin remodeling has been proposed in the CNS, clues for how this happens during synapse refinement at the NMJ may provide important conceptual and even mechanistic insights for the CNS. Thus, this is a significant problem.

The authors bring a variety of experimental approaches to investigate the problem. First, they show that myelin preferentially occurs on 'winner' branches. This is not surprising but an important observation. As another surrogate measure of branch maturity, the authors examine node formation. They find the same result: that winner branches have more stable nodal components. The question is: why do winner branches become preferentially myelinated and differentiate? Before answering this question the authors show that myelination does not simply occur because of a larger axon diameter, and they show that myelination onset did not correlate with synaptic territory.

The authors then blocked NMJ activity and showed this impaired synapse pruning (i.e. competition) and this also delayed myelination. Thus, NMJ activity is critical for the winner axon to become myelinated. To further define how this happens, the authors show that the maturity of the

microtubule network and vesicle transport are critical. They show that stabilizing microtubules inhibits branch removal and promotes myelination and axon differentiation (more nodes). Inhibition of axonal transport using a dominant negative kinesin-1 heavy chain impaired myelination of the winner axon. The authors suggest that NRG1 type III may be one of the factors that is transported to promote myelination. They show that overexpression of NRG1 type III also promoted remodeling, myelination and node of Ranvier formation - but this occurred in both doubly- and singly-innervated axon branches. Overexpression of NRG1 type III also promoted pErk signaling in Schwann cells along winner branches.

The results presented are very well done and are compelling. Conceptually, this paper provides evidence that neuronal activity driven branch refinement also eventually drives glial differentiation and myelination. Thus, this is a great example for how activity-dependent NMJ-derived signals promote presynaptic differentiation. Although the precise nature of these muscle-derived signals are not identified here, the results are nonetheless important. With these positive comments in mind, I suggest a few additional questions that should be answered or addressed:

1. The result showing overexpression of NRG1 type III promotes refinement (fig. 5F) is somewhat confusing because it implies that faster myelination can promote refinement. This is opposite to what they claim in Fig. 3 - that myelination does not affect competition. From line 139: "Together, the data suggest a unidirectional relationship with ongoing axon remodeling delaying axon-glial maturation, but not the reverse." Fig. 5F shows that promoting myelination by overexpression of NRG1 type III expression promotes branch removal and refinement. The authors should address this apparent inconsistency.
2. Several papers suggest that NRG1 promotes NMJ differentiation/stability. How do the authors uncouple the effect of NRG1 signaling on Schwann cells from NRG1 signaling on the NMJ itself?

Reviewer #2 (Comments to the Authors (Required)):

This is an interesting study that demonstrates myelination is coordinated with motor axon branch elimination and suggests this coordination results from accelerated anterograde transport and expression of promyelinating signals, e.g. neuregulin (NRG), into the remaining axon branch. The foundational observation that myelination is correlated/coordinated to terminal branch elimination is convincing and has broader implications for synching myelination with axon remodeling. However, the remainder of the study, which includes a lot of interesting data, has some real gaps and makes some assumptions that if filled in, would make the overall study stronger and more compelling.

In particular, the notion that anterograde transport in the DIN branches is rate limiting for expression of a pro-myelinating signal/NRG is at best indirect. In marshalling support, the authors show increasing microtubular mass by targeting spastin accelerates myelination in both SIN and DIN branches (Fig. 4H-N) and that blocking transport inhibits myelination of both SIN and DIN branches (Fig. 5 B/C). However, it would be more convincing and more direct if they demonstrated that transport rates in the SIN (or emergent DIN winner) are in fact greater than in the DIN and if increasing tubulin III by cKO of spastin in fact increases transport rates. In the case of the spastin KOs, it would also be useful to know if the DIN branches increase their diameter (as potentially hinted at in Fig. 4L) or not - as this might contribute.

The data that NRG1 is deficient in the DIN branches and therefore underlies the delay in myelination is plausible but not compelling. Increased NRG1 levels in the transgenics that promotes

myelination in all branches (Fig. 5G) supports this but could also increase myelination by an independent mechanism. Direct evidence that NRG is rate limiting in the DIN branches is limited. NRG1 is both a mitogen and a differentiation signal and a deficiency of NRG1 on DIN vs SIN branches might manifest as a reduced density of Schwann cells in DIN branches - Schwann cell density would be useful to document either way. While staining for NRG is challenging, antibody staining might prove helpful; an alternate potential option is to use erbB-Fc binding/staining as a readout for expression on the axons. pERK staining in Fig. 5 is not necessarily a specific readout for NRG. The increase in pERK staining itself shown in panels 5I and 5J is not entirely convincing and appears blotchy rather than cytoplasmic or nuclear. A double stain with a Schwann cell marker would be useful to show that this staining is in fact within overlying Schwann cells. Finally, if spastin cKOs work by increasing transport and NRG levels, some increase in pERK (or NRG staining) should be evident.

Fig. 1D shows a decrease of doubly innervated NMJs from ~ 40% to 0% from P7 to P11 with a concomitant increase of Caspr+ myelin segments on single branches from ~ 10% to almost 80%. This would indicate that at P7 10% of the 60% of SIN branches have myelin whereas by P11, about 80% of such branches have myelin. If this is correct, it indicates there is a significant lag in myelination of such branches and the temporal correlation is not as tight as implied, presumably due to a lag in axon maturation. The authors should comment/discuss.

Minor points the authors should consider:

Fig. 2 shows recovery from FRAP is faster on DIN and is taken as sign of node immaturity consistent with their data showing delayed appearance. Might this difference reflect that there are more heminodes on the DINs vs nodes on the SIN as that might also account for different recovery times?

Fig. 3D: seems to show an increase in the axon diameter by territory for the unmyelinated branches but not for the myelinated - the authors may wish to comment/discuss on this difference. It is also unclear what the double asterisk and associated p value correspond to

On line 199, the length measure for the transport rates are missing, e.g 1.8 ?/min

Reviewer #3 (Comments to the Authors (Required)):

The manuscript by Wang et al, attempts to answer a long-standing question in the field - does myelination cease axonal remodeling, or does the cessation of axonal remodeling allow for myelination to occur? The data presented makes a case for the latter. The authors nicely describe how myelination coincides with the end of axonal branch competition in the PNS neuromuscular junction (NMJ) and then show that they can extend the competition period by blocking AChRs with bungarotoxin (BTX) or preventing the cleavage of microtubules along branches by knocking out spastin. However, while BTX treatment does lead to decreased myelination, the spastin KOs show increased myelination. The authors resolve the discrepancy by showing that these manipulations have differential effects on microtubular mass, BTX reduces it while knocking out spastin increases it, and they claim that the changes in myelination observed must occur via these cytoskeletal changes. They claim that the degree of myelination observed correlates with increased microtubule mass, which is not clearly shown, and go on to show that disrupting transport along microtubules, using a dominant negative kinesin-binding domain approach, also delays myelination. Finally, the

authors surmise that the transport of Nrg1 type III, which is well-known to regulate myelination in the PNS, must be limited until the end of branch competition and show that overexpression of Nrg1 type III in competing branches promotes earlier myelination. In the end the authors present a story where the maturation of axonal branches, as reflected by increased microtubule mass, induces myelination through increasing transport of Nrg1 type III. However, several pieces in this story are not clearly connected and much remains to be shown. My major comments/concerns are outlined below.

1) If the authors claims are correct, the experiments in Figure 4 that delay competition, BTX treatment and spastin KOs, should also reveal changes in Nrg1 type III that reflect the microtubule and myelination changes observed. Can the authors show that Nrg1 type III levels are reduced following BTX administration and increased in the spastin KOs?

2) The authors use bIII-tubulin fluorescence staining intensity to measure increases in microtubular mass, however fluorescence staining intensity is often not quantitative and the authors need to show that the changes in fluorescence intensity that they are measuring do indeed reflect changes in microtubular mass. They cite a previous paper (Brill, 2016) where they show that decreases in bIII-tubulin staining intensity of retreating axon branches reflect decreases in microtubule numbers seen on EM images, but it is not clear whether the differences in bIII-tubulin staining intensity they currently see between competing branches and winner branches reflect increases in microtubules. Is this a linear relationship?

3) The authors mention that increased microtubule mass indicates a more mature cytoskeleton with increased axon transport. Can the authors show that axon transport is increased in winner branches over competing branches? Specifically transport of Nrg1 type III?

4) In the discussion, the authors state that their work reveals an activity-dependence of myelination in the PNS (lines 270-271), but this is a stretch. The authors do not show any activity-dependence of myelination.

5) In Figure 3 the authors measure axon diameters by measuring the entire terminal branch area then dividing it by the length of the branches, however this is a very crude estimate and does not reflect actual diameter measurements, especially since diameters can vary greatly along the same branch (see images in Figure 3E,F).

6) In Figure 4 the authors show a large decrease in Caspr+ branches following BTX administration. Can this be due to non-specific effects on Schwann Cells (SC)? Is there a decrease in SC numbers?

7) In Figure 4K, the authors show that there is no increase in Caspr+ branches in sin spastin KO branches, but there is an increase in bIII-tubulin (Figure 4M). If increased microtubular mass promotes myelination, why isn't there an increase in sin Caspr+ branches in the spastin KOs?

8) In Figure 5H-K, the authors attempt to measure Erk signaling in SCs, but since SCs are not being identified, it is not clear where the signal they are measuring is originating.

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To

**Prof. Elior Peles**, PhD, Monitoring Editor
**Tim Spencer**, PhD, Executive Editor
*The Journal of Cell Biology*

Munich, November 20th 2020

**Resubmission of JCB manuscript #201911114**
**"*Completion of neuronal remodeling prompts myelination along developing motor axon branches*"**

Dear Drs. Peles and Spencer,

Thank you very much for reviewing our manuscript and conveying the Reviewers' assessment. We were pleased to see that you and the Reviewers found our work in principle suitable for *The Journal of Cell Biology*, and that would consider a revised version of the manuscript.

We have now completed all the revisions that we discussed in July. We are very satisfied that – not least thanks to your insistence on providing a direct visualization of Nrg1 – we found a way to immunostain for HA-tagged Nrg1 in situ (the ErB fusion proteins did not work in our hands). By this, we are able to substantially corroborate our suggested mechanism, namely that neuregulin is preferentially delivered to singly innervating axon branches, compatible with the view that this differential delivery underlies the different myelination probability between competing vs. 'winner' branches.

With these new insights, as well as the comprehensive revisions and substantial additional data outlined at the beginning of our point-to-point response, we feel that we have improved the study considerably.

We really appreciate the constructive comments of the Reviewers and your helpful guidance. We hope that you and the Reviewers will now find our work suitable for *The Journal of Cell Biology.*

With best regards,

Dr. Monika Brill  Prof. Thomas Misgeld

## Editor:

**E.1**: "[…] We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here. You will see that although all three reviewers find the study to be interesting and timely, they each raise a number of substantive issues that will need to be resolved before the paper would be suitable for publication in JCB. We hope that you will be able to address each of the reviewers' concerns in full in a revised manuscript. […]"

Many thanks to you and the Reviewers for the overall positive assessment of our work and the constructive and helpful comments. As detailed above, we have in the meantime been able to address the Reviewer's key concerns by performing numerous new experiments and analyses, and by thoroughly revising the text and figures.

Specifically, we have added the following new data (the corresponding panels are also shown in the responses to Reviewers' comments below; additional 'Reviewer figures' are added to the letter where we answer more specific or technical questions by the Reviewers, which in our view would not add to the flow of the manuscript):

**Fig. 3D, E**: Caliber of retraction bulb-tipped axon branches related to local myelination (local absence/ presence of MPZ immunostaining).

**Fig. 5B**: Transport of Nav-GFP$^{+}$ particles in *Thy1*-Nav-GFP mice.

**Fig. 6E, F**: Innervation status and node formation in *Thy1*-Nrg1 type III mice at P7 in addition to P9.

**Fig. 7**: Staining of HA-tagged Nrg1 on terminal axon branches of *Thy1*-Nrg1 type III mice and pAKT staining (to complement pERK) around 'din' and 'sin' branches. Effect of BTX blockade on Nrg1-HA content and pERK signalling. Postsynaptic development in *Thy1*-Nrg1 type III mice.

**Fig. S2**: Schwann cell number along terminal axon branches after BTX blockade. Tubulin content and Schwann cell number related to myelination and innervation status.

**Fig. S3D**: Axon branch caliber in control vs. spastin cKO axons

**E.2**: "[…] Text limits: Character count for an Article is < 40,000, not including spaces. […] Figures: Articles may have up to 10 main text figures. […] Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. […] A summary of all supplemental material should appear at the end of the Materials and methods section. […] When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript. […]"

The manuscript conforms to the editorial guidelines. Major changes are highlighted in yellow. Please find our point-by-point responses below.

## Reviewer #1:

**R1.0**: "[…] While much is known about the neuron-dependent cues that control NMJ and muscle differentiation, much less is known about the cues that drive presynaptic specializations. Among these presynaptic specializations are myelination. Since myelin remodeling has been proposed in the CNS, clues for how this happens during synapse refinement at the NMJ may provide important conceptual and even mechanistic insights for the CNS. Thus, this is a significant problem. […] The results presented are very well done and are compelling. Conceptually, this paper provides evidence that neuronal activity driven branch refinement also eventually drives glial differentiation and myelination. Thus, this is a great example for how activity-dependent NMJ-derived signals promote presynaptic differentiation. Although the precise nature of these muscle-derived signals are not identified here, the results are nonetheless important."

We thank the Reviewer for the kind assessment of the novelty and interest of our study. We concede that we did not undertake an effort to identify the muscle-derived signals that likely govern the presynaptic maturation of the axon-glial unit during and after remodeling – this is a long-standing problem, and while some progress has emerged over the past years, no single dominant factor has been identified. So this remains a fascinating and open question as the Reviewer points out. However, it is not the topic we chose to address here, as we are more concerned with the axon-to-glia signaling that is initiated, once the competition at the tripartite synapse is resolved.

**R1.1**: "The result showing overexpression of NRG1 type III promotes refinement (fig. 5F) is somewhat confusing because it implies that faster myelination can promote refinement. This is opposite to what they claim in Fig. 3 - that myelination does not affect competition. From line 139: "Together, the data suggest a unidirectional relationship with ongoing axon remodeling delaying axon-glial maturation, but not the reverse." Fig. 5F shows that promoting myelination by overexpression of NRG1 type III expression promotes branch removal and refinement. The authors should address this apparent inconsistency."

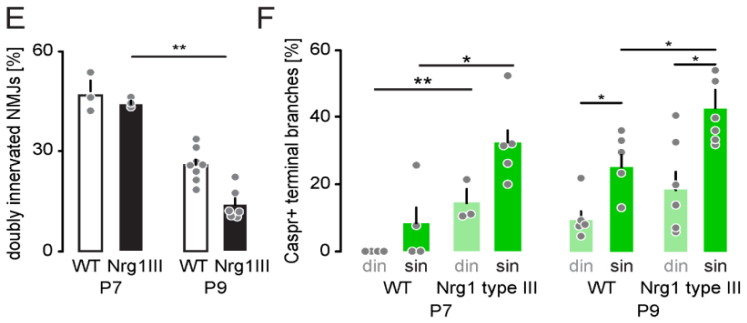
The Reviewer addresses an important point, referring to our data on *Thy1*-Nrg1 type III transgenic mice. Indeed, our interpretation of this result is premised on the previous observations in wild-type animals that we have obtained with physiological Nrg1 type III levels. Here, we find clearly that myelination *does not* interfere with remodeling and does not *per se* convey a competitive advantage (see data in original **Fig. 3 C**).

In the artificial Nrg1 overexpression situation, secondary effects emerge that explain the apparent discrepancy – and that have been described previously by Wes Thompson's lab. Indeed, our observations on the *increased* competition speed in *Thy1*-Nrg1 type III is in line with *Lee et al. Proc. Natl. Acad. Sci. 2016*, which reports acceleration of synapse elimination in different muscles (cf. Fig. 2, *soleus* and *sternomastoid*). *Lee et al.*'s work explains the effects of Nrg1 type III on synapse elimination with a hyper-activated state of terminal Schwann cells that invade the synapse, without any resort to collateral hypermyelination. But even if ascribed to axonal rather than synaptic effects, the synapse elimination phenotype of the neuregulin overexpression certainly further refutes the hypothesis that myelination would 'freeze' axons in place and hence would delay or even abolish axonal remodeling.

So in the context of our work, the *Thy1*-Nrg1 type III mice solely test the question, whether competing axons are fundamentally non-receptive to myelination, or whether a sufficiently strong pro-myelination signal can overcome the physiological delay. Moreover, in a newly added data set, we also use these mice to visualize Nrg1 distribution. To highlight the non-physiological nature of the *Thy1*-Nrg1 type III phenotype, which due to the *Thy1*-promotor activity profile have artificially high Nrg1 levels only late postnatally (see *Lee et al., 2016*), we now added synapse elimination and myelination onset data from postnatal day (P)7 (**Fig. 6E, F**). These show that the number of doubly innervated neuromuscular synapses is initially not

altered, so that the accelerated synapse remodeling only becomes apparent later. Thus, this acceleration at least does not appear to be due to general prematurity of the axon-glial unit.

We have now revised the **Discussion** to be upfront about the issue raised by the Reviewer and to make our thoughts on the neuregulin overexpression effects and its resolution by prior work more transparent (in addition, the new time-course data are now included in **Fig. 6E, F**).




**New data in Fig. 6E, F: (E)** Polyinnervation at postnatal day (P)7 and P9 in wild-type (WT) vs. *Thy1*-Nrg1 type III mice (n ≥ 3 mice per genotype, ≥ 99 axons per animal). **(F)** Quantification of Caspr-positive terminal branches in P7 and P9 WT vs. *Thy1*-Nrg1 type III mice (n ≥ 3 mice per genotype, ≥ 40 axons per animal; **, P < 0.01, Mann-Whitney test).

**R1.2**: "Several papers suggest that NRG1 promotes NMJ differentiation/stability. How do the authors uncouple the effect of NRG1 signaling on Schwann cells from NRG1 signaling on the NMJ itself?"

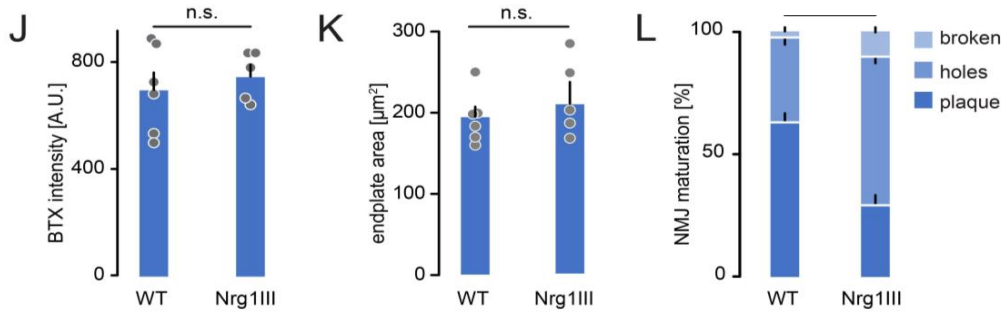
Again the Reviewer raises an important point related to the previous issue, and is certainly aware of the challenges that have in the past surrounded the attempts of assigning a clear role to specific cellular sources of Nrg1 in the postsynaptic development of the NMJ.

In a nutshell, we have no realistic means to exclude indirect signaling e.g. a scenario where axonal Nrg1 at the preterminal would influence terminal Schwann cells, which then signal backwards to the axonal Schwann cells in the chain sheathing one of the inputs. This would have to involve a complex system, as we have previously shown that at the NMJ no single terminal Schwann cell is assigned to the individual inputs that innervate a junction (*Brill et al. J. Cell Biol. 2011*); but the prior history of neuromuscular junction development has certainly taught the lesson that the developmental processes do not have to conform to our sense of simplicity.

The central problem here is that for terminal (vs. axonal myelinating) Schwann cells no specific tool exists for cell or gene ablation. Being aware of this limitation, we have tried to remedy this problem over the years that we have been working on this project. Based on an intriguing insertional effect that selectively labels terminal Schwann cells with a neuronal promotor (*Thy1*-Brainbow-1.0 line B in *Livet et al. Nature 2007*), we have mapped the insertion and tried to generate a CreER knock-in to be able to selectively deplete terminal Schwann cells or generate a selective deletion e.g. of Erb receptors. Unfortunately, this effort has proven unsuccessful as we never managed to generate the desired knock-in.

So, while we cannot give a direct experimental answer to the Reviewer's question, we still felt that it would be necessary to characterize the effects of altered neuregulin signaling on postsynaptic and glial development in more detail, especially as also Reviewer 2 (R2.2) raised a related point (about Schwann cell proliferation). To determine effects of neuregulin signaling on postsynaptic maturation at the NMJ in the *triangularis sterni* muscle, we now measured (1) acetylcholine receptor (AChR) distribution (along the 'plaque-to-pretzel' transition as a

measure of maturation; *Marques et al. J. Neurosci. 2000*), (2) AChR density and AChR area (as a measure of postsynaptic responsiveness to neurotransmission) in *Thy1*-Nrg1 type III mice compared to littermates on P9. While we find that the shape of the postsynaptic membrane changes prematurely in line with accelerated synaptic remodeling via a contribution of terminal Schwann cells (as proposed by *Lee et al. Proc. Natl. Acad. Sci. 2016*, see R1.1, above), the density of acetylcholine receptors (quantified by bungarotoxin, BTX intensity), as well as the area of the postsynaptic membrane at the NMJ, did not differ between wild-type and Nrg1 transgenic littermates, suggesting that the effects are likely not mediated via increased muscular responsiveness. Together these results confirm that in *Thy1*-Nrg1 type III mice changes in terminal Schwann cells accelerate synaptic remodeling, but that there is no generalized pre-maturity. These results underscore the point that the Reviewer raised, which we now address in more detail in the **Discussion section**; the new data are contained in the new panels of **Fig. 7J–L**.




**New panels of Fig. 7J–L:** Postsynaptic maturation of wild-type (WT) vs. *Thy1*-Nrg1 type III littermates at P9 (n ≥ 5 mice per group, ≥ 14 NMJ per animal). *, P < 0.05, Mann-Whitney test.

### Reviewer #2:

**R2.0**: "This is an interesting study that demonstrates myelination is coordinated with motor axon branch elimination and suggests this coordination results from accelerated anterograde transport and expression of promyelinating signals, e.g. neuregulin (NRG), into the remaining axon branch. The foundational observation that myelination is correlated/coordinated to terminal branch elimination is convincing and has broader implications for synching myelination with axon remodeling. However, the remainder of the study, which includes a lot of interesting data, has some real gaps and makes some assumptions that if filled in, would make the overall study stronger and more compelling."

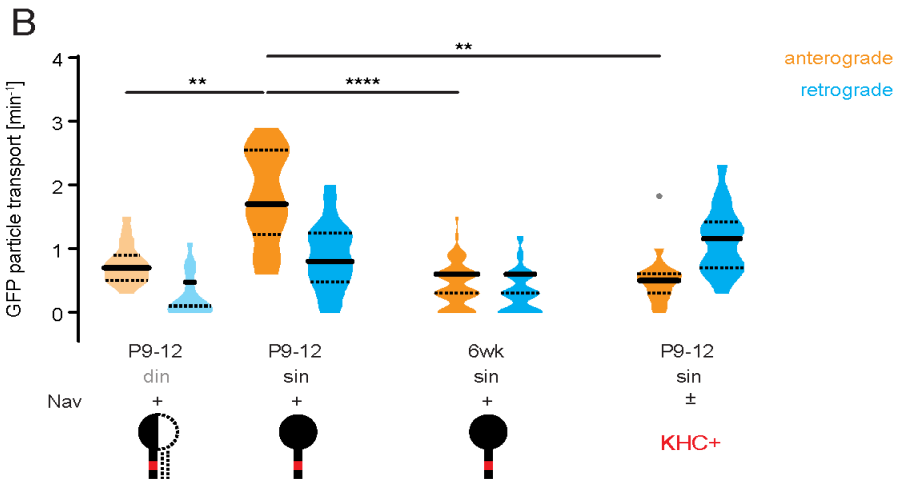
We thank the Reviewer for the positive assessment of the topic, significance of our study and the interest of our reported data. The Reviewer rightly points out some gaps in the mechanistic analysis in the second half of our manuscript. Guided by the Reviewer' critique, we performed a number of experiments and analyses to fill these gaps, as detailed below.

**R2.1**: "In particular, the notion that anterograde transport in the DIN branches is rate limiting for expression of a pro-myelinating signal/NRG is at best indirect. […] it would be more convincing and more direct if they demonstrated that transport rates in the SIN (or emergent DIN winner) are in fact greater than in the DIN and if increasing tubulin III by cKO of spastin in fact increases transport rates.

In the case of the spastin KOs, it would also be useful to know if the DIN branches increase their diameter (as potentially hinted at in Fig. 4L) or not - as this might contribute."

We agree with the Reviewer that knowing whether transport rates in competing 'din' branches are indeed lower than in victorious 'sin' axons, would be an important aspect of our mechanistic analysis that we failed to provide. We had actually measured this in previous work, where we characterized mitochondrial transport during axonal remodeling (*Brill et al. Neuron 2016*) and apologize for not referring to this more clearly. As shown in *Brill et al. Neuron 2016*, Fig. S1B, axonal transport of mitochondria correlates to the synaptic territory an axon possesses. Moreover, 'din' branches (1–99% synaptic territory) have substantially less transport than 'winner' ('sin', 100% synaptic territory) branches.

Still, mitochondria are perhaps not a very relevant cargo in this context. To also address the trafficking of axonal cargos directly related to myelination, we measured transport of Nav-GFP$^+$ particles in 'winner' ('sin') vs 'competing' ('din') branches in *Thy1*-Nav-GFP transgenic mice. Indeed, for these particles, anterograde transport is increased in 'sin' vs. 'din' branches once they have initiated myelination (**new Fig. 5B**). Notably, the anterograde delivery of such particles ceases in adult mice, in line with our original FRAP experiments (**Fig. 2**) – and is absent in branches that have not initiated node formation. These additional data are now integrated into the new **Fig. 5B**.



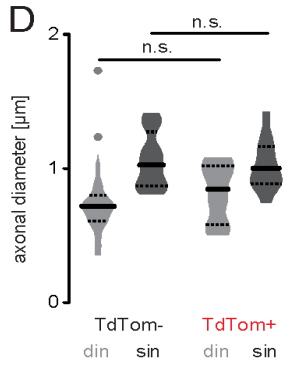

**New panels in Fig. 5B:** Antero- and retrograde transport of Nav-GFP+ particles in *Thy1*-Nav-GFP mice (n ≥ 16 axons, ≥ 5 mice per group; **, P < 0.01; ****, P < 0.0001, Mann-Whitney test. Outlier determined by Tukey test).

Further, the Reviewer asked the interesting question, whether increased βIII-tubulin content would lead to higher transport rates in spastin knock-out mice. Indeed, we had already shown in previous work that increasing microtubule mass in terminal motor axon branches pharmacologically can increase mitochondrial transport (*Brill et al. Neuron 2016*, Fig. S5). We fully appreciate that a comprehensive analysis, e.g. of branch-specific Nav-GFP$^+$ transport in conditional spastin knock-outs (cKO) as suggested by the Reviewer, would be valuable in addition; however, given the number of alleles involved (five – three for cKO and one for Nav-GFP and a cytoplasmic label to identify 'sin' vs. 'din' branches), this seems prohibitive.

Moreover, the effect of spastin deletion on myelination was restricted to 'din' inputs (original **Fig. 4K**), while the increase of βIII-tubulin was general ('sin' and 'din'; original **Fig. 4M**). This

suggests that other factors (such as cargo limitations) are also in play (see below, comment to R3.7) and that the prediction for this experiment is not critical for our model.

Finally, the reviewer asked, whether axon caliber is increased in spastin knock-out mice. In conditional spastin knock-out (TdTom+) vs. internal-control unaffected axons (TdTom-), we could not detect caliber changes in 'sin' or 'din' branches and conclude that caliber does not contribute. We inserted these data into a **new panel, Fig. S3D**.



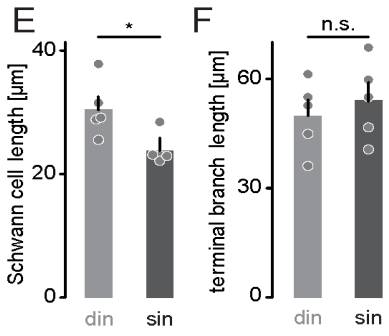

**New panel Fig. S3D:** Axon branch caliber in TdTomato-negative (TdTom-) vs. spastin cKO (TdTom+, n ≥ 10 axons per group, n = 5 mice; Mann-Whitney test).

**R2.2**: "The data that NRG1 is deficient in the DIN branches and therefore underlies the delay in myelination is plausible but not compelling. […] NRG1 is both a mitogen and a differentiation signal and a deficiency of NRG1 on DIN vs SIN branches might manifest as a reduced density of Schwann cells in DIN branches - Schwann cell density would be useful to document either way.

While staining for NRG is challenging, antibody staining might prove helpful; an alternate potential option is to use erbB-Fc binding/staining as a readout for expression on the axons. pERK staining in Fig. 5 is not necessarily a specific readout for NRG. The increase in pERK staining itself shown in panels 5I and 5J is not entirely convincing and appears blotchy rather than cytoplasmic or nuclear. A double stain with a Schwann cell marker would be useful to show that this staining is in fact within overlying Schwann cells.

Finally, if spastin cKOs work by increasing transport and NRG levels, some increase in pERK (or NRG staining) should be evident."

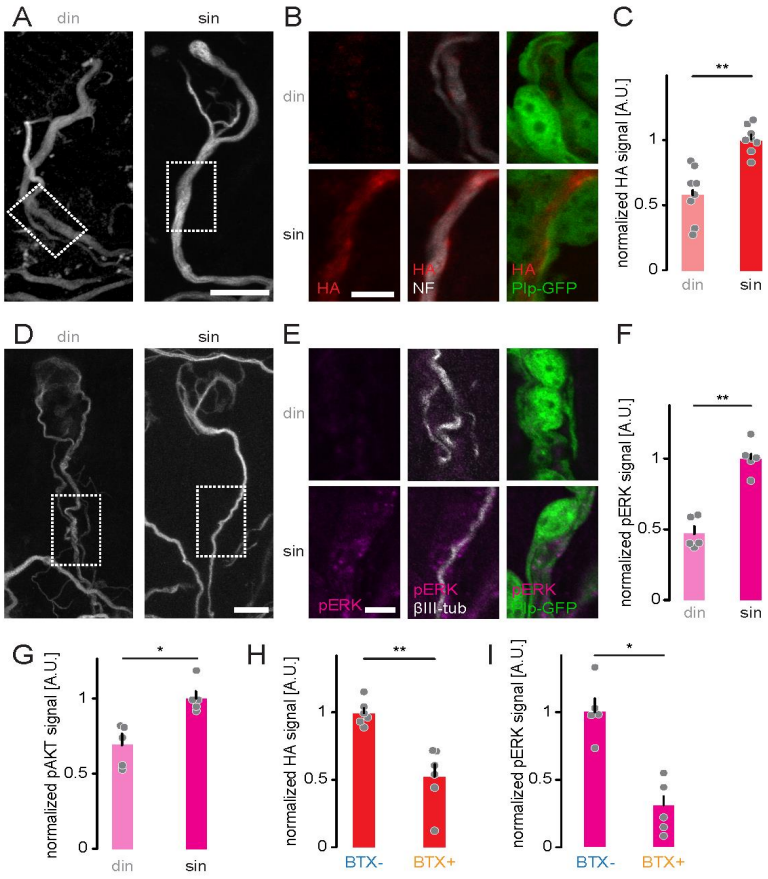
Following the Reviewer's suggestion, we documented axonal Schwann cell length and number on 'din' vs 'sin' axons in *Plp*-GFP mice, where all Schwann cells are labeled (*Mallon et al. J. Neurosci. 2002*). Indeed, the Schwann cells appear to be on average longer on 'din' axons (as the branches are fully sheathed, this equals to more Schwann cells per axon length along 'sin' branches as predicted by the Reviewer). These data are now shown in **Fig. S2 E** and **F**; the overall effect size is small, however (Schwann cells along singly innervating branches are 22% shorter than along doubly innervating branches).




**New Fig. S2 E, F:** (**E**) Schwann cell length and (**F**) axon branch length along 'winner' ('sin') and 'competing' ('din') branches (≥ 16 axons per animal in n = 5 mice; *, P < 0.05, Mann-Whitney test).

As the Reviewer predicted, we found Nrg1 immunostaining taxing. We followed his/her lead towards ErbB-Fc ligands, and tried several versions of ErbB3/4-Fc proteins (R&D Systems: mouse ErbB3/Her3 Fc #4518-RB-050; mouse ErbB4/Her4 Fc #4387-ER; human ErbB3/Her3 Fc #10368-RB) on wild-type and *Thy1*-Nrg1 type III transgenic mice without success (i.e. no difference in staining between wild-type and

Nrg1 KO mice and no specific membrane signal on *Thy1*-Nrg1 type III axons). In the end, however, we are able to visualize neuregulin by immunostaining for the HA-tag in *Thy1*-Nrg1 type III transgenic mice with an improved protocol (see revised **Material & Methods** for detail). In addition, we achieved convincing immunostaining results in *Thy1*-Nrg1 type III transgenic mice of an additional down-stream effectors of Nrg1, pAKT (**new panel, Fig. 7G**). Together, we found increased HA-tagged Nrg1, pERK and pAKT staining along 'sin' branches compared to competing 'din' branches (**new panels, Fig. 7F–I**).



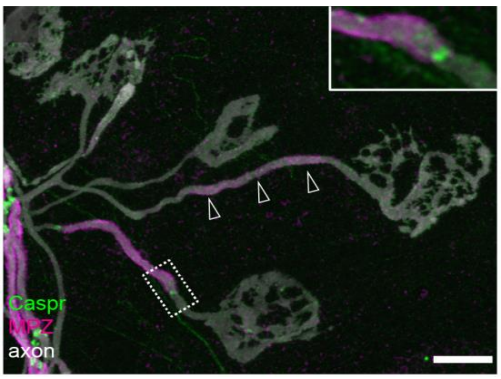

**New Fig. 7A–I: (A, B)** Example confocal images of HA staining (red) in terminal axon branches of *Thy1*-Nrg1 type III mice; **(D, E)** Schwann cells (*Plp*-GFP; green) around terminal axon branches in *Thy1*-Nrg1 type III mice showing pERK staining (red). **(C, F and G)** Quantification of HA, pERK and pAKT staining along 'din' and 'sin' branches respectively. **(H, I)** Changes in HA-tagged Nrg1 and pERK immunostaining after BTX-mediated blockade of neurotransmission (≥ 13 axons per group in n ≥ 5 mice; *, $P < 0.05$; **, $P < 0.01$; Mann-Whitney test). Scale bar, 10 µm in (A) and (D), 5 µm in (B) and (E).

We appreciate the Reviewer's observation that these immunostaining have a 'blotchy' quality – these stainings are TSA-amplified, which generally results in a punctate pattern due to the precipitation reaction. To place the localization more clearly into Schwann cells, we now repeated these immunostainings in *Plp*-GFP x *Thy1*-Nrg1 type III transgenic mice as suggested: As expected, HA-tagged neuregulin locates to axons, while pERK and pAKT show Schwann cell localization (**new panels, Fig. 7A–G**).

Finally, the Reviewer suggests to repeat this analysis while altering synapse elimination speed. The specific suggestion is for the spastin cKO, but as there appears to be chromosomal incompatibility between the Nrg1 transgene and spastin (at least we failed to assemble these alleles despite a substantial effort that should suffice if Mendelian ratios prevailed), we resorted to testing the converse prediction: We show reduced HA-tagged Nrg1 and pERK immunostainings when synapse elimination is delayed by blocking neurotransmission (injecting BTX into Nrg1 type III transgenic mice; **new panel, Fig. 7H, I**).

**R2.3**: "Fig. 1D shows a decrease of doubly innervated NMJs from ~ 40% to 0% from P7 to P11 with a concomitant increase of Caspr+ myelin segments on single branches from ~ 10% to almost 80%. This would indicate that at P7 10% of the 60% of SIN branches have myelin whereas by P11, about 80% of such branches have myelin. If this is correct, it indicates there is a significant lag in myelination of such branches and the temporal correlation is not as tight as implied, presumably due to a lag in axon maturation. The authors should comment/discuss."

We appreciate the Reviewer's comment and hope we interpreted it right. We concur that our data point to a lag, but do not see this in conflict with our interpretation; myelination is a protracted process that will take time to develop – and indeed, we observe different stages of the process along the maturing motor axon branches (see e.g. **Reviewer Fig. 1**). We think this is due to the time it takes for the cytoskeleton to mature, the delivered pro-myelination signalling to gain strength and for first signs of myelination to become detectable with our methods – as we do not have simple means to reliably visualize the very first moment of myelination onset and limited temporal sampling. We now explicitly address this lag in the **Discussion section**.



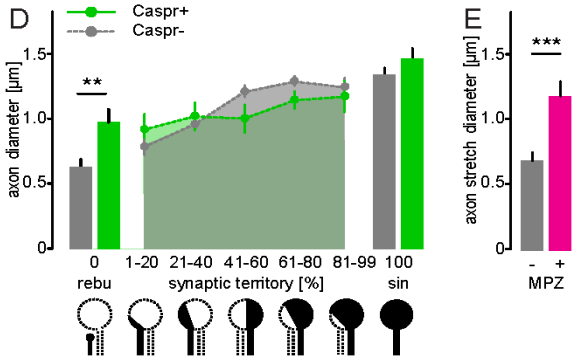

**Reviewer Fig. 1:** Progression of initial myelination in terminal axon branches. Immunostaining for Caspr (green, paranode), and MPZ (magenta, myelin) in single terminal axon branches of Thy1-XFP mice (axons, white). Hollow arrowheads point to a terminal branch with only MPZ staining.

**R2.4 - minor**: "Fig. 2 shows recovery from FRAP is faster on DIN and is taken as sign of node immaturity consistent with their data showing delayed appearance. Might this difference reflect that there are more heminodes on the DINs vs nodes on the SIN as that might also account for different recovery times?"

We thank the Reviewer for making this point. We failed originally to clarify that all of the nodes analyzed on immature axons in our FRAP experiments are heminodes (i.e. unpaired Caspr$^+$ accumulations), as even on 'sin' branches at postnatal day 9, fully developed nodes are rare and we avoided them to ensure consistency. We now state this in the text, to rule out the possible confounding influence that the Reviewer points out.

**R2.5 - minor**: "Fig. 3D: seems to show an increase in the axon diameter by territory for the unmyelinated branches but not for the myelinated - the authors may wish to comment/discuss on this difference. It is also unclear what the double asterisk and associated p value correspond to?"

The Reviewer is right, the asterisk was not clearly revealing the origin of the indicated statistical significance. Indeed, this exclusively derives from the fact that the few retreating axon branches that carry myelin are less atrophic than non-myelinated ones – typically in a very localized fashion (see original **Fig. 3F**). This supports the idea that myelination determines, or at least preserves, axon diameter locally. We now removed this bin from the graph, as it represents a distinct category; none of the other bins shows a significant difference.




**Revised panels Fig. 3D, E:** (**D**) No significant difference in axon branch caliber between terminal branches of all synaptic territories that have (green) and have not (gray) initiated myelination. (**E**) Caliber of retraction bulb-tipped axon branches related to local myelination (local absence / presence of MPZ immunostaining, n ≥ 8 axons, ≥ 4 mice per group; ***, P < 0.001, Mann-Whitney test).

In addition, we now measured the local caliber along retreating axon branch segments that are either myelinated or unmyelinated, showing this local influence – this is included as revised **Fig. 3 E**, while **D** contains the original graph with retraction bulbs separated off.

**R2.6 - minor**: "On line 199, the length measure for the transport rates are missing, e.g 1.8 ?/min"

This is the number of Nav-GFP particles per time (i.e. flux, not speed), and hence the numerator is unit-less "(Nav-GFP particles)/min". We revised this to "GFP particle transport [$min^{-1}$]".

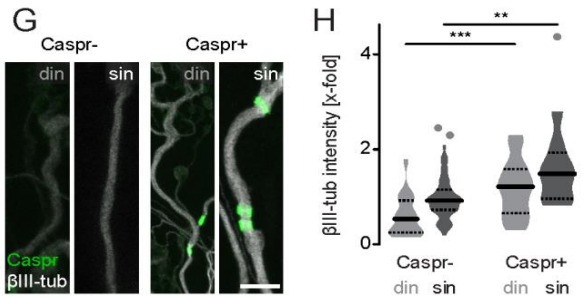
## Reviewer #3:

**R3.0**: "The manuscript by Wang et al, attempts to answer a long-standing question in the field - does myelination cease axonal remodeling, or does the cessation of axonal remodeling allow for myelination to occur? The data presented makes a case for the latter. The authors nicely describe how myelination coincides with the end of axonal branch competition in the PNS neuromuscular junction (NMJ) […]. However, several pieces in this story are not clearly connected and much remains to be shown. My major comments/concerns are outlined below."

We thank the Reviewer for the acknowledgement of the importance of the question addressed and the strength of the data relating myelination onset to the end of axon remodeling. We appreciate the Reviewer's fair assessment that there are some links in our chain of argument that needed to be strengthened. In our point-to-point answers to the specific points raised by this Reviewer and throughout this letter, we have tried to fortify these links and believe that we have been able to address most of the specific weaknesses that were pointed out.

**R3.1**: "They claim that the degree of myelination observed correlates with increased microtubule mass, which is not clearly shown."

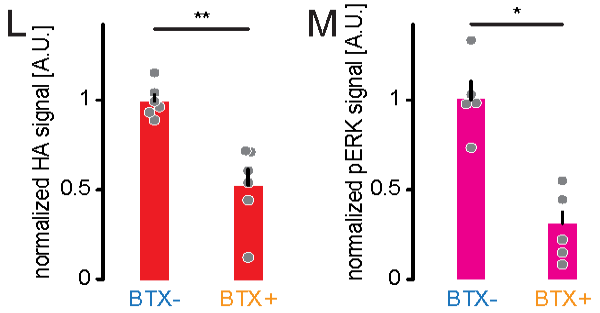
The Reviewer is right, this specific claim should be illustrated with an additional data set, which we now included as **new Fig. S2, panels G and H**. Here we directly show that terminal axons, which have initiated myelination (Caspr+) have significantly higher tubulin levels than caspr-negative counterparts in the same animal – this is true for 'winner' ('sin') and 'competing' ('din') axons.




**New Fig. S2G, H:** (**G**) Illustration of βIII-tubulin levels in 'sin' and 'din' branches that lack (left) or show (right) early signs of myelination (accumulations of Caspr, green). (**H**) Quantification of βIII-tubulin levels I terminal axon branches without (Caspr-) or with (Caspr+) indications of myelination (n ≥ 18 axons per group in n = 3 mice; **, P < 0.01; ***, P < 0.001, Mann-Whitney test). Scale bar, 10 µm in (E).

**R3.2**: "If the authors claims are correct, the experiments in Figure 4 that delay competition, BTX treatment and spastin KOs, should also reveal changes in Nrg1 type III that reflect the microtubule and myelination changes observed. Can the authors show that Nrg1 type III levels are reduced following BTX administration and increased in the spastin KOs?"

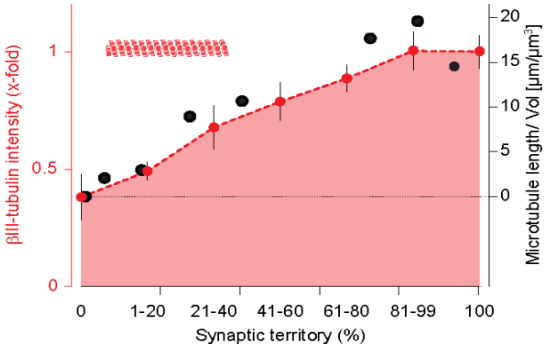
See also comments R2.2 and R3.3 for the specific challenges of breeding the spastin cKO x *Thy1*-Nrg1 type III transgenic mice. However, we now show HA-tagged neuregulin staining, as well as the down-stream effectors, pERK and pAKT in *Thy1*-Nrg1 type III transgenic mice. As shown above (R2.2) and as requested by this Reviewer, both HA-tagged neuregulin and pERK signals are reduced following block of neurotransmission (injection of bungarotoxin, BTX, into *Thy1*-Nrg1 type III mice). These data are shown in **new panels, Fig 7L, M**.




**New panel Fig. 7L, M:** Changes in HA-tagged Nrg1 and pERK immunostaining after BTX-mediated blockade of neurotransmission in *Thy1*-Nrg1 type III mice (≥ 13 axons per group in n ≥ 5 mice; *, P < 0.05, **, P < 0.01, Mann-Whitney test).

**R3.2**: "The authors use bIII-tubulin fluorescence staining intensity to measure increases in microtubular mass, however fluorescence staining intensity is often not quantitative and the authors need to show that the changes in fluorescence intensity that they are measuring do indeed reflect changes in microtubular mass. They cite a previous paper (Brill, 2016) where they show that decreases in bIII-tubulin staining intensity of retreating axon branches reflect decreases in microtubule numbers seen on EM images, but it is not clear whether the differences in bIII-tubulin staining intensity they currently see between competing branches and winner branches reflect increases in microtubules. Is this a linear relationship?

We thank the Reviewer for pointing out this omission. We should have stated this more clearly in the present study – and our reference to *Brill et al. Neuron 2016* was indeed incomplete, as the direct relationship between immunostainings and EM were not plotted there.




**Reviewer Fig. 2:** Relationship of microtubular length per axonal volume (EM; black dots) and βIII tubulin immunostainings (red dots) and synaptic territory (data replotted from *Brill et al. Neuron 2016*).

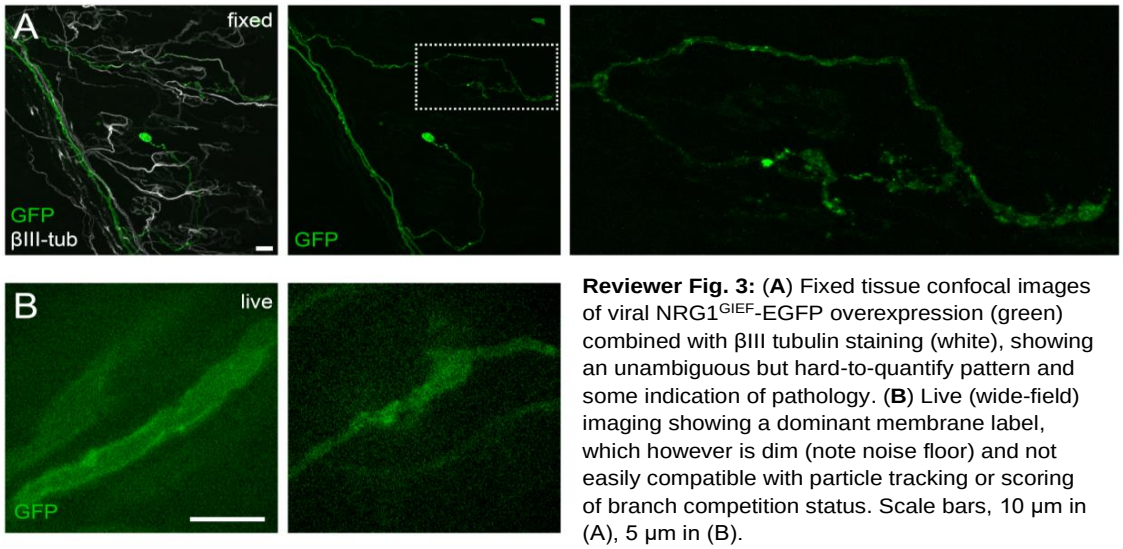
We now replot Fig. 1G and Fig. 3G of *Brill et al. Neuron 2016* as a **Reviewer Fig. 2**, showing the direct correlation between light-microscopic measurement of βIII-tubulin levels and the microtubular length per axonal volume by electron microscopy (EM). Indeed, we find a linear relationship between the measures in EM (black dots) and light-microscopy (red dots). There is, however, an offset in the immunostainings, probably indicative of some non-filamentous tubulin – hence we generally underestimate the size of effects using immunostainings. We inserted a statement referring to this in the **Methods section**.

**R3.3**: "The authors mention that increased microtubule mass indicates a more mature cytoskeleton with increased axon transport. Can the authors show that axon transport is increased in winner branches over competing branches? Specifically transport of Nrg1 type III?"

This Reviewer is in line with Reviewer 1 in suggesting that our proposed mechanism would be strengthened by visualizing and ideally even tracking the delivery of Nrg1 into the terminal branches. For immune- or ligand-based stainings, we have detailed our new results in response to comment R2.2. For Nrg1 particle tracking, we generated an AAV9 virus encoding a cre-dependent FLEXed Nrg1$^{GIEF}$-EGFP (AAV-hSyn-DIO-Nrg1$^{GIEF}$-EGFP, *Velanac et al. Glia 2012*), and injected this AAV into neonatal *ChAT*-Cre mice (**Reviewer Fig. 3A**). We attempted to analyze fixed tissue samples, but found transduced motor units to appear unhealthy with non-physiological axonal swellings and caliber variations (**Reviewer Fig. 3A, right**). Moreover, in live samples, we were also unable to measure Nrg1$^{GIEF}$-EGFP as the fluorescence levels are low and the membrane-located signal obscures the signal from any transported particles (**Reviewer Fig. 3B**).

However, we address the Reviewer's first question by showing increased delivery of Nav-GFP$^{+}$ particles into winner branches and the corresponding enhanced accumulation of HA-tagged Nrg1, as well as increased downstream pERK/ pAKT signaling in Schwann cells around such branches (see above, answers to R2.1 and R2.2). Therefore, while we are not able to track Nrg1 directly (and to our knowledge this has not been achieved in intact tissue

preparations so far), we can provide direct evidence for increased transport in general, specific cargo accumulation and downstream signaling.



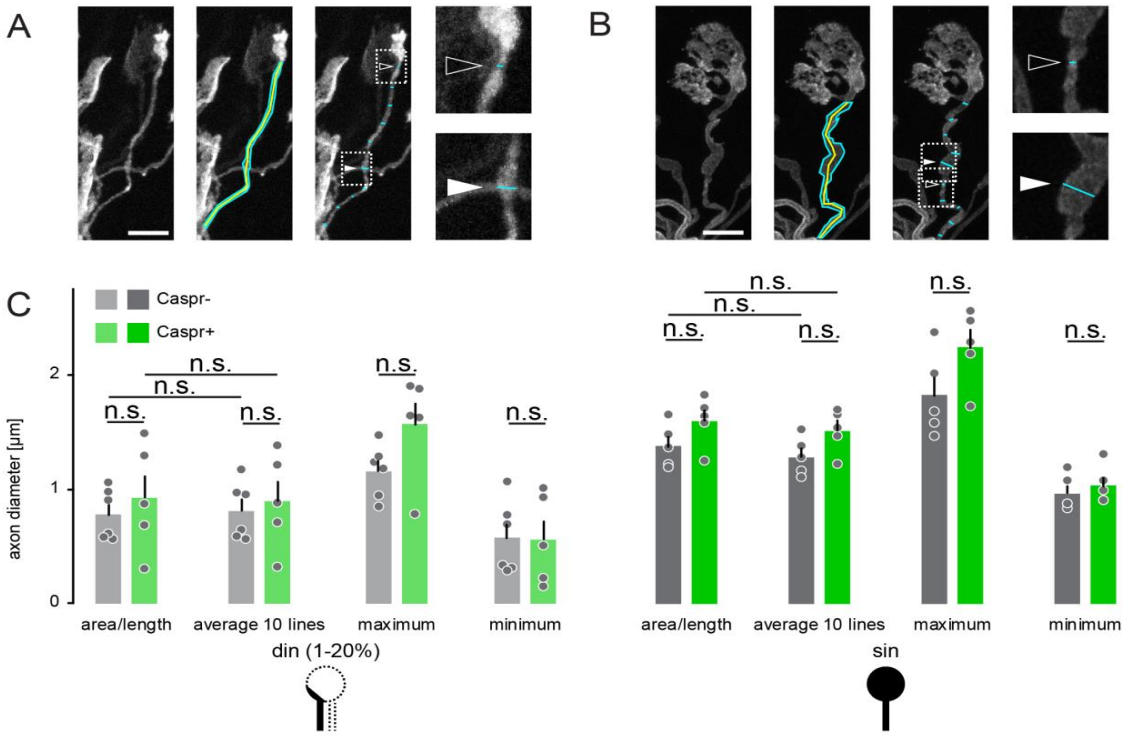

**Reviewer Fig. 3:** (**A**) Fixed tissue confocal images of viral NRG1$^{GIEF}$-EGFP overexpression (green) combined with βIII tubulin staining (white), showing an unambiguous but hard-to-quantify pattern and some indication of pathology. (**B**) Live (wide-field) imaging showing a dominant membrane label, which however is dim (note noise floor) and not easily compatible with particle tracking or scoring of branch competition status. Scale bars, 10 µm in (A), 5 µm in (B).

**R3.4**: "In the discussion, the authors state that their work reveals an activity-dependence of myelination in the PNS (lines 270-271), but this is a stretch. The authors do not show any activity-dependence of myelination."

The Reviewer is correct. We have revised the corresponding statement.

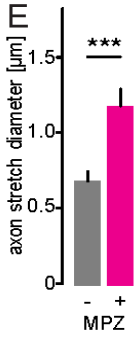
**R3.5**: "In Figure 3 the authors measure axon diameters by measuring the entire terminal branch area then dividing it by the length of the branches, however this is a very crude estimate and does not reflect actual diameter measurements, especially since diameters can vary greatly along the same branch (see images in Figure 3E,F)."

We thank the Reviewer for this comment. Indeed, diameters of motor axons vary along terminal branches; this has several reasons, including the presence of Schwann cell nuclei, local axon motility and pruning-related atrophy – this variability is most drastic along axon branches that have lost contact to a synapse and retreat (see below). We think therefore that there is no single 'correct' way of measuring an axon caliber. Thus, we have now performed different measurements on five Caspr-positive vs. five Caspr-negative branches for both the 'din' (on average 18% territory in both groups) and 'sin' category. Caspr-positive and -negative axons do not statistically differ in measured caliber in all the different approaches we tested (see **Reviewer Fig. 4**). We now modified the **Methods section** to reflect these controls.




**Reviewer Fig. 4:** (**A, B**) Examples of performed axon caliber measurements for 'din (**A**) and 'sin' (**B**). (**C**) Axon caliber as determined with various measures differ in absolute value but not in relationship to myelination status (Caspr+/-, n ≥ 5 axons). Scale bar, 10 µm in (A) and (B). Mann-Whitney test.

Explanation of approaches - area/length: The original measure; average 10 lines: Axon caliber was equated to the average of ten positions equally spaced along the branch; maximum/ minimum: the thickest/ thinnest diameter along the branch.

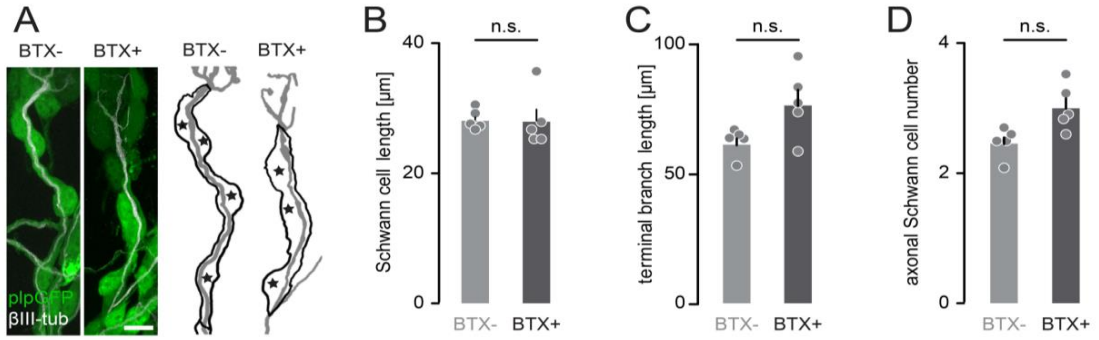



**Revised panels Fig. 3E:** Caliber of retraction bulb-tipped axon branches related to local myelination (local absence / presence of MPZ immunostaining, n ≥ 8 axons, ≥ 4 mice per group; ***, P < 0.001, Mann-Whitney test).

There remains the special case of axon branches that have formed a retraction bulb, with local atrophy along the branch (see *Bernstein & Lichtman Curr. Opin. Neurobiol.1999,* original **Fig. 3F**). We have now quantified the local axon diameter along such pruning branches in the non-myelinated vs. myelinated stretches and show a highly significant difference between these different parts of the same axon branch – providing evidence for a local impact of myelination on axon caliber (see comment R2.5). This is now shown in a **new panel**, **Fig. 3E**.

**R3.6**: "In Figure 4 the authors show a large decrease in Caspr+ branches following BTX administration. Can this be due to non-specific effects on Schwann Cells (SC)? Is there a decrease in SC numbers?"

To address the Reviewer's concern, we injected BTX into *Plp*-GFP mice, where all Schwann cells are labeled (*Mallon et al. J. Neurosci. 2002*) and quantified axonal Schwann cell length along winner ('sin') branches. We did not find a significant change in SC length, indicating that BTX injection does not change SC numbers. This is shown in a **new Fig. S2A–D**).



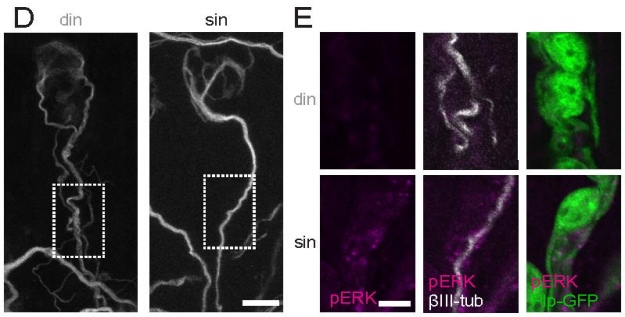

**New Fig. S2A–D:** (**A**) Example confocal images of the Schwann cells (*Plp*-GFP) around terminal axon branches without or with BTX injection (BTX-/+). Schematics to the right depict measured terminal axon length (gray) and Schwann cell outline with cell nuclei marked with asterisks. (**B–D**) Quantification of (**B**) Schwann cell length, (**C**) terminal branch length and (**D**) Schwann cell number along singly innervating branches, showing no significant difference after BTX treatment (≥ 10 axons per animal in n = 5 mice). Scale bar, 10 µm in (A). Mann-Whitney test.

**R3.7**: "In Figure 4K, the authors show that there is no increase in Caspr+ branches in sin spastin KO branches, but there is an increase in bIII-tubulin (Figure 4M). If increased microtubular mass promotes myelination, why isn't there an increase in sin Caspr+ branches in the spastin KOs?"

We appreciate the Reviewer's point; our discussion of this aspect was insufficient. In fact, artificially increasing microtubular mass (i.e. track availability) will only increase myelination, if the number of Nrg1-transport vesicles is non-limiting (i.e. if there are extra vesicles in the stem axons that could be rerouted by additional tracks). This might not be the case, as Nrg1 indeed is assumed to be a limiting factor (*Michailov et al., Science 2004; Taveggia et al., Neuron 2005*). Hence we assume, that 'sin' branches already in wildtype conditions have sufficient tracks to deliver the full amount of pro-myelination signaling factors. Along these lines, we have observed previously that under baseline conditions, spastin deletion per se does not increase e.g. mitochondrial transport in 'sin' axon branches (*Marahori, 2020*). We added these considerations to the **Discussion** to make our reasoning easier to follow.

**R3.8**: "In Figure 5H-K, the authors attempt to measure Erk signaling in SCs, but since SCs are not being identified, it is not clear where the signal they are measuring is originating."

We followed the Reviewer's suggestion and performed pERK staining in *Plp*-GFP mice (*Mallon et al. J. Neurosci. 2002*) crossed to *Thy1*-Nrg1 type III mice, where Schwann cells are labeled and the location of the signal can be appreciated. This now shown in **new panels, Fig. 7D, E** (see R2.2).




**New Fig. 7D, E:** Schwann cells (*Plp*-GFP; green) around terminal axon branches in *Thy1*-Nrg1 type III mice showing pERK staining (magenta). Scale bar, 10 µm in (D), 5 µm in (E).

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December 18, 2020

RE: JCB Manuscript #201911114R

Prof. Thomas Misgeld
Institute of Neuronal Cell Biology
Technische Universitaet Muenchen Biedersteiner Str. 29
Muenchen 80802
Germany

Dear Prof. Misgeld:

Thank you for submitting your revised manuscript entitled "Completion of neuronal remodeling prompts myelination along developing motor axon branches". Your paper has now been seen again by the original reviewers, all of whom now recommend acceptance. Therefore, we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

As you will see, reviewers #2 and #3 have raised some final (relatively minor) concerns which we would like for you to address in your final revision. It should be possible to address these comments with changes to the text and/or figure layout; no new experiments will be required.

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles and Tools is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends. You are below the limit at this time but please bear it in mind when revising.

2) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used

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4) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

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c. Temperature
d. imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

7) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

8) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental figures. At the moment, you are below this limit but please bear it in mind when revising.
Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

9) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

10) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

11) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

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Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Elior Peles, PhD
Monitoring Editor
Journal of Cell Biology

Tim Spencer, PhD
Executive Editor
Journal of Cell Biology

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Reviewer #1 (Comments to the Authors (Required)):

The authors have added additional new data addressing my concerns. I think this is very nice paper that provides new conceptual insights into the mechanisms of branch maturation and myelination in motor axons.

Reviewer #2 (Comments to the Authors (Required)):

The authors have been very responsive to the concerns raised and substantially addressed most of my concerns - filling in previous gaps on differences in transport rates, NRG content, and Schwann cell signaling in the SIN vs. DIN branches as well as showing effects of axonal transport on the onset of myelination. These changes have substantially strengthened the manuscript and their model of how NMJ remodeling is linked to myelination. I have two minor suggestions they should consider:

Fig 6A, B: the colors of these panels are very different and it is quite difficult to see how much of this is due to increased HA staining (red). It would help to separate the channels - or at least show the HA/red channel separately or as an insert inside each panel

Fig. 7A, D. I would add NF in white to each panel to indicate the staining shown is for neurofilament

Reviewer #3 (Comments to the Authors (Required)):

The authors of the manuscript by Wang et al addressed all of my concerns, but one. It is disappointing that the authors could not produce any Thy1-Nrg1 type III transgenic, spastin cKOs, which would show whether increasing microtubular mass leads to an earlier/greater accumulation of Nrg1 type III along branches and thereby greatly support the authors conclusions that maturation of the cytoskeleton allows for axon-glial signaling to occur. However, the evidence they do present including new data showing that maintaining an immature cytoskeleton by prolonging branch competition with BTX leads to decreases in Nrg1 type III accumulation along branches, as their model predicts, is convincing. Perhaps, the inability to produce the Nrg1 type III transgenic, spastin cKOs could be discussed in the manuscript to provide this knowledge to the readership who may wonder about that particular experiment. Ultimately, this body of work presents an intriguing model in which the completion of axon remodeling in the PNS results in cytoskeletal maturation which then allows for myelination to occur, and it will be of great interest to the field.