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Dynamic remodeling of ribosomes and endoplasmic reticulum in axon terminals of motoneurons

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MS TITLE: Dynamic remodeling of ribosomes and endoplasmic reticulum in axon terminals of motoneurons

AUTHORS: Michael Sendtner, Chunchu Deng, Mehri Moradi, Sebastian Reinhard, Soeren Doose, Luisa Hennlein, Sibylle Jablonka, and Markus Sauer
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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise some substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. after reviewing them, I anticipate that you satisfactorily address these criticisms on revision. If this is the case, I would be delighted to handle a revised manuscript in the very near future.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The authors performed super-resolution microscopic analyses to show that BDNF stimulation induced dynamic remodeling of presynaptic ER through actin and microtubule crosstalk. They examined chemical treatment studies and showed that presynaptic filopodia ER movement is mainly mediated by actin-based transport.
Most noteworthy, double-immunocytochemical analyses revealed that BDNF treatment enhanced the colocalization of ribosomes and ER in presynaptic area, suggesting that the site of local translation is instantly composed by BDNF stimulation in presynaptic area. These findings are intriguing, and the data of SIM analyses in this study are impressive.

Comments for the author

Previous studies showed that several myosin motors, such as myosin II, V, and VI are involved in local transport system at postsynaptic area. This reviewer is quite interested in what kind of myosin motor proteins are involved in the dynamic remodeling of presynaptic ER. It would strongly support this study when the authors examine additional chemical treatment studies by using (±)-blebbistatin MyoVin-1, and 2,4,6-triiodophenol.

Reviewer 2

Advance summary and potential significance to field

The manuscript “Dynamic remodeling of ribosomes and endoplasmic reticulum in axon terminals of motoneurons” by Deng et al shows that the ER within motorneuron growth cones is highly dynamic, and ribosomes rapidly assemble and associate with this ER upon BDNF treatment. While the role of BDNF in protein synthesis is not new, the rapid association of ribosomes with growth cone ER is. They further show that the observed ER dynamics are reduced upon nocodazole/ cytochalasin treatment. How dynamic is KDEL-cherry upon BDNF treatment? No data/ comment on this is provided, and the title curiously only refers to the ER/ ribosome dynamics, not to the BDNF stimulation. It would be good to see these two aspects tied together. I have a few further comments and queries regarding the manuscript, as detailed below:

Comments for the author

The authors repeatedly refer to “presynaptic” ER (eg p5, 9), however, no evidence of synapse formation in these young cultures is shown, instead growth cones are in focus. It would be helpful to rephrase as “axonal” or show proximity to established presynaptic sites.
Figure 1 A shows a rather punctate distribution for an organelle that is thought of as a continuous tubular network - what is the axial resolution in this setup? Please provide a 3D reconstruction.
Figure 1 B, C: the co-localisation of ER and actin as well as ER and microtubules should be quantified. Given the high density of cytoskeletal components in these structures, is the co-localisation really greater than what is expected by chance? EM might help showing how tight the association is.

Figure 1 D: please comment on the almost complete overlap between actin and ER – is this due to lack of resolution in these images, which are not SIM (why not?), and/ or consistently seen?
Figure 2: please give concentrations and times of treatment for all drug treatments. To what degree is the reduction in ER motility due to a reduction in movement of the entire growth cone structure/ collapse of the growth cone and retraction of filopodia as a result of treatments, versus

specific to the ER? Visualisation of an independent structure, or even high quality phase / DIC contrast images would be informative to interpret the observation.
Figure 3: please give details on how the growth cone was outlined to measure the mean intensity of the area – it would be helpful to show the independent channel used to delineate the structure of interest.

The striking increase in signal, both for ribosomal subunits and for total TrkB, with in 10s is curious and faster than expected by motor based transport – especially since this signal extends into the most distal structures. Please refer to data showing that the antibodies for Y10B and RPL8 are conformation specific (as suggested on p8). What is the explanation for TrkB? Supporting live imaging data for at least one of the three proteins showing the dynamics upon treatment would be useful.
Figure 6 would really benefit from a complementing EM image.
Figure 2G, H: image is taken at 10s stimulation, quantification at 10min or also 10s?

Reviewer 3

Advance summary and potential significance to field

The manuscript by Deng et al. investigates the dynamics of the ER in the growth cones of motoneurons. The authors use confocal and SIM microscopy to show ER dynamics in relation to actin dynamics, and use pharmacological approaches to show that ER dynamics depend on both actin filaments and microtubules. In motoneurons treated with BDNF, the authors observe rapid changes in the ER, rapid assembly of active ribosomes, and rapid local translation. Finally, they provide evidence that there is also local association of ribosomes with ER to form RER in the distal axon.

This work addresses an important yet understudied question, and provides timely and potentially important insights into the dynamics of ER, ribosomes, and local translation in axons. The most exciting and novel aspects of the manuscript are the experiments showing the very rapid and dynamic response induced by BDNF. The work in general is thoughtfully performed and includes important controls. As such, it clearly merits consideration for publication in the Journal of Cell Science.

However, a number of points require further analysis and/or controls, some of the conclusions are over-stated, and further editing could streamline the story and thus increase its impact. Thus, I recommend the authors consider the following points in their revision of the work, and resubmit a more compelling manuscript on this interesting topic.

Comments for the author

Major points:
1. The most interesting data reported here involve the rapid changes induced by BDNF in ribosome activation and local protein synthesis. These data are the strongest part of the story. In contrast, the initial two figures on the relationship between the ER, actin filaments, and microtubules are not as interesting, novel, nor strong. I recommend condensing much of this initial discussion and/or moving much of this work to the supplement.

2. A weakness of the first part of the manuscript is the authors' conclusion that they have shown that ER dynamics are dependent on "crosstalk" between the actin and microtubule cytoskeletons, or on a "coordinated actin/microtubule cytoskeleton". This may be true, but their work doesn't prove it – instead they show that depolymerization of either actin filaments or microtubules alters ER morphology in growth cones. This is not an unexpected finding and gives very little insight into the underlying mechanisms. If they wanted to strengthen this aspect of the manuscript, they could either test the effects of perturbing specific proteins implicated in cytoskeletal crosstalk such as drebrin, or they could test the effects or dampening cytoskeletal dynamics short of full-scale depolymerization. But I'm not sure this is the most interesting direction to go so I recommend instead that they focus on a possible role for actin dynamics in mediating the rapid ribosome activation and local translation that is the focus of the second part of the manuscript.

3. The final statement in the Introduction is too strong – I disagree that they provide evidence of a novel function of axonal ER in local protein synthesis.
If they were to show more directly that there is local synthesis of membrane/secreted protein, this point would be stronger.

4. The results on beta-actin are very interesting, but this part of the manuscript would be stronger if they could include data on the increased local synthesis of another protein of interest. Ideally, data on a membrane-associated or secreted protein to support their observation on RER in the presynaptic compartment.

5. How is actin involved in the RER dynamics they observe?

6. I appreciate that the authors sought to extend their study to in vivo observations, but this part of the manuscript is weak. If they wish to make this point, they need to solidify this observation with more supporting data.

7. The association of ribosomes with ER in the presynaptic compartment seems transient – have they measured off-rates?

Minor points:
1. Change “life cell imaging” to “live cell imaging” in the abstract.
2. The manuscript does not provide enough information on the use of ICS for the reader to appreciate how robust this analysis is. Inclusion of additional controls would strengthen this part of the manuscript. For example, what is the error over time if they image and analyze fixed cultures?
3. The authors refer to presynaptic compartments in their motoneuron cultures. It would be good to include data showing that their cultures are synaptically connected at the time points examined.
4. The authors state that 1 micron/sec is the fastest measured microtubule-dependent axonal transport speed, but this is not accurate – transport along the axon has been measured at rates 3-4 times this speed.
5. Some of the literature discussion on ER in the axon feels out of date – this is a fast moving field, and references from 1986-2002 are of interest, but not up to date.
6. I have some concerns about their fixation of microtubules in the images shown in Figure 1, they look fragmented.

First revision

Author response to reviewers' comments

We have made a series of additional experiments, as suggested by the reviewers, including experiments with myosin-inhibitors (Rev. 1, Rev. 2), with Drebrin-knockdown, and an analysis of synthesis of the alpha-1-beta subunit of the N-type Ca2+ channel in axon terminals, as a marker for a presynaptic transmembrane protein that is produced locally at the rough endoplasmic reticulum. All of these new data support the conclusions of our paper that the ER in axon terminals is highly mobile and moved by actin/myosin within filopodia of axonal growth cones, and that rough endoplasmic reticulum forms and allows synthesis of transmembrane proteins.

I also would like to thank you for your suggestions and ideas to revise our work, and we hope very much that this revised version is now acceptable for publication in JCS.

Reviewer 1:
Advance Summary and Potential Significance to Field:
The authors performed super-resolution microscopic analyses to show that BDNF stimulation induced dynamic remodeling of presynaptic ER through actin and microtubule crosstalk. They examined chemical treatment studies and showed that presynaptic filopodia ER movement is mainly mediated by actin-based transport. Most noteworthy, double-immunocytochemical analyses revealed that BDNF treatment enhanced the colocalization of ribosomes and ER in presynaptic

area, suggesting that the site of local translation is instantly composed by BDNF stimulation in presynaptic area. These findings are intriguing, and the data of SIM analyses in this study are impressive.

Reviewer 1 Comments for the Author:
Previous studies showed that several myosin motors, such as myosin II, V, and VI, are involved in local transport system at postsynaptic area. This reviewer is quite interested in what kind of myosin motor proteins are involved in the dynamic remodeling of presynaptic ER. It would strongly support this study when the authors examine additional chemical treatment studies by using (±)-blebbistatin, MyoVin-1, and 2,4,6-triiodophenol.

We thank this reviewer for his positive comments. We have now tested the role of myosin II, V and VI for ER dynamics and included these data in the new Fig. 3. These experiments show that in particular myosin VI and to some extent also myosin V inhibition has a major effect on ER dynamics in axon terminal filopodia, and also some effects on ER dynamics in the core of axonal growth cones. In contrast, the effects of myosin II inhibition were minor and did not reach statistical significance. These data show that myosin VI is particularly relevant for ER dynamics along actin fibers in filopodia.

Reviewer 2:
We would also like to thank this reviewer for making a series of points that help us to improve our manuscript.

General comment: *"How dynamic is KDEL-cherry upon BDNF treatment?"*
The specific function of BDNF signaling on the dynamics of the ER was not the central topic of this paper. We used BDNF for our experiments, because it is a well-known neurotrophic factor for activation of tyrosine kinase receptor induced remodeling in axon terminals. It needs to be emphasized that our experiments were performed with motoneurons that were cultured on laminin-2/merosin, in order to differentiate motoneurons and presynaptic structures as much as possible. In a recent paper, we could show that laminin-2 induces presynaptic maturation: Clusters of presynaptic Cav2.2 channels form and assemble with other presynaptic active zone components such as Piccolo or Bassoon (Jablonka et al., 2007). However, this specific culture condition also reduces axon extension and in general also actin and tubulin dynamics in axons (Dombert et al., 2017). Thus, the effect of BDNF on axon extension and movement of axonal growth cones is lower than in early embryonic motoneurons that are cultured on a conventional laminin-1 substrate which promotes maximal axon extension. Therefore, BDNF has much less effects on the shape of the axons and growth cones themselves, thus allowing us to investigate ER dynamics within these structures. We have added a short paragraph to the introduction to make the intention of our paper clearer: "We used culture conditions with laminin-2 which promotes differentiation of presynaptic structures in axon terminals." In such differentiated growth cones with presynaptic structures, ribosomes relocate to the ER where they can accomplish local translation of membrane- associated and secreted proteins such as N-type Ca2+ channels.

Specific points of reviewer 2:
The authors repeatedly refer to "presynaptic" ER (eg p5, 9), however, no evidence of synapse formation in these young cultures is shown, instead growth cones are in focus. It would be helpful to rephrase as "axonal" or show proximity to established presynaptic sites.

We exchanged the term "presynaptic ER" by "ER in axon terminals" to avoid any misunderstanding. We have also made reference to the previous paper published in JCB (Jablonka et al., 2007) where we showed that the specific culture conditions also used in this paper allow assembly of presynaptic active zones structures.

Figure 1 A shows a rather punctate distribution for an organelle that is thought of as a continuous tubular network - what is the axial resolution in this setup? Please provide a 3D reconstruction.

As the reviewer indicates, the punctuated distribution reflects the relatively high axial resolution of SIM microscopic analyses. We have done a 3D reconstruction and have included these data in Suppl. Fig. S1.

Figure 1 B, C: the co-localisation of ER and actin as well as ER and microtubules should be quantified. Given the high density of cytoskeletal components in these structures, is the co-localisation really greater than what is expected by chance? EM might help showing how tight the association is.

We have done quantification of the co-localization of ER, actin and microtubules by line scan analyses of the three channels. These data are now included in Fig. S1 and clearly show that actin and ER closely overlap in filopodia. Overlap with alpha-tubulin is low in filopodia but prominent in the core of growth cones. At this location, overlap of ER with actin is much less than in filopodia. In any case, the co-localization is much higher than expected by chance. To rule out this possibility, we rotated the ER channel by 90° and could detect only a small overlap between the ER and either F-actin or alpha-Tubulin (Fig. S1D)

We did not do EM analyses, because these experiments are highly prone to fixation artefacts, and it was also hard to detect ribosomal structures under such conditions. Moreover, EM analysis does not allow distinguishing ER-like structures from other vesicular and tubular structures such as endosomes. Therefore, we decided to extend our live imaging and high resolution SIM microscopic analyses to add evidence for the co-localization of ER and actin in filopodia and growth cone core.

Figure 1 D: please comment on the almost complete overlap between actin and ER – is this due to lack of resolution in these images, which are not SIM (why not?), and/ or consistently seen?

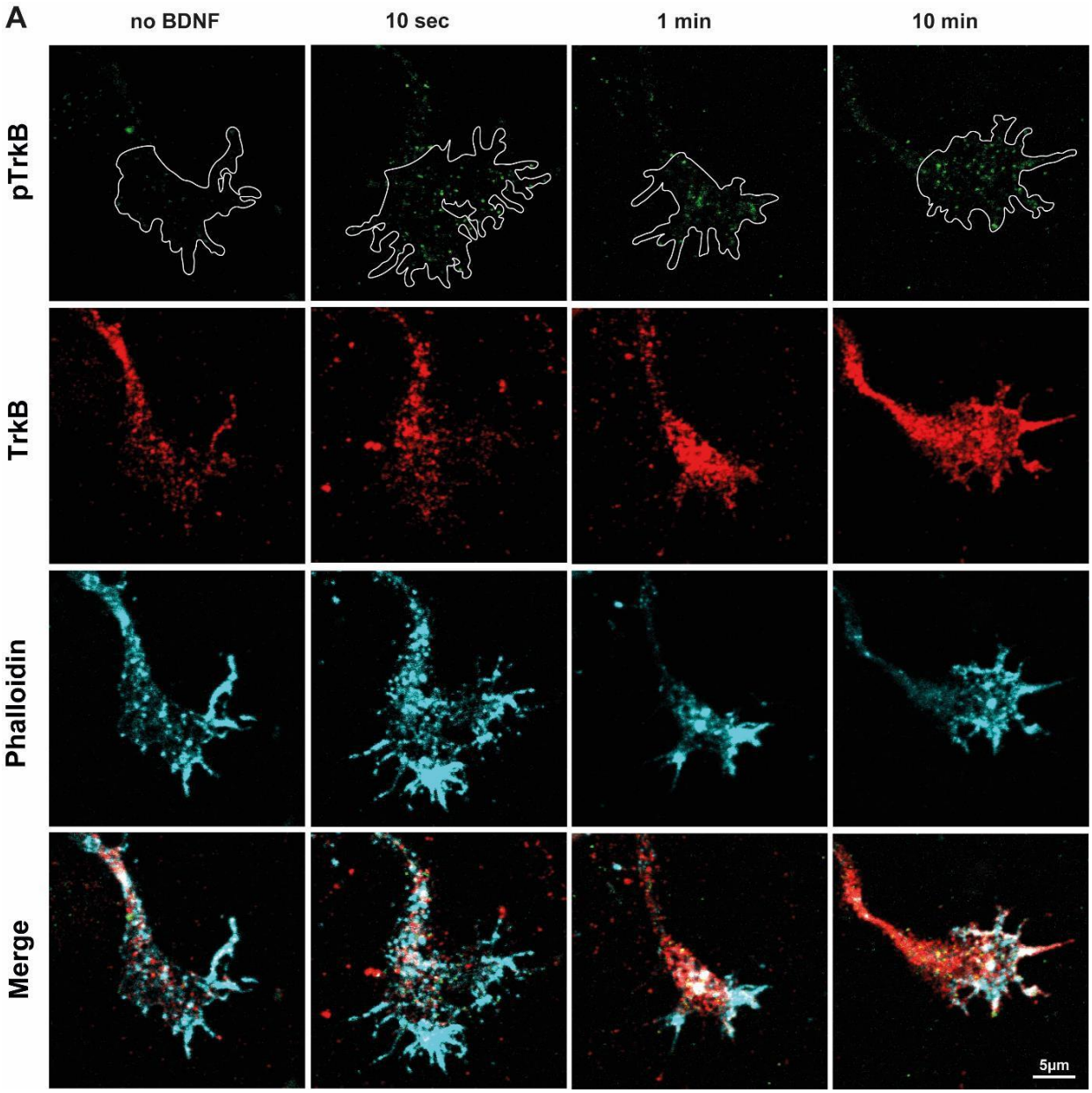
The almost complete overlap between actin and ER is due to the relatively lower resolution of conventional live cell imaging in comparison to SIM microscopy of fixed neurons. These data were included to provide evidence with dynamic kymograms, in order to demonstrate that actin and ER dynamically move together, to support the evidence of co-localization from static high resolution SIM pictures. We have deleted these kymograms and replaced analyses of colocalization from live cell imaging by co-localization analyses with image correlation spectroscopy (ICS). These analyses showed that ER entered only a fraction of highly dynamic filopodia in axonal growth cones during periods of 8 min observation, despite high actin dynamics in all of these filopodia, indicating that ER does not move everywhere where actin is moving. To our mind, this also provides evidence that movement of the ER is not by default coupled to movements of filopodia structures. If this would be the case, we would expect mCherry-KDEL staining in all filopodia. These new data are now included in the revised Fig. 1B.

Figure 2: please give concentrations and times of treatment for all drug treatments. To what degree is the reduction in ER motility due to a reduction in movement of the entire growth cone structure/ collapse of the growth cone and retraction of filopodia as a result of treatments, versus specific to the ER? Visualisation of an independent structure, or even high quality phase / DIC contrast images would be informative to interpret the observation.

We have now added concentrations and times of treatment for all drug treatments. In order to test whether the reduction in ER motility is due to a reduction in movement of the entire growth cone structure, we performed new experiments after co-transduction of motoneurons with GFP as a marker for the whole structure, and mCherry-ER as a marker of the endoplasmic reticulum within this structure. Image correlation spectroscopy of these two channels clearly showed that ER moved independently from the complete structure within growth cone filopodia (new Fig. 1A and B).

Figure 3: please give details on how the growth cone was outlined to measure the mean intensity of the area – it would be helpful to show the independent channel used to delineate the structure of interest.

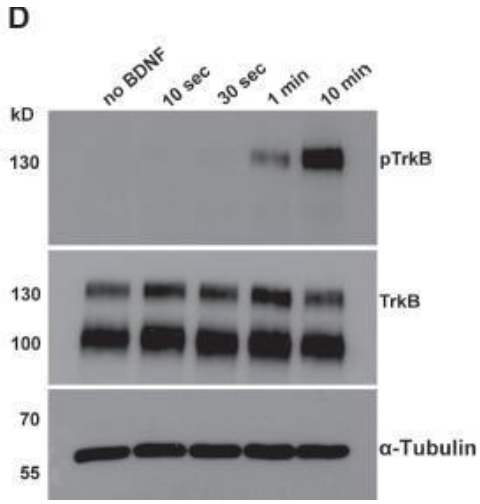
We have now outlined an example of the growth cones used for analysis in the revised Fig. 5 (original Fig. 3) and the growth cones used for visualizing TrkB. We also performed an additional experiment in which we used phalloidin to visualize outlines of the growth cones and added these data below. TrkB labeled outlines of growth cones are almost the same with those marked by F-actin.



The striking increase in signal, both for ribosomal subunits and for total TrkB, with in 10s is curious and faster than expected by motor based transport – especially since this signal extends into the most distal structures. Please refer to data showing that the antibodies for Y10B and RPL8 are conformation specific (as suggested on p8). What is the explanation for TrkB? Supporting live imaging data for at least one of the three proteins showing the dynamics upon treatment would be useful.

There is no indication in the literature that these antibodies are conformation specific. It needs to be pointed out that the alterations for ribosomal subunits and TrkB occur very fast and peak at 1 min. We hypothesize that the increased immunoreactivity for Y10B and RPL8 observed at 10 s and 1 min after BDNF stimulation is due to conformational changes in ribosomes that leads to increased exposure of epitopes to antibodies. This increase is blocked by inhibition of actin dynamics, as shown in Fig. 5L and M. In contrast, as shown in Fig. 5K, blocking of axonal transport and inhibition of translation does not change the increased staining for Y10B within 10 s or 1 min, which excludes the possibilities of increased transport of ribosomes from axon and soma to growth cones as well as local synthesis of new ribosomal components. This supports the hypothesis of conformational changes.

We have previously shown that activation of TrkB leads to a fast translocation of this receptor within seconds to the cell surface (Puehringer et al., 2013), and we believe that the rapid increase in TrkB signal just reflects this fast movement to the cell surface where it becomes more accessible for the immunostaining. Western blot analyses of motoneuron extracts from parallel cultures do not show such an increase of TrkB levels, which supports this view.



In the case of TrkB, specificity of TrkB and pTrkB antibodies is confirmed by immunostaining using TrkB knockout mice (Fig. S4A,B). Whereas total levels of TrkB do not change in Western blots of motoneuron lysates during a stimulation period with BDNF for 10 min (Fig. 5D), the immune signal increases rapidly in growth cones within less than one minute. This could be explained most likely by release of this receptor from intracellular stores but also by changes in receptor conformation after ligand binding which then favors antigen-binding (Fig. 5A). This is now clearly outlined on p. 9 in our revised manuscript.

We generated several lentiviral constructs of GFP- or mCherry-fused L10A and RPL26 for live cell imaging of ribosomes. However, these fused proteins were trapped in the nucleus of transduced motoneurons and were not detectable outside of the nucleus. We reasoned that the fluorescent tag might affect the export of ribosomes out of nucleus. For live cell imaging of TrkB, we constructed a TrkB-eGFP lentivirus and observed a fast retrograde transport of TrkB-eGFP molecules in transduced neurons after BDNF stimulation. These data are not included in this manuscript, since the retrograde transport of the TrkB is not relevant for this study.

Figure 6 would really benefit from a complementing EM image.

As outlined above, we did not include any EM images, since fixation of cultured motoneurons appeared in our hands as highly prone to generate artefacts that do not allow detection of ER structures or precise localization of TrkB or ribosomal subunits.

Figure 2G, H: image is taken at 10s stimulation, quantification at 10min or also 10s?

This was a mistake in the original version of the manuscript and has been corrected.

Reviewer 3:

We also thank this reviewer for his positive comments and specific points that helped us to improve our manuscript.

1. The most interesting data reported here involve the rapid changes induced by BDNF in ribosome activation and local protein synthesis. These data are the strongest part of the story. In contrast, the initial two figures on the relationship between the ER, actin filaments, and microtubules are not as interesting, novel, nor strong. I recommend condensing much of this initial discussion and/or moving much of this work to the supplement.

We have followed the advice and moved the original Fig. 1 into the supplement as Fig. S1.

2. A weakness of the first part of the manuscript is the authors' conclusion that they have shown that ER dynamics are dependent on "crosstalk" between the actin and microtubule cytoskeletons, or on a "coordinated actin/microtubule cytoskeleton". This may be true, but their work doesn't prove it – instead they show that depolymerization of either actin filaments or microtubules alters ER morphology in growth cones. This is not an unexpected finding and gives very little insight into the underlying mechanisms. If they wanted to strengthen this aspect of the manuscript, they could either test the effects of perturbingspecific proteins implicated in cytoskeletal crosstalk such as drebrin, or they could test the effects or dampening cytoskeletal dynamics short of full-scale depolymerization. But I'm not sure this is the most interesting direction to go, so I recommend instead that they focus on a possible role for actin dynamics in mediating the rapid ribosome activation and local translation that is the focus of the second part of the manuscript.

In order to address this point, we have now performed experiments with drebrin A and drebrin E knockdown. These experiments are now included as the new Fig. 4. They clearly show that drebrin A massively reduces ER dynamics in filopodia, and to some degree also reduces it in the core of growth cones. Interestingly, additional depletion of drebrin E has no further effect, indicating that drebrin A, which is predominant in the adult nervous system, contributes to the coordination of microtubule and actin cytoskeleton in the context of ER movement. In addition, we have also included experiments with inhibition of different myosin isoforms in the new Fig. 3, showing that in particular myosin-VI inhibition reduces ER dynamics in filopodia, whereas inhibition of myosin-II is much less efficient. Again, these effects are much more pronounced in filopodia in comparison to growth cone core.

3. The final statement in the Introduction is too strong – I disagree that they provide evidence of a novel function of axonal ER in local protein synthesis. If they were to show more directly that there is local synthesis of membrane/secreted protein, this point would be stronger.

We have now changed this sentence to "Thus, we provide evidence of a novel function of axonal ER in local protein synthesis of transmembrane proteins such as the alpha-1B subunit of presynaptic N-type Ca2+ channels."

4. The results on beta-actin are very interesting, but this part of the manuscript would be stronger if they could include data on the increased local synthesis of another protein of interest. Ideally, data on a membrane- associated or secreted protein to support their observation on RER in the presynaptic compartment.

We have now included data on increased synthesis of the alpha-1-beta subunit of the presynaptic N-type Ca2+ channels. These new data in Fig. 7E and F show that Cav2.2 levels increase within one minute of BDNF stimulation, and that this increase can be completely blocked by anisomycin (Fig. 7G) but not by nocodazole (Fig. 7H) treatment, indicating that this increase depends on local synthesis of the Cav2.2 transmembrane subunits, rather than anterior transport from somatic and proximal axonal compartments via microtubule- dependent transport.

5. How is actin involved in the RER dynamics they observe?

To address this point, we performed immunostaining of motoneurons with antibodies against RPL24, RPS6 and mCherry-ER after treatment with cytochalasin D. Results, as shown in revised Fig. 6D and Fig. 8C, revealed that inhibition of actin polymerization impedes the assembly of 80S ribosomes and also abolishes the BDNF-induced attachment of 80S ribosomes to the ER in axon terminals.

6. I appreciate that the authors sought to extend their study to in vivo observations, but this part of the manuscript is weak. If they wish to make this point, they need to solidify this observation with more supporting data.

Extension of the in vivo work would broaden this manuscript, resulting in lack of focus, and therefore we decided to take out the in vivo data.

7. The association of ribosomes with ER in the presynaptic compartment seems transient – have they measured off-rates?

We have now performed new experiments with 30 min BDNF stimulation and found that the levels of RPL24/RPS6/ER co-localization return close to baseline levels at 30 min (included in new Fig. 6B and 8B). This analysis did not allow determination of any off-rates. However, the observation of the return close to baseline levels is very pronounced, and we hope the reviewer and all readers are convinced that this indicates that the effects of assembly of RPL24/RPS6/ER are rapid and only transient.

Minor points:
1. Change "life cell imaging" to "live cell imaging" in the abstract.

Done.

2. The manuscript does not provide enough information on the use of ICS for the reader to appreciate how robust this analysis is. Inclusion of additional controls would strengthen this part of the manuscript. For example, what is the error over time if they image and analyze fixed cultures?

We have added this information by including a scheme as new Fig. 1E to demonstrate how image correlation spectroscopy works and was performed in the context of our study. In order to control the specificity and background levels, we also included analyses of fixed cells which should not show any movement. These ICS data are close to zero, as shown in the new Fig. 2C. This should provide evidence on the specificity and validity of this method.

3. The authors refer to presynaptic compartments in their motoneuron cultures. It would be good to include data showing that their cultures are synaptically connected at the time points examined.

This is a misunderstanding: As already mentioned in the response to the other reviewers, the motoneurons used for these experiments have been cultured on laminin-2/merosin under conditions used previously to differentiate presynaptic active zone structures, as shown by clustering of Cav2.2 and other active zone components such as Piccolo and Bassoon (Jablonka et al., 2007).

4. The authors state that 1 micron/sec is the fastest measured microtubule-dependent axonal transport speed, but this is not accurate – transport along the axon has been measured at rates 3-4 times this speed.

This has been corrected

5. Some of the literature discussion on ER in the axon feels out of date – this is a fast moving field, and references from 1986-2002 are of interest, but not up to date.

We have added new references regarding ER in axons: (Fernandopulle et al., 2021), (Cohen et al., 2018), (Lu et al., 2009), (Wozniak et al., 2009), (Farah et al., 2005) and (Du et al., 2006).

6. I have some concerns about their fixation of microtubules in the images shown in Figure 1, they look fragmented.

These pictures have now been replaced by technically better ones in Suppl. Fig. S1A and B.

Cited literature:

Cohen, S., A.M. Valm, and J. Lippincott-Schwartz. 2018. Interacting organelles. *Curr Opin Cell Biol*. 53:84-91.

Dombert, B., S. Balk, P. Luningschror, M. Moradi, R. Sivadasan, L. Saal-Bauernschubert, and S. Jablonka. 2017. BDNF/trkB Induction of Calcium Transients through Cav2.2 Calcium Channels in Motoneurons Corresponds to F-actin Assembly and Growth Cone Formation on beta2-Chain Laminin (221). *Frontiers in molecular neuroscience*. 10:346.

Du, Y., L. Walker, P. Novick, and S. Ferro-Novick. 2006. Ptc1p regulates cortical ER inheritance via Slt2p. *Embo j*. 25:4413-4422.
Farah, C.A., D. Liazoghli, S. Perreault, M. Desjardins, A. Guimont, A. Anton, M. Lauzon, G. Kreibich, J. Paiement, and N. Leclerc. 2005. Interaction of microtubule-associated protein-2 and p63: a new link between microtubules and rough endoplasmic reticulum membranes in neurons. *J Biol Chem*. 280:9439-9449.
Fernandopulle, M.S., J. Lippincott-Schwartz, and M.E. Ward. 2021. RNA transport and local translation in neurodevelopmental and neurodegenerative disease. *Nature Neuroscience*. 24:622-632.
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Lu, L., M.S. Ladinsky, and T. Kirchhausen. 2009. Cisternal organization of the endoplasmic reticulum during mitosis. *Mol Biol Cell*. 20:3471-3480.
Puehringer, D., N. Orel, P. Lüningschrör, N. Subramanian, T. Herrmann, M.V. Chao, and M. Sendtner. 2013. EGF transactivation of Trk receptors regulates the migration of newborn cortical neurons. *Nat Neurosci*. 16:407-415.
Wozniak, M.J., B. Bola, K. Brownhill, Y.C. Yang, V. Levakova, and V.J. Allan. 2009. Role of kinesin-1 and cytoplasmic dynein in endoplasmic reticulum movement in VERO cells. *Journal of cell science*. 122:1979-1989.

Second decision letter

MS ID#: JOCES/2021/258785

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AUTHORS: Michael Sendtner, Chunchu Deng, Mehri Moradi, Sebastian Reinhard, Changhe Ji, Sibylle Jablonka, Luisa Hennlein, Patrick Lueningschroer, Soeren Doose, and Markus Sauer
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

We have received feedback from two reviewers (another one is long overdue), who are very supportive for publication of your manuscript. However, one of them raised a minor point which requires a small amendment to your manuscript. Could you please consider this change and let me know? I would like to be able to accept your paper without further delays.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to

all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The authors carried out additional experiments and found that the inhibition of myosin VI strongly affects on ER dynamics in axon terminal filopodia. They also showed that the effects of myosin II inhibition were minor and did not reach statistical significance. These findings indicate that myosin VI is particularly relevant for ER dynamics along actin fibers in filopodia.

Comments for the author

The authors examined additional experiments to address this reviewer's coments and adequately revised the manuscript.

Reviewer 2

Advance summary and potential significance to field

The authors show that the ER is highly dynamic within motor neuron growth cones, a process regulated by actin and myosin V and VI at the filopodia, and both actin and tubular within the growth cone centre. They also describe a role for the ER in regulating local protein translation of secreted proteins.

Comments for the author

I thank the authors for carefully addressing all my queries and only have one additional comment to make regarding the wording: The abstract and introduction describe the ER as continuous, whereas the authors'
imaging suggests tubular/ vesicular fragmentation in the growth cone (Figure S1), could the authors address this observation in the text?

Second revision

Author response to reviewers' comments

Dear GiPi,

Thank you very much for the good news that our manuscript is in principle acceptable for publication.

Reviewer 2 made a comment regarding wording: “The abstract and introduction describe the ER as continuous, whereas the authors’ imaging show tubular/vesicular fragmentation in the growth cone (Fig. S1). Could the authors address this observation in the text?”

The appearance of ER as tubular/vesicular fragmented structure in Fig. S1 is caused by the optical sectioning along the Z-axis. I agree with the reviewer that this does not allow a conclusion on whether the stained structure is a continuous tubular space or more fragmented into individual tubules and vesicles. We have therefore changed our wording in the abstract: “In neurons, endoplasmic reticulum forms a highly dynamic network that enters axons…” and we deleted “and continuous”. In the introduction, we also deleted “continuous” in the first sentence “In neurons, the endoplasmic reticulum (ER) provides a luminal space throughout the cytoplasm which extends into dendrites and axons. Within presynaptic terminals, the ER forms a network with predominant

tubular appearance close to the active zone, which is highly dynamic and undergoes constant movement and reorganization, and regularly forms contact sites with the plasma membrane".

I hope very much that this adequately covers the reviewer´s point.

Thank you very much again for your very helpful comments and suggestions to revise our paper. We are very happy that our work has received such a positive response at the Journal of Cell Science.

With best regards,

Michael

Third decision letter

MS ID#: JOCES/2021/258785

MS TITLE: Dynamic remodeling of ribosomes and endoplasmic reticulum in axon terminals of motoneurons

AUTHORS: Michael Sendtner, Chunchu Deng, Mehri Moradi, Sebastian Reinhard, Changhe Ji, Sibylle Jablonka, Luisa Hennlein, Patrick Lueningschroer, Soeren Doose, and Markus Sauer
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.