JCB

# Transcytosis and transsynaptic retention by postsynaptic ErbB4 underlie axonal accumulation of NRG3

Tanveer Ahmad, Detlef Vullhorst, Rituparna Chaudhuri, Carlos Guardia, Nisha Chaudhary, Irina Karavanova, Juan Bonifacino, and Andres Buonanno

*Corresponding Author(s): Andres Buonanno, National Institutes of Health*

## Review Timeline:

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|---|---|
| Submission Date: | 2021-10-28 |
| Editorial Decision: | 2021-12-09 |
| Revision Received: | 2022-04-18 |
| Editorial Decision: | 2022-04-20 |
| Revision Received: | 2022-04-26 |

*Monitoring Editor: Louis Reichardt*

*Scientific Editor: Andrea Marat*

## 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.202110167**

December 9, 2021

Re: JCB manuscript #202110167

Dr. Andres Buonanno
National Institutes of Health
Section on Molecular Neurobiology
35 Lincoln Drive
Bldg. 35, Room 2C-1000
Bethesda, Maryland 20892

Dear Dr. Buonanno,

Thank you for submitting your manuscript entitled "Transcytosis and trans-synaptic retention by postsynaptic ErbB4 underlie axonal accumulation of NRG3". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. Happily, both reviewers find the work to be interesting although each has comments that need to be addressed in a revision. Therefore, we invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

Concerning those of reviewer #1, his/her third paragraph suggests reorganization of your bar graphs. I think this is just an optional suggestion and you should use your best judgement. Regarding next comment (Fig 2C), I think it would be reasonable for you to comment on this in the text. Regarding, the following paragraph starting with "Results: What does it mean.....), I agree that the meaning of "abundantly expressed" is not very clear. The next two paragraphs also have some thoughts that I think are useful. I do agree that there should be validations of the light-induced cleavage as well as the issues associated with overexpression. All the comments listed under "discussion" seem reasonable to me and ones that I hope you can incorporate. Most important is the final comment regarding citation of primary references.

Concerning those of the second reviewer, I think that the second paragraph is intended to be helpful and urge you to consider it seriously. The comments in the third paragraph raises the same issue raised by the first reviewer, biochemical validation of cleavage. Comments regarding Figures 1, 2 and 3 are all intended to avoid confusion by readers and seem straightforward for you to address with small textually modifications. I agree that you need to be clearer on the experiments presented in Figure 4 if you are only looking at static images. The most important of the remaining comments is that addressing the Rab dominant negative experiments. I would urge you to take the time to repeat this by assessing impact on endogenous neuregulin.

Overall, then, there are only two items that cannot be addressed textually. It seems important to validate cleavage using a biochemical assay and to repeat the Rab experiments while monitoring endogenous neuregulin. I hope that you can consider the reviewers' comments, all aimed at enhancing your study's clarity, do these two experiments and return a revision to the journal office as quickly as possible. I expect to be able to evaluate it quickly, almost certainly without involving the reviewers. To help me, I would appreciate a copy of the manuscript with your changes marked with a yellow highlighter pen or in some other obvious way. I look forward to seeing your revision.

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, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

***IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.***

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Since your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible.
Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the files will be directly linked to specific figures in the published article.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing measures that limit spread of COVID-19 also pose challenges to scientific researchers. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. 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,

Louis Reichardt, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

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

The manuscript by Buonanno and colleagues explores the mechanisms underlying NRG3 axonal transport and presynaptic accumulation. The study uses elegant cell biological and imaging approaches to investigate the spatial-temporal dynamics of NRG3 processing and trafficking in neurons. The authors report that following BACE 1-cleavage, NRG3 is allocated to somato-dendritic plasma membrane and re-endocytosed into endosomes, sorted, and transported into axons via transcytosis. Their data leads them to conclude that trans-synaptic retention of NRG3 at glutamatergic presynaptic terminals depends on interactions with its postsynaptic receptor ErbB4 on GABAergic interneurons.

Overall, this an elegant study with well-designed experiments and a well-written manuscript. The reasons to study NRG3 are well presented, and the conclusions are mostly supported by the data. However, there are several points that need clarification to make this a compelling story.

Figures: the bar graphs showing intensity density (1D, 1F, 2, C etc) present the data for the NTF and CTFs side-by-side. However, since the intensities of 2 different fluorophores cannot be compared to each other, and each fluorophore is used to look at a specific molecule, I suggest they put all the CTF bars on one graph and the NTF on another. It would make the points clearer and avoid any potential confusion by a reader.

Fig 2 C: is there a reduction in NTF fluorescence in compartments other than the PM?

Results: What does it mean by NRG3 being "abundantly expressed" (first paragraph of Results section)? Can it be express as relative to other molecules?

It would be important to validate the light-induced cleavage of the constructs by western blot.

It will be important for the authors to acknowledge that the experiments use over-expression that could lead to artifacts in the localization of the molecules.

Discussion: the authors should consider discussing the physiological implication of their findings in vivo.

Have the authors observed morphological changes in pyramidal neurons in dark condition, compared with light exposure? Is NRG3 axonal localization relevant for neurite elongation/morphology

Since NRG3 appears to be crucial for oligodendrocyte survival, it would be important to discuss if the findings are relevant to oligodendrocytes and how it would occur?

Considering the present findings and previous findings from Wang and coworkers (2018) showing that increased levels of NRG3 impairs glutamatergic, but not GABAergic release, could the authors point to the likelihood of the impact of NRG3 trafficking and transcytosis defects on neuronal physiology/circuit network?

References: should use primary references instead of referencing their own work (e.g. reference for RNA scope).

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

This paper by Ahmad et al. is rigorous with good controls, and the conclusion are largely warranted. I have some comments on data presentation and interpretation. In some cases, clearer evidence is needed to make the conclusions stronger:

- Overall, when multiple different experimental paradigms are described in same figure, consider adding a schematic corresponding to that specific experiment and visually segregating the panels in some way so it's easier to follow the logic.

- Have the authors shown that the tagged NRG3 can be cleaved by BACE1 using biochemistry? Applies for all tagged proteins.

- Fig 1: In B, why do the dendrites look so thin? Looks like these are DIV 8, so the dendrites should be more tapered (unless the neurons are unhealthy). A more detailed examination of this phenomenon (that NTF is only present in axon) at earlier and later DIVs will strengthen the case.

- Fig. 1C - low power images showing the entire neuron would be better.

- Fig. 1D - here and elsewhere, please specify what the y axis represents (int density of what?).

- "These preliminary experiments were performed in HEK293 cells". These are not "preliminary", but experiments in HEK cells, where the tools are tested first before applying to neurons.

- Are Figs 2B-C in HEK cells? This is confusing as the other experiments are in neurons (see first point). These an be moved to Supp to improve flow.

- Fig. 2D - It's not clear if after light-induced cleavage, the NTFs are also going into dendrites. This must be the case (strengthens the point that this is transcytosis), but it is not clear from the language and data shown. Again, some low power images showing the entire neuron (or a lot of it) will be a much better way to present these data (like the original Sampo/Banker VAMP2 transcytosis paper).

- Fig. 3 - again, A-D is HEK and rest are in neurons, and there are no visual cues for the reader to follow the logic. No schematics either.

- Fig. 4: How are these experiments exactly done? It seems that axonal puncta - and not moving particles - were quantified, in which case its not correct to call these "trafficking" vesicles.

- Fig. 6D-E should be combined as they seem to make a similar point. How are the authors sure that these are GABAergic interneurons? Why is that important? Is the NRG2 increased upon NRG3 knockout? What about the other NRG? How will that affect the data?

- Some kind of control experiment is needed to show that the overexpressed syntaphilin is not messing with normal trafficking.

- Pg. 11 top: The experiments are not clearly described. What are the authors exactly doing? For ex - "...neurons were transfected with x/y and after z days, q was imaged....".

- Many images (for example Fig. 3 I and J) are too small to see. This hurts the authors.

- Fig. 4 - Assuming the authors are looking at static images, how can they say there is "distinct axonal trafficking"? They are not looking at "trafficking".

- The Rab DN experiments would be better if the authors looked at endogenous NRG using an antibody, since too many things are being overexpressed in these experiments.

- Explain clearly: "....(P349KTDSILSDPTDHLGIEFMES369), an important requirement for caging by the J helix with LOV2 under dark conditions (Zimmerman et al., 2016)...".

- Clarify: "...only recently have its processing, subcellular distribution...". "...Consistent with earlier reports (Dey et al., 2017), we found that..." This sounds like Dey et al showed that NRG4/Rab4 colocalize and the authors are just confirming this...is that the case?

## Responses to Reviewers

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

Overall, this an elegant study with well-designed experiments and a well-written manuscript. The reasons to study NRG3 are well presented, and the conclusions are mostly supported by the data. However, there are several points that need clarification to make this a compelling story.
Response: We are very pleased to hear that the Reviewer found our work to be elegant and well-designed, that the manuscript is well written and the conclusions are supported by our data.

1. Figures: the bar graphs showing intensity density (1D, 1F, 2, C etc) present the data for the NTF and CTFs side-by-side. However, since the intensities of 2 different fluorophores cannot be compared to each other, and each fluorophore is used to look at a specific molecule, I suggest they put all the CTF bars on one graph and the NTF on another. It would make the points clearer and avoid any potential confusion by a reader.
Response: We understand the general point raised by the reviewer and have modified many of the figures as suggested. However, in some cases when we rearranged the panels as suggested, we felt that the emphasis of the data shown was diminished rather than improved. In our view, the color coding helps the reader understand that comparisons are with-in group (i.e. red vs. red) and not across groups (i.e., red vs. green).

2. Fig 2 C: is there a reduction in NTF fluorescence in compartments other than the PM?
Response: There is a general increase in the NTF fluorescence in all the compartments upon light activation, but the signal is substantially higher on the PM at any given point of time.

3. Results: What does it mean by NRG3 being "abundantly expressed" (first paragraph of Results section)? Can it be express as relative to other molecules?
Response: We have rewritten this sentence (page 5) to convey more clearly that, relative to NRG1 and NRG2, NRG3 transcripts are expressed more broadly and at relatively higher levels during prenatal and postnatal brain neurodevelopment.

4. It would be important to validate the light-induced cleavage of the constructs by western blot.
Response: As requested, in the revised manuscript we now show by Western blot that cleavage of the LOV-containing light-inducible NRG3 construct (LA-NRG3) transfected in HEK293 cells is promoted by exposure of cells to blue light, relative to the cells kept in the dark. Please refer to the supplementary Figure S4B.

5. It will be important for the authors to acknowledge that the experiments use over-expression that could lead to artifacts in the localization of the molecules.
Response: We have added a comment in the Discussion addressing this point (page 17) and the measures we have taken to reduce this possibility. In an additional experiment we performed for the revision of the manuscript using an independent approach (Figure 7G,H), we demonstrate that reduction of *endogenous Rab4* function results in a decreased accumulation of *endogenous NRG3* at axonal terminals.

6. Discussion: the authors should consider discussing the physiological implication of their findings in vivo.
Response: The final paragraph of our Discussion (page 18/19) addressed several physiological implications of our findings, which included a role of NRG3-ErbB4 in the formation, maintenance, maturation and function of glutamatergic synapses selectively onto GABAergic interneurons. We also went on to discuss the similarities and differences between NRG3 and the CRD-NRG1 type III variant (page 18). In response to the Reviewer's request, based on observations by Carteron et al, 2006 from the Dr. Cabedo's lab, we added a comment in the Discussion (page 18) about the potential implication of our findings to oligodendrocyte survival.

7. Have the authors observed morphological changes in pyramidal neurons in dark condition, compared with light exposure? Is NRG3 axonal localization relevant for neurite elongation/morphology .
Response: We have not noticed any morphological/elongation differences in dark vs. light conditions, but those type of changes could be difficult to observe without utilizing specific labeling and quantitative analytical procedures.

8. Since NRG3 appears to be crucial for oligodendrocyte survival, it would be important to discuss if the findings are relevant to oligodendrocytes and how it would occur?
Response: In our Introduction and revised Discussion (see response to Point #6 above) we refer to work from Dr. H. Cabedo's lab (Carteron et al., 2006) that reports on the effects of NRG3 on oligodendrocyte survival; however, we feel uncomfortable speculating on "how it would occur".

9. Considering the present findings and previous findings from Wang and coworkers (2018) showing that increased levels of NRG3 impairs glutamatergic, but not GABAergic release, could the authors point to the likelihood of the impact of NRG3 trafficking and transcytosis defects on neuronal physiology/circuit network?
Response: As mentioned above, we have speculated about the relevance of our findings to glutamatergic synapse development and stabilization (see Point #6). It is worth noting that, contrary to publications from Carmen Birchmeier's group (Muller et al. 2018) and our lab (Vullhorst, Ahmad et al., 2017; this manuscript), and as we elaborate in our Discussion, Wang et al. proposed that proNRG3 exerts its biological effects on glutamate release via its C-terminal domain.

10. References: should use primary references instead of referencing their own work (e.g. reference for RNA scope).
Response: Thank you for catching this oversight, we have included the reference to the original literature from ADC published in the Journal of Molecular Diagnosis (page 5). The prior reference is used when referring to the specific methodology used for mouse brain sample preparation and analysis.

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

This paper by Ahmad et al. is rigorous with good controls, and the conclusion are largely warranted. I have some comments on data presentation and interpretation. In some cases, clearer evidence is needed to make the conclusions stronger:
Response: We are very pleased the Reviewer found our work to be rigorous and the conclusions supported by the data.

2. Overall, when multiple different experimental paradigms are described in same figure, consider adding a schematic corresponding to that specific experiment and visually segregating the panels in some way so it's easier to follow the logic.
Response: Thank you for the useful suggestions. We have added new schematics to Figures 1 (panel I), 3 (panel B) and 5 (panel J), whereas Figures 2 & 6 already had schematic representations. Furthermore, we have simplified Figure 2 by moving the HEK293 part (panels B,C) to the supplemental materials (now Figures S3D and S4A, respectively) and separated previous Figure 3 into two figures (Figures 3 and 4), each of which showing single experimental approaches. Lastly, in Figures 3 and S4, we visually separated experiments performed in HEK293 cells from experiments performed in neurons.

3. Have the authors shown that the tagged NRG3 can be cleaved by BACE1 using biochemistry? Applies for all tagged proteins.
Response: As requested, we performed additional experiments that demonstrate by Western blotting that exposure of transfected HEK293 cells to blue light, relative to the cells kept in the dark, enhances the cleavage of the LOV-containing light-inducible LA-NRG3 (Figure S4). This new biochemical data, in addition to imaging experiments demonstrating that: 1) the degree of fluorescence overlap from the N-terminal GFP and C-terminal mCherry tags is reduced following blue-light exposure (Fig. 2B-G), 2) treatment of LA-NRG3 transfected neurons with the BACE-IV inhibitor results in a higher degree of fluorescence overlap following light exposure (Fig. S2A) and 3) a cleavage-resistant LA-NRG3 construct harboring a mutated BACE cleavage site retains overlapping fluorescence upon illumination (Fig. 2H, Fig. S4E), make a compelling case that the LOV-containing LA-NRG3 is cleaved by BACE1 in response to blue light.

In addition, we are now demonstrating BACE-dependent processing of GFP-NRG3$^{BBS}$ (used for surface labeling experiments shown in Figure 3) by Western blotting and added the corresponding panel to Figure 3C. Processing of V5 epitope-tagged NRG3 was already demonstrated in the original submission (now Figure S2A). Importantly, Figure S3A also showed that the cleavage-resistant (cr) NRG3 construct harboring a mutated BACE1 site is not cleaved.

4. Fig 1: In B, why do the dendrites look so thin? Looks like these are DIV 8, so the dendrites should be more tapered (unless the neurons are unhealthy). A more detailed examination of this phenomenon (that NTF is only present in axon) at earlier and later DIVs will strengthen the case.
Response: In response to this comment, the panel has been replaced with a more representative image that allows the reader to see more clearly dendrites and axons.

5. Fig. 1C - low power images showing the entire neuron would be better.

Response: In response to reviewer's suggestion, we have provided a new lower power image that shows an entire neuron.

6. Fig. 1D - here and elsewhere, please specify what the y axis represents (int density of what?).
Response: We have mentioned the protein(s) for which the integrated density values were calculated. The text now reads as "Integrated density [NTF / CTF]. Similar changes were made to the Y-axis labels in figure panels 5G,I, 6H and 7F.

7. "These preliminary experiments were performed in HEK293 cells". These are not "preliminary", but experiments in HEK cells, where the tools are tested first before applying to neurons.
Response: Thank you for bringing this point to our attention, the Reviewer is correct and we have revised the text accordingly.

8. Are Figs 2B-C in HEK cells? This is confusing as the other experiments are in neurons (see first point). These can be moved to Supp to improve flow.
Response: Thank you for the comment. As suggested, we have moved the HEK293 results to supplementary data (new Figure S3D and S4A, respectively) to improve the flow of the paper.

9. Fig. 2D - It's not clear if after light-induced cleavage, the NTFs are also going into dendrites. This must be the case (strengthens the point that this is transcytosis), but it is not clear from the language and data shown. Again, some low power images showing the entire neuron (or a lot of it) will be a much better way to present these data (like the original Sampo/Banker VAMP2 transcytosis paper).
Response: The panel (now Figure 2B) already has a low-power images of an entire neuron showing the NRG3 NTF in soma and neurites before and after photoactivation. The images clearly illustrate the release of the NTF from the TGN and is broad distribution throughout the neuron.

10. Fig. 3 - again, A-D is HEK and rest are in neurons, and there are no visual cues for the reader to follow the logic. No schematics either.
Response: Thank you for the comment. As suggested, we have visually separated HEK293 from neuron data (same for Figure S4).

11. Fig. 4: How are these experiments exactly done? It seems that axonal puncta - and not moving particles - were quantified, in which case its not correct to call these "trafficking" vesicles.
Response: We have included additional details and revised the writing to clarify that these are not "trafficking vesicles".

12. Fig. 6D-E should be combined as they seem to make a similar point. How are the authors sure that these are GABAergic interneurons? Why is that important? Is the NRG2 increased upon NRG3 knockout? What about the other NRG? How will that affect the data?
Response: Panels D and E make related, yet distinct points, as (D) shows that the ErbB4 shRNA effectively reduces ErbB4 protein and (E) shows that ErbB4 knockdown reduces NRG3 signals in GABAergic interneurons, which in these cultures are the only cells to co-express ErbB4 and NRG2 puncta (Longart et al., 2007 [PMID: 17562386], Vullhorst et al., 2015 [PMID: 26027736]).

Therefore, in ErbB4 knockdown experiments, we used NRG2 puncta to identify GABAergic interneurons. We have not observed any overt changes in NRG2 signal intensities due to ErbB4 knockdown. Our data are in full agreement with the hypothesis of NRG3-ErbB4 retention, as NRG3 signals on NRG2-positive GABAergic interneurons are strongly reduced with ErbB4 KD, whereas no effect of ErbB4 KD was observed in NRG2-negative pyramidal neurons.

13. Some kind of control experiment is needed to show that the overexpressed syntaphilin is not messing with normal trafficking.
Response: Wild-type syntaphilin is involved in docking axonal mitochondria to the microtubule-based cytoskeleton. The GFP-tagged variant we have used in this work (GFP-SNPHΔMTB; developed by Kang et al., 2008 [PMID: 18191227]) associates with axonal mitochondria but fails to immobilize them due to its inability to interact with microtubules, thereby rendering it nonfunctional. Importantly, we have previously used GFP-SNPHΔMTB in transfected hippocampal neurons to verify the identity of neurites showing NRG3 accumulation and did not observe any adverse effect of GFP-SNPHΔMTB compared to soluble GFP (Vullhorst et al., 2017 [PMID: 28432142]).

14. Pg. 11 top: The experiments are not clearly described. What are the authors exactly doing? For ex - "...neurons were transfected with x/y and after z days, q was imaged....".
Response: We have incorporated the requested experimental details in Methods (page 22).

15. Many images (for example Fig. 3 I and J) are too small to see. This hurts the authors.
Response: Thank you for this helpful comment. Because Figures 2 and 3 were very data heavy, we decided to move the HEK293 panels B,C in Figure 2 to Suppl Info (Figures S3D and S4A). We also separated Figure 3 into two figures. In addition, we increased the micrographs in former Figures 4 and 5 (now Figures 5 and 6).

16. Fig. 4 - Assuming the authors are looking at static images, how can they say there is "distinct axonal trafficking"? They are not looking at "trafficking".
Response: The Reviewer's point is well taken. The text has been modified throughout the manuscript to address this concern.

17. The Rab DN experiments would be better if the authors looked at endogenous NRG using an antibody, since too many things are being overexpressed in these experiments.
Response: Because of experimental limitations we could not perform precisely the suggested experiment. That being said, we are excited to add a new (and arguably more informative) experiment that directly addresses the functional importance of Rab4 activity for *endogenous NRG3* axonal transport by analyzing NRG3 presynaptic accumulation in cultured neurons transduced with AAVs expressing either wild-type (wt) or dominant-negative (dn) Rab4. Consistent with our earlier data using NRG3 overexpression (see Figure 5), we found that presynaptic accumulation of *endogenous NRG3* puncta on ErbB4+ GABAergic interneurons was markedly reduced in cultures transduced with dn Rab4 compared to wt Rab4. This new experiment has been added to the manuscript (Fig. 7 G, H), and we believe it has strongly enhances the conclusions of our findings.

18. Explain clearly: "....(P349KTDSILSDPTDHLGIEFMES369), an important requirement for caging by the J helix with LOV2 under dark conditions (Zimmerman et al., 2016)...".
Response: Thank you for bringing this to our attention. In response, we have revised the text to simplify the language (page 7).

19. Clarify: "...only recently have its processing, subcellular distribution...". "...Consistent with earlier reports (Dey et al., 2017), we found that..." This sounds like Dey et al showed that NRG4/Rab4 colocalize and the authors are just confirming this...is that the case?
Response: Thank you for the comment. We have revised the text to point out more clearly that our observation of NRG3 sorting into Rab4+ vesicles in axons, is consistent with the reported role of Rab4-associated vesicles in axonal anterograde transport affecting synapse organization (page 10).

April 20, 2022

RE: JCB Manuscript #202110167R

Dr. Andres Buonanno
National Institutes of Health
Section on Molecular Neurobiology
35 Lincoln Drive
Bldg. 35, Room 2C-1000
Bethesda, Maryland 20892

Dear Dr. Buonanno:

Thank you for submitting your revised manuscript entitled "Transcytosis and trans-synaptic retention by postsynaptic ErbB4 underlie axonal accumulation of NRG3". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

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 is < 40,000, not including spaces. Count includes abstract, introduction, results, discussion, and acknowledgments. Count does not include title page, figure legends, materials and methods, references, tables, or supplemental legends.

2) Figures limits: Articles may have up to 10 main text figures.

3) 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.

4) 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 (either in the figure legend itself or 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 parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

6) 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 in the text for readers who may not have access to referenced manuscripts.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
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.).

9) 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.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. 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.

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

12) 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."

13) 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.

14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

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