Opposing functions for retromer and Rab11 in extracellular vesicle traffic at presynaptic terminals

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Review #1

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Cannot tell / Not applicable

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

This manuscript describes the transport mechanisms by which fragments of APP, specifically the C terminal region are deposited into the extracellular space through neuronal extracellular vesicles. To do this work, the authors describe a transgene of APP with a GFP tag that is then used to track APP fragment secretion into EVs. The initial part of the
manuscript describes the use of this reagent to study EV based APP deposition at the Drosophila NMJ. The second part of the paper describes the complex pathways of membrane trafficking in the neuron ad maps the rout of APP containing EVs in this context. The final conclusion presented is that a Rab11 and retromer dependent process specifically controls APP containing EV trafficking at the NMJ.

**Major comments:**

*Are the key conclusions convincing?*

The paper presents an absolutely enormous amount of data analysing many aspects of trafficking at the NMJ. While each bit of analysis in itself seems fine, it is very hard for the general reader to appreciate a global picture of what exactly is going on. There is analysis of multiple rab compartments, retromer function, etc but the paper was hard to assimilate. The authors may be better served by removing non-essential data or minimising it. This will allow the key message to be better presented and appreciated.

The problem of specific cargo trafficking within a cell in the midst of a sea of ongoing trafficking reactions is the nub of the problem here. This is very hard to clearly demonstrate and while much of the data presented is an effort to get to this goal, right now it is very confusing.

*Should the authors qualify some of their claims as preliminary or speculative, or remove them altogether?*

The presentation of data to show that there is a specific defect in APP-EV trafficking which is distinctive from a general endo-lysosomal dysfunction on loss of retromer is hard for the reader to assimilate.

*Would additional experiments be essential to support the claims of the paper? Request additional experiments only where necessary for the paper as it is, and do not ask authors to open new lines of experimentation.*

No, indeed I would suggest removing all non-essential data or simplifying its presentation.
Also the entire analysis of retromer (except one assay using Vps26) is based on a mutant in Vps35. If the key element being analysed here is the retromer complex, I suggest that 2-3 key phenotypes in the study be verified with more than one retromer component.

*Are the data and the methods presented in such a way that they can be reproduced?*

yes

*Are the experiments adequately replicated and statistical analysis adequate?*

yes

**Minor comments:**

*Specific experimental issues that are easily addressable.*

nil except for retromer component analysis comment.

*Are prior studies referenced appropriately?*

yes

*Are the text and figures clear and accurate?*

mostly fine other than that there is too much data
3. Significance:

Significance (Required)

*Describe the nature and significance of the advance (e.g. conceptual, technical, clinical) for the field.*

There are some key technical advances here. The use of the C-terminal GFP tagged APP transgene will be a valuable tool for this and future studies. From the point of Alzheimer's disease therapy which the manuscript speaks to in the introduction, there will be a need to find components or pathways that work relatively specifically and distinct from the general trafficking machinery of neurons, if there is to be a hope of developing a therapeutic molecule.

*Place the work in the context of the existing literature (provide references, where appropriate).*

Many aspects of retromer and Rab function described here are already well known from other studies even in neurons.

*State what audience might be interested in and influenced by the reported findings.*

A clear definition of how APP fragments are specifically trafficked into EV, distinct from other cargoes.

*Define your field of expertise with a few keywords to help the authors contextualize your point of view. Indicate if there are any parts of the paper that you do not have sufficient expertise to evaluate.*

Cellular neuroscience, membrane trafficking, Drosophila, brain disorders
**Referees cross commenting**

Agree with the summary comment added by reviewer 3.

**Review #2**

1. How much time do you estimate the authors will need to complete the suggested revisions:

   Estimated time to Complete Revisions (Required)

   (Decision Recommendation)

   Between 1 and 3 months
2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

The technical quality of the paper appears to me to be high, the reproducibility of a similarly high quality (the stats appear sound to me) and the microscopy of leading edge quality. The question that the authors are addressing places the traffic of cargo in a nexus where the ingress and egress is multiple, making the clarity of the paper an issue, but one that the authors have done well to address. In essence the area is complex, and explaining the experiments and their outcomes complex. I feel they have done an admirable job. The schematic in Figure 9 could be improved with minor adjustments: indicate on the figure the early endosome, MVB, recycling endosome etc. Also, In Fig 1a, give an indication of cytoplasm and extracellular orientation. The uninitiated may not be aware that ICD stands for intracellular domain. Perhaps also give a key to the domain structure (E1, E2 etc) in the figure legend.

My technical comments would be the following:

For the GFP intensity measurements, might the difference in intensity be generated by inclusion of the EV in a low pH compartment, or an alteration of the pH in the compartment? The tkv, syt4 and syt1 data argues against this, but the images within the axon and the cell body could be due to this effect.

Is the recycling of APP (or other EV cargoes) entirely through the slow (Rab11) compartment? What about the Rab4 dependent recycling compartment?

I'm uncomfortable with the Rab11/Vps35 double mutant. It would be preferable to be working with null alleles to allow firmer conclusions, and the Rab11 93Bi allele is not a null.

The majority of the trafficking imaging is based on ectopically expressed APP-eGFP and genomically tagged Syt4-GFP. The forced expression of the APP, and the tagging of Syt4 lead to doubts about the natural trafficking of these proteins in the conditions used in the experiments. Is there an antibody against an endogenously expressed EV cargo that could be used in the Figure 8 experiments to back up the conclusions from this figure? (Anti-Arc1? See https://pubmed.ncbi.nlm.nih.gov/29328915/).
3. Significance:

Significance (Required)

The significance of the paper is a subtle issue. My interpretation lies in my last comment for the reproducibility section. The title of the article speaks of retromer and Rab11 at pre-synaptic terminals, and I accept many of the conclusion with the caveat that much of the data rests on either ectopic expression of APP, or a genomically GFP-tagged Syt4. If the title was about APP trafficking, I feel that the level of analysis and conclusions would be excellent as another model of APP traffic in a tractable neuron, albeit with an ectopic expression, and I feel it would be a welcome addition to the APP literature. But if the paper is making the case for retromer and Rab11 generally, I would prefer to see an endogenous un-tagged marker used to back up the conclusions made. Nonetheless, the understanding of membrane traffic at the distal end of a very elongated structure is a very important one, and one that requires study in a functional synapse which this study provides. I feel to some degree that if I was to say 'so what, how does this impinge on synapse function and growth?' would be a bit severe, but the answer is made in the evident changes to synapse structure in Figure 4 and perhaps the authors could emphasise this more?

**Referees Cross commenting**

I would agree with reviewer #3, a re-focus on the claims of the paper allied to a trimming of the material to drill down on either the Rab11 v retromer or APP traffic.
1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Less than 1 month

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

In their manuscript, the authors demonstrate that APP is secreted from drosophila motor neurons in extracellular vesicles. This route of secretion is shared by Syt4 and Tkv but not Syt1 and increased with defects in the retromer component Vps35.

Vps35 defects elicit a wide range of phenotype that can be separated genetically.

Vps35 defects elicit differential changes in endolysosomal vesicle pools: fewer Rab5 puncta, more Rab7 puncta, fewer but larger Rab11 puncta. Expressing DN and CA mutants of Rab5 and Rab11 change the EV pool more strongly than mutant Rab7. Also, the pool of Rab7-positive vesicles is not sensitive to Snx1 and Snx6, which control the EV branch of Vps35 signalling.
Vps35 defect increases the fraction of APP in Rab11 compartment.

Vps35 defect increases the fraction of Syt4 puncta (presynaptic and EV) whereas Rab11 decreases this fraction (not shown for APP).

I must admit that I am new to the area of pre-submission review. Therefore this review is a mixture of advice I would give colleagues when reading their manuscript before submission and a post submission review.

The data is sound and the writing is overall good. There are several points where I think writing can be improved or things explained better. They are detailed below.

To some extent the manuscript contains two stories and the authors have not entirely decided which one they are telling. One is the story of APP secretion in extracellular vesicles. The other story is about the different pathways regulated by Vps35 in dorosiphila motor neurons.

For the first story, Figures 4 and 5 are dispensable, but it needs to be demonstrated that the quintessential experiment (Figure 8D/E) - that the excessive secretion of EVs by the Vps35 defect can be rescued by Rab11 - also applies to APP.

Conversely, if the story is mainly about the different facets of Vps35, one could show the new role for retromer at the example of Syt4 and in a second step demonstrate that APP is also a substrate of this pathway. The title and the very nice mechanistic work elucidating the consequences of Vps35 defect in dorosiphila neuromuscular junctions argue for the second story.

Hence, the key conclusion from the title is shown convincingly for Syt4, but the long introduction about APP and Figure 1 raise the expectation that the antagonism of Vps35 and Rab11 are also shown for APP and not only Syt4. I cannot say whether this experiment are realistic. It is so obvious that I almost think that the authors tried but that it was technically challenging or produced inconclusive results.

There are only minor issues of overinterpretation detailed below and minor points where I think methods or processing of data could be explained better.

**Minor comments:**

p.2: "By contrast, a cytosolic presynaptic protein, Complexin (Cpx), was excluded from postsynaptic APP-EGFP puncta, indicating that they arise from specific presynaptic sorting events rather than from non-specific shedding of presynaptic membrane and cytoplasm (Fig. 1B)."
I consider this overinterpretation. In small structures such as EVs or spines, there is comparably much membrane and little cytosol, so it is natural that the puncta are positive for membrane markers and negative for the cytosolic marker.

Revise language in legend to Figure 1B: p.2: (B) Maximum intensity projection (MaxIP) of motor-neuron-derived APP-EGFP localization presynaptically and to extraneuronal puncta that exclude the presynaptic cytoplasmic protein Complexin (Cpx).

Clarify language: p.2: "Thus, a large proportion of presynaptic APP is trafficked into postsynaptic EV-like structures that are associated with the neuronal membrane." -> large proportion: do you refer to the extraneuronal vesicles only or to all of them? associated with neuronal membrane: do you mean that the EVs remain in contact with the axon or do you mean that they contain neuronal membrane markers?

p.4: "Taken together, these results indicate that APP-EGFP, and particularly a CTF of this protein, are trafficked into EVs by multiple established criteria"

-> I would tone it down to say "can be trafficked into EVs in S2 cells" since this experiment - while important - does not show that they are EVs in flies.

Figure 2 A-C: This is not explained in sufficient detail. What are the yellow lines in Figure 2A? How were the thresholds defined? What are the VPS mutants? Which is the postsynaptic compartment?

Figure 2D: Not sufficiently explained what the two planes (?) of confocal images are, what the yellow line is surrounding.

Figure 2F: The alternating colors make it hard to understand. Since this is false colors anyway, I suggest to keep the same color for e.g. HRP. Also, the labels on the left of the images are hard to read, but they are the key to understanding the figure. I suggest to write them horizontally or in two lines to make the figure easier to grasp. This is one of the most important figures of the manuscript.

Why is there no quantification of postsynaptic Syt1? - Discuss in the text or legend. Why is there no quantification for CD8? -> either add the quantification or remove the images.

p.5: "We noted a significant increase in postsynaptic 50-100 nm vesicles (consistent with the expected size for endosomally-derived exosomes) in the
muscle SSR immediately surrounding the neuron (Fig 3A,B), suggesting that retromer mutant NMJs release more EVs."

-> This sounds inconsistent with the earlier statement "p.5: Using structured illumination microscopy (SIM), we resolved individual presynaptic puncta and found that increased APP-EGFP levels occurred as larger and more intense puncta, rather than as a greater density of puncta (Fig S2A)" -> Discuss possible explanations.

Does the term "postsynaptic" really refer to the EVs? If so, it is somewhat misleading -> use "extrasynaptic" or "extracellular" instead?

Figure 5: The Vps superscripts are very hard to read, consider changing the way they are displayed, e.g. to brackets.

Explain SIM abbreviation in legend to Figure 6.

Quite often, there are too many references cited without specifying what they are cited for. It is good practice to give credit to everyone, but this massive amount is not helpful for the reader. An extreme example is this: "p.9: Depending on the specific cell type and cargo, loss of retromer causes differential effects on cargo flux through the endolysosomal pathway, and can result in altered cargo levels either in early endosomes, late endosomes, or on the plasma membrane. (Chen et al., 2013; Hussain et al., 2014; Jimenez-Orgaz et al., 2017; Loo et al., 2014; Pocha et al., 2011; Steinberg et al., 2013; Strutt et al., 2019; Tian et al., 2015; Vazquez-Sanchez et al., 2018; Wang et al., 2014; Wang et al., 2013; Zhou et al., 2011). "

Explain why Mander's coefficients were used for Rab8 and not Pearson's, given that the latter is more common.

3. Significance:

Significance (Required)

The story about the role of Vps35 and Rab11 in drosophila motor neurons is
an important step forward in understanding how endosomal vesicles are regulated - in particular since the authors cite a large body of work demonstrating that many aspects of the investigated pathway differ between cell types. Because this signaling pathway touches on many aspects of physiology and disease, it will be of interested to a wide range of readers.

The translational aspect that puts the finding in the context of APP processing and hence Alzheimer's disease of course increases significance, but this story is unfortunately incomplete, so the authors need to decide whether to finish it or shift their focus on the physiology.

**Referees cross commenting**

My feeling is that the three reviews each have a specific take, but they address the same issue and do not contradict each other.

I think that all agree that it is excellent data that should be published, but I think that all three would discourage the authors from moving on with the current text/figures and would rather encourage them to revise the flow of the manuscript before submission, focusing either on the translational issue of APP processing or on the more mechanistic/cell biological focus of Rab11 and retromer.
Response to reviews

Overall Positives: The reviewers appreciated the overall impact and quality of our work:

- The importance of understanding how cargoes are trafficked into EVs, specifically in neurons
- The novelty of our finding that retromer functions in EV cargo traffic, and that we were able to provide mechanistic insight, showing that it plays a highly specific role counteracting recycling rather than general role in endolysosomal traffic.
- The relevance of our work to understanding APP traffic and the insights it provides for specifically targeting the EV pathway in Alzheimer’s Disease.
- The overall quality and rigor of our data and analyses

Main criticism: The reviewers felt that the paper contained too much data for a single story. Please note that a previous version of this manuscript was evaluated by JCB in June of 2019 (manuscript ID #201906027) – the editors were in principle interested in our findings but requested that we add more mechanistic studies before agreeing to send it out for review. Our revised and expanded manuscript, as submitted to Review Commons, contains these additional mechanistic studies (on which the reviewers were satisfied regarding depth and rigor), but as a consequence they felt that the story became quite long and dense.

The reviewers suggested selecting one of two main stories to focus on: (1) Traffic of APP or (2) the mechanism of retromer function in EV traffic and its balance with Rab11-mediated trafficking. Our intention is to tell the latter story, using APP as one of several example cargoes.

We propose two alternative solutions to this issue, and would appreciate the editors’ input into which would be best:

1. Go ahead with submitting the entire manuscript (incorporating specific reviewer suggestions) to JCB, with the understanding that Figure 1 is included primarily for validation of APP as a cargo in this system, and perhaps moving this figure to Supplemental.
2. Coordinate consideration of the mechanistic retromer-Rab11 story at JCB with a companion paper, perhaps to Life Science Alliance. This paper would be entitled “A toolkit for investigating extracellular vesicle cargo traffic in the Drosophila nervous system”. It would include a detailed description and validation of tools for evaluating traffic of APP, Syt4, and neuroglian (see below, another newly identified cargo that would address a reviewer concern for the mechanism-oriented manuscript, but would add yet more validation data were we to include it in a single large paper). Figures 1 and S1 from the current manuscript would be moved to this paper. Importantly the reviewers recruited by Review Commons for the original submission could incorporate this second manuscript into their re-revision, and it would address their request that the remaining story be more
This manuscript describes the transport mechanisms by which fragments of APP, specifically the C terminal region are deposited into the extracellular space through neuronal extracellular vesicles. To do this work, the authors describe a transgene of APP with a GFP tag that is then used to track APP fragment secretion into EVs. The initial part of the manuscript describes the use of this reagent to study EV based APP deposition at the Drosophila NMJ. The second part of the paper describes the complex pathways of membrane trafficking in the neuron ad maps the rout of APP containing EVs in this context. The final conclusion presented is that a Rab11 and retromer dependent process specifically controls APP containing EV trafficking at the NMJ.

**Major comments:**

*Are the key conclusions convincing?*

The paper presents an absolutely enormous amount of data analysing many aspects of trafficking at the NMJ. While each bit of analysis in itself seems fine, it is very hard for the general reader to appreciate a global picture of what exactly is going on. There is analysis of multiple rab compartments, retromer function, etc but the paper was hard to assimilate. The authors may be better served by removing non-essential data or minimising it. This will allow the key message to be better presented and appreciated.

The problem of specific cargo trafficking within a cell in the midst of a sea of ongoing trafficking reactions is the nub of the problem here. This is very hard to clearly demonstrate and while much of the data presented is an effort to get to this goal, right now it is very confusing.

*Should the authors qualify some of their claims as preliminary or speculative, or remove them altogether?*

The presentation of data to show that there is a specific defect in APP-EV trafficking which is distinctive from a general endo-lysosomosal dysfunction on loss of retromer is hard for the reader to assimilate.

*Would additional experiments be essential to support the claims of the paper? Request additional experiments only where necessary for the paper as it is, and do not ask
authors to open new lines of experimentation.*

No, indeed I would suggest removing all non-essential data or simplifying its presentation. Also the entire analysis of retromer (except one assay using Vps26) is based on a mutant in Vps35. If the key element being analysed here is the retromer complex, I suggest that 2-3 key phenotypes in the study be verified with more than one retromer component.

We can add some of the following experiments in a Vps26 mutant to complement our Vps35 results:

R1 Experiment 1: To complement our finding that Vps26 mutants show increased levels of the EV cargo Syt4, we have examined an independent EV cargo, neuroglian. We have already done this experiment, and Vps26 mutants show the expected increase in pre and postsynaptic levels of this cargo.

![Image](image)

However, neuroglian has not yet been published as an EV cargo (though there are hints in the literature), and including this experiment would require us to add even more validation data (though we do have it in hand) showing that Neuroglian is an EV cargo. While we are happy to include yet another supplemental figure, another possibility is to coordinate publication of this validation (as well as the current figure 1 validating APP) in a tools paper, for example in Life Science Alliance

R1 Experiment 2: Rab11 compartment characterization in vps26 mutants
R1 Experiment 3: EV cargo levels (Syt4 and Neuroglian) in Vps26 rab11 double mutants to support the conclusion that these pathways act in opposition.

These experiments can be completed in the next month
*Are the data and the methods presented in such a way that they can be reproduced?*

yes

*Are the experiments adequately replicated and statistical analysis adequate?*

yes

**Minor comments:**

*Specific experimental issues that are easily addressable.*

nil except for retromer component analysis comment.

*Are prior studies referenced appropriately?*

yes

*Are the text and figures clear and accurate?*

mostly fine other than that there is too much data

Reviewer #1 (Significance (Required)):

*Describe the nature and significance of the advance (e.g. conceptual, technical, clinical) for the field.*

There are some key technical advances here. The use of the C-terminal GFP tagged APP transgene will be a valuable tool for this and future studies. From the point of Alzheimer’s disease therapy which the manuscript speaks to in the introduction, there will be a need to find components or pathways that work relatively specifically and distinct from the general trafficking machinery of neurons, if there is to be a hope of developing a therapeutic molecule.

*Place the work in the context of the existing literature (provide references, where appropriate).*

Many aspects of retromer and Rab function described here are already well known from other studies even in neurons.

*State what audience might be interested in and influenced by the reported findings.*

A clear definition of how APP fragments are specifically trafficked into EV, distinct from
other cargoes.

*Define your field of expertise with a few keywords to help the authors contextualize your point of view. Indicate if there are any parts of the paper that you do not have sufficient expertise to evaluate.*

Cellular neuroscience, membrane trafficking, Drosophila, brain disorders

**Referees cross commenting**

Agree with the summary comment added by reviewer 3.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

The technical quality of the paper appears to me to be high, the reproducibility of a similarly high quality (the stats appear sound to me) and the microscopy of leading edge quality. The question that the authors are addressing places the traffic of cargo in a nexus where the ingress and egress is multiple, making the clarity of the paper an issue, but one that the authors have done well to address. In essence the area is complex, and explaining the experiments and their outcomes complex. I feel they have done an admirable job. The schematic in Figure 9 could be improved with minor adjustments: indicate on the figure the early endosome, MVB, recycling endosome etc. Also, In Fig 1a, give an indication of cytoplasm and extracellular orientation. The unininitiated may not be aware that ICD stands for intracellular domain. Perhaps also give a key to the domain structure (E1, E2 etc) in the figure legend.

We will make these changes to the figures.

My technical comments would be the following:

For the GFP intensity measurements, might the difference in intensity be generated by inclusion of the EV in a low pH compartment, or an alteration of the pH in the compartment? The tkv, syt4 and syt1 data argues against this, but the images within the axon and the cell body could be due to this effect.

We don’t believe that this is likely since the samples are fixed and permeabilized, removing the issue of compartment pH. We have also overstained with anti-GFP nanobody in SIM images, which would not be subject to pH-dependent fluorescence changes. However, we can add an experiment showing the effects of Retromer mutants on endogenous, untagged neuroglian, which would also address this reviewer’s concern about examining tagged proteins (See data above for R1 Experiment 1).
Is the recycling of APP (or other EV cargoes) entirely through the slow (Rab11) compartment? What about the Rab4 dependent recycling compartment?

We have not examined the Rab4 compartment, and we are hesitant to add more data to an already heavy manuscript, but we are happy to mention this possibility in the Discussion.

I'm uncomfortable with the Rab11/Vps35 double mutant. It would be preferable to be working with null alleles to allow firmer conclusions, and the Rab11 93Bi allele is not a null.

Unfortunately this is not possible as rab11 nulls are embryonic lethal (Dollar 2002). We can clarify in the Discussion that this is not a clean epistasis experiment, and rather shows competition between the two pathways.

The majority of the trafficking imaging is based on ectopically expressed APP-eGFP and genomically tagged Syt4-GFP. The forced expression of the APP, and the tagging of Syt4 lead to doubts about the natural trafficking of these proteins in the conditions used in the experiments. Is there an antibody against an endogenously expressed EV cargo that could be used in the Figure 8 experiments to back up the conclusions from this figure? (Anti-Arc1? See [https://pubmed.ncbi.nlm.nih.gov/29328915/]).

Unfortunately, anti-Arc, anti-syt4, and anti-Appl antibodies are difficult to work with (ie often fail to produce any staining at all), and when they do they have a high punctate nonspecific background. They are therefore not suitable for measuring EVs. Instead, we can address this concern with excellent and specific antibodies against the endogenous EV cargo neuroglian. The request to validate Figure 8 experiments can be addressed by R1 Experiment 1 (already done) and also R1 Experiment 3.

Reviewer #2 (Significance (Required)):

The significance of the paper is a subtle issue. My interpretation lies in my last comment for the reproducibility section. The title of the article speaks of retromer and Rab11 at pre-synaptic terminals, and I accept many of the conclusion with the caveat that much of the data rests on either ectopic expression of APP, or a genomically GFP-tagged Syt4. If the title was about APP trafficking, I feel that the level of analysis and conclusions would be excellent as another model of APP traffic in a tractable neuron, albeit with an ectopic expression, and I feel it would be a welcome addition to the APP literature. But if the paper is making the case for retromer and Rab11 generally, I would prefer to see an endogenous un-tagged marker used to back up the conclusions made. Nonetheless, the understanding of membrane traffic at the distal end of a very elongated structure is a very important one, and one that requires study in a functional synapse which this study provides. I feel to some degree that if I was to say 'so what, how does this impinge on synapse function and growth?' would be a bit severe, but the
answer is made in the evident changes to synapse structure in Figure 4 and perhaps
the authors could emphasise this more?

Again, we can add an experiment testing the balance between Retromer and Rab11 for
an endogenous, untagged protein (Neuroglian), identical to R1 Experiment 3.

We are also happy to expand our discussion of the physiological significance of
retromer-dependent EV cargo traffic. In particular, we reiterate that our data adds new
interpretation to phenotypes and physiological functions of retromer previously
described in many papers.

**Referees Cross commenting**

I would agree with reviewer #3, a re-focus on the claims of the paper allied to a trimming
of the material to drill down on either the Rab11 v retromer or APP traffic.

The reviewers were concerned that the data describing APP as an EV cargo was
distracting from the main story of opposing roles of retromer and Rab11. Options
include moving Fig 1 to supplement or removing it to a new tools paper and
coordinating publication.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

In their manuscript, the authors demonstrate that APP is secreted from drosophila motor
neurons in extracellular vesicles. This route of secretion is shared by Syt4 and Tkv but
not Syt1 and increased with defects in the retromer component Vps35.
Vps35 defects elicit a wide range of phenotype that can be separated genetically.
Vps35 defects elicit differential changes in endolysosomal vesicle pools: fewer Rab5
puncta, more Rab7 puncta, fewer but larger Rab11 puncta. Expressing DN and CA
mutants of Rab5 and Rab11 change the EV pool more strongly than mutant Rab7. Also,
the pool of Rab7-positive vesicles is not sensitive to Snx1 and Snx6, which control the
EV branch of Vps35 signalling.
Vps35 defect increases the fraction of APP in Rab11 compartment.
Vps35 defect increases the fraction of Syt4 puncta (presynaptic and EV) whereas
Rab11 decreases this fraction (not shown for APP).

I must admit that I am new to the are of pre-submission review. Therefore this review is
a mixture of advice I would give colleagues when reading their manuscript before
submission and a post submission review.
The data is sound and the writing is overall good. There are several points where I think
writing can be improved or things explained better. They are detailed below.

To some extent the manuscript contains two stories and the authors have not entirely
decided which one they are telling. One is the story of APP secretion in extracellular
vesicles. The other story is about the different pathways regulated by Vps35 in drosophila motor neurons.

We intended to tell the second story, but had to validate APP as a disease-relevant APP cargo first. We have offered some potential solutions to this confusion in the overall response.

For the first story, Figures 4 and 5 are dispensable, but it needs to be demonstrated that the quientessential experiment (Figure 8D/E) - that the excessive secretion of EVs by the Vps35 defect can be rescued by Rab11 - also applies to APP.

See next response.

Conversely, if the story is mainly about the different facets of Vps35, one could show the new role for retromer at the example of Syt4 and in a second step demonstrate that APP is also a substrate of this pathway. The title and the very nice mechanistic work elucidating the consequences of Vps35 defect in drosophila neuromuscular junctions argue for the second story. Hence, the key conclusion from the title is shown convincingly for Syt4, but the long introduction about APP and Figure 1 raise the expectation that the antagonism of Vps35 and Rab11 are also shown for APP and not only Syt4. I cannot say whether this experiment are realistic. It is so obvious that I almost think that the authors tried but that it was technically challenging or produced inconclusive results.

This experiment (GAL4 driving APPGFP in the Vps35 rab11 double mutant) is technically challenging as it involves combining 6 genetic elements and five chromosomes. We are willing to try to generate the appropriate strains for this experiment, but cannot guarantee that it will work, especially with the constraints of ongoing periodic lab shutdowns due to COVID-19 cases at our institute. Alternatively, we will test Syt4 and Neuroglian levels in Vps26 Rab11 double mutant (identical to R1 Experiment 3), which will provide a complementary dataset for balance between these pathways, and will be suitable for the Retromer mechanism-focused story.

There are only minor issues of overinterpretation detailed below and minor points where I think methods or processing of data could be explained better.

**Minor comments:**

p.2: "By contrast, a cytosolic presynaptic protein, Complexin (Cpx), was excluded from postsynaptic APP-EGFP puncta, indicating that they arise from specific presynaptic sorting events rather than from non-specific shedding of presynaptic membrane and cytoplasm (Fig. 1B).

-> I consider this overinterpretation. In small structures such as EVs or spines, there is comparably much membrane and little cytosol, so it is natural that the puncta are positive for membrane markers and negative for the cytosolic marker.
Revise language in legend to Figure 1B: p.2: (B) Maximum intensity projection (MaxIP) of motor-neuron-derived APP-EGFP localization presynaptically and to extraneuronal puncta that exclude the presynaptic cytoplasmic protein Complexin (Cpx).

Clarify language: p.2: "Thus, a large proportion of presynaptic APP is trafficked into postsynaptic EV-like structures that are associated with the neuronal membrane." -> large proportion: do you refer to the extra-neuronal vesicles only or to all of them? associated with neuronal membrane: do you mean that the EVs remain in contact with the axon or do you mean that they contain neuronal membrane markers?

p.4: "Taken together, these results indicate that APP-EGFP, and particularly a CTF of this protein, are trafficked into EVs by multiple established criteria" -> I would tone it down to say "can be trafficked into EVs in S2 cells" since this experiment - while important - does not show that they are EVs in flies.

Figure 2 A-C: This is not explained in sufficient detail. What are the yellow lines in Figure 2A? How were the thresholds defined? What are the VPS mutants? Which is the postsynaptic compartment?
Figure 2D: Not sufficiently explained what the two planes (?) of confocal images are, what the yellow line is surrounding.
Figure 2F: The alternating colors make it hard to understand. Since this is false colors anyway, I suggest to keep the same color for e.g. HRP. Also, the labels on the left of the images are hard to read, but they are the key to understanding the figure. I suggest to write them horizontally or in two lines to make the figure easier to grasp. This is one of the most important figures of the manuscript.
Why is there no quantification of postsynaptic Syt1? - Discuss in the text or legend. Why is there no quantification for CD8? -> either add the quantification or remove the images.

p.5: "We noted a significant increase in postsynaptic 50-100 nm vesicles (consistent with the expected size for endosomally-derived exosomes) in the muscle SSR immediately surrounding the neuron (Fig 3A,B), suggesting that retromer mutant NMJs release more EVs."
-> This sounds inconsistent with the earlier statement "p.5: Using structured illumination microscopy (SIM), we resolved individual presynaptic puncta and found that increased APP-EGFP levels occurred as larger and more intense puncta, rather than as a greater density of puncta (Fig S2A)" -> Discuss possible explanations.

Does the term "postsynaptic" really refer to the EVs? If so, it is somewhat misleading -> use "extrasynaptic" or "extracellular" instead?

Figure 5: The Vps superscripts are very hard to read, consider changing the way they are displayed, e.g. to brackets.
Explain SIM abbreviation in legend to Figure 6.
Quite often, there are too many references cited without specifying what they are cited for. It is good practice to give credit to everyone, but this massive amount is not helpful for the reader. An extreme example is this: "p.9: Depending on the specific cell type and cargo, loss of retromer causes differential effects on cargo flux through the endolysosomal pathway, and can result in altered cargo levels either in early endosomes, late endosomes, or on the plasma membrane. (Chen et al., 2013; Hussain et al., 2014; Jimenez-Orgaz et al., 2017; Loo et al., 2014; Pocha et al., 2011; Steinberg et al., 2013; Strutt et al., 2019; Tian et al., 2015; Vazquez-Sanchez et al., 2018; Wang et al., 2014; Wang et al., 2013; Zhou et al., 2011)."

*We can easily make all the above changes to the text and cite reviews rather than primary literature.*

Explain why Mander’s coefficients were used for Rab8 and not Pearson’s, given that the latter is more common.

*Mander’s coefficients are more appropriate to measure co-occurrence (how much of X is in Y structures), while Pearson’s is more appropriate for measuring correlation (how does X intensity scale with Y intensity) (see Bolte 2016 PMID 17210054, Aaron 2018 PMID 29439158). In this case we are not asking if APP is brightest in the brightest Rab11 spots, but instead what fraction of APP and Rab11 are in/on the same compartments, in control versus Retromer mutants, and therefore Mander’s coefficients are more suitable.*

Reviewer #3 (Significance (Required)):

The story about the role of Vps35 and Rab11 in drosophila motor neurons is an important step forward in understanding how endosomal vesicles are regulated - in particular since the authors cite a large body of work demonstrating that many aspects of the investigated pathway differ between cell types. Because this signaling pathway touches on many aspects of physiology and disease, it will be of interested to a wide range of readers.

The translational aspect that puts the finding in the context of APP processing and hence Alzheimer’s disease of course increases significance, but this story is unfortunately incomplete, so the authors need to decide whether to finish it or shift their focus on the physiology.

*Again, our goal is to focus on a new function for retromer, using APP as one physiologically and disease-relevant cargo. Our manuscript addresses only the trafficking, not the processing of APP.*

**Referees cross commenting**

My feeling is that the three reviews each have a specific take, but they address the same issue and do not contradict each other.
I think that all agree that it is excellent data that should be published, but I think that all three would discourage the authors from moving on with the current text/figures and would rather encourage them to revise the flow of the manuscript before submission, focusing either on the translational issue of APP processing or on the more mechanistic/cell biological focus of Rab11 and retromer.

Below please find the title and abstract of our proposed tools paper, perhaps for coordinated publication at Life Science Alliance. This manuscript can be completed in 1-2 weeks; all the data are in hand.

A toolkit for investigating extracellular vesicle cargo traffic in the Drosophila nervous system

Kate Koles*, Agata N. Becalska*, Erica C. Dresselhaus, Cassie R. Blanchette, Julia Apiki, Amy Scalera, Monica Quinones-Frias, Matthew J. Zunitch, Anna Yeh, Avital A. Rodal†

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Key words: Amyloid Precursor Protein, Synaptotagmin -4, Neuroglian, Drosophila, extracellular vesicle, synapse, endosome, exosome

Abstract
Extracellular vesicles (EVs) serve as an important means of communication between cells in the nervous system, as well as for disposal of materials from donor cells. However, we still do not understand how membrane-associated cargoes are directed to the EV pathway during their transit through the plasma membrane and endolysosomal system. Here we generate and characterize new tools for analyzing EV cargo traffic in the genetically amenable nervous system of Drosophila. We show that at Drosophila larval motor neuron synaptic terminals, neuronally-expressed human Amyloid Precursor
Protein (hAPP-GFP) is released in EVs. Using tissue-specific tagging of endogenous Synaptotagmin 4, we demonstrate that Syt4 protein (rather than RNA) is trafficked into EVs. Finally, we find that the cell adhesion molecule Neuroglian (Nrg/L1-CAM) is a novel EV cargo. Importantly, these cargoes traffic into both overlapping and distinct populations of EVs, indicating that their packaging may be spatially and/or temporally controlled. These tools will provide a platform for future studies of trafficking pathways that specify which cargoes are destined for an EV fate, and for analyzing how developmental, activity-dependent, and disease-related cues control EV cargo traffic, release and function.
January 11, 2021

Re: JCB manuscript #202012034T

Dr. Avital Rodal
Brandeis University
Biology
415 South St. MS29
Waltham, MA 02453

Dear Dr. Rodal,

Thank you for submitting your manuscript entitled "Opposing functions for retromer and Rab11 in extracellular vesicle cargo traffic at presynaptic terminals" from Review Commons. We have assessed the comments from the reviewers along with your proposed revision plan in detail. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

We appreciate that a main strength of your study is the detailed characterization of the mode of EV release. However, the molecular insight into the relationship between Retromer and Rab11 remains limited. Therefore, thoroughly addressing the reviewer points including additional controls and validations such as testing an additional Retromer subunit, along with testing a potential contribution of the Rab4 dependent recycling compartment, seems essential. We hope you agree that providing a complete analysis of EV trafficking will serve as a solid foundation for future studies investigating the mechanistic relationships. Editorially, we disagree with splitting the study into a separate Tools study as the methodology is an important component as a JCB paper. Please however consider carefully editing the text for length and clarity.

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 Transfer 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: Transfers 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. Transfers 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.

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 and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. 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

---------------------------------------------------------------------------
New experiments in the revised manuscript are addressed in italic red text below, and responses to minor text criticisms are addressed in italic blue text below.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

This manuscript describes the transport mechanisms by which fragments of APP, specifically the C terminal region are deposited into the extracellular space through neuronal extracellular vesicles. To do this work, the authors describe a transgene of APP with a GFP tag that is then used to track APP fragment secretion into EVs. The initial part of the manuscript describes the use of this reagent to study EV based APP deposition at the Drosophila NMJ. The second part of the paper describes the complex pathways of membrane trafficking in the neuron ad maps the rout of APP containing EVs in this context. The final conclusion presented is that a Rab11 and retromer dependent process specifically controls APP containing EV trafficking at the NMJ.

**Major comments:**

*Are the key conclusions convincing?*

The paper presents an absolutely enormous amount of data analysing many aspects of trafficking at the NMJ. While each bit of analysis in itself seems fine, it is very hard for the general reader to appreciate a global picture of what exactly is going on. There is analysis of multiple rab compartments, retromer function, etc but the paper was hard to assimilate. The authors may be better served by removing non-essential data or minimising it. This will allow the key message to be better presented and appreciated.

The problem of specific cargo trafficking within a cell in the midst of a sea of ongoing trafficking reactions is the nub of the problem here. This is very hard to clearly demonstrate and while much of the data presented is an effort to get to this goal, right now it is very confusing.

*Should the authors qualify some of their claims as preliminary or speculative, or remove them altogether?*

The presentation of data to show that there is a specific defect in APP-EV trafficking which is distinctive from a general endo-lysosomal dysfunction on loss of retromer is hard for the reader to assimilate.

*Would additional experiments be essential to support the claims of the paper? Request additional experiments only where necessary for the paper as it is, and do not ask authors to open new lines of experimentation.*

No, indeed I would suggest removing all non-essential data or simplifying its presentation.
After adding all the new requested data (see below), we have simplified presentation of the data in the figures, streamlined the references, and minimized the required text to 40000 characters (including title, authors, keywords, summary, abstract, introduction, results, discussion, acknowledgments, and figure legends). We have also included a version of the manuscript with track changes.

Also the entire analysis of retromer (except one assay using Vps26) is based on a mutant in Vps35. If the key element being analysed here is the retromer complex, I suggest that 2-3 key phenotypes in the study be verified with more than one retromer component.

We have added the following experiments in a Vps26 mutant to complement our Vps35 results:

**R1 Experiment 1:** To complement our finding that Vps35 and Vps26 mutants show increased levels of the EV cargo Syt4, we have examined an independent EV cargo, neuroglian. Vps26 mutants show the expected increase in pre and postsynaptic levels of this cargo (New Fig 3B). Since Nrg has not previously been published as an EV cargo, we have also added a supplemental figure (New Fig. S3) validating its localization to EVs.

**R1 Experiment 2:** To complement our finding that Vps35 mutants show larger Rab11 and more abundant Rab7 compartments, we have measured both Rab7 and Rab11 compartments in Vps26 mutants, and found the same phenotypes as for Vps35 (New Fig S5A-B).

*Are the data and the methods presented in such a way that they can be reproduced?*

yes

*Are the experiments adequately replicated and statistical analysis adequate?*

yes

**Minor comments:**

*Specific experimental issues that are easily addressable.*

nil except for retromer component analysis comment.

*Are prior studies referenced appropriately?*

yes

*Are the text and figures clear and accurate?*

mostly fine other than that there is too much data
Reviewer #1 (Significance (Required)):

*Describe the nature and significance of the advance (e.g. conceptual, technical, clinical) for the field.*

There are some key technical advances here. The use of the C-terminal GFP tagged APP transgene will be a valuable tool for this and future studies. From the point of Alzheimer's disease therapy which the manuscript speaks to in the introduction, there will be a need to find components or pathways that work relatively specifically and distinct from the general trafficking machinery of neurons, if there is to be a hope of developing a therapeutic molecule.

*Place the work in the context of the existing literature (provide references, where appropriate).*

Many aspects of retromer and Rab function described here are already well known from other studies even in neurons.

_We agree that we have leveraged many existing observations about Rab and retromer function in neuronal and non-neuronal cells, applied them for the first time to the study of neuronal exosomes at presynaptic terminals, and discovered new paradigms for cargo sorting._

*State what audience might be interested in and influenced by the reported findings.*

A clear definition of how APP fragments are specifically trafficked into EV, distinct from other cargoes.

*Define your field of expertise with a few keywords to help the authors contextualize your point of view. Indicate if there are any parts of the paper that you do not have sufficient expertise to evaluate.*

Cellular neuroscience, membrane trafficking, Drosophila, brain disorders

**Referees cross commenting**

Agree with the summary comment added by reviewer 3.
Reviewer #2 (Evidence, reproducibility and clarity (Required)):

The technical quality of the paper appears to me to be high, the reproducibility of a similarly high quality (the stats appear sound to me) and the microscopy of leading edge quality. The question that the authors are addressing places the traffic of cargo in a nexus where the ingress and egress is multiple, making the clarity of the paper an issue, but one that the authors have done well to address. In essence the area is complex, and explaining the experiments and their outcomes complex. I feel they have done an admirable job. The schematic in Figure 9 could be improved with minor adjustments: indicate on the figure the early endosome, MVB, recycling endosome etc. Also, In Fig 1a, give an indication of cytoplasm and extracellular orientation. The uninitiated may not be aware that ICD stands for intracellular domain. Perhaps also give a key to the domain structure (E1, E2 etc) in the figure legend.

We have made these changes to Figures 1 and S1 and replaced the model with a clearer version (now Fig 10).

My technical comments would be the following:

For the GFP intensity measurements, might the difference in intensity be generated by inclusion of the EV in a low pH compartment, or an alteration of the pH in the compartment? The tkv, syt4 and syt1 data argues against this, but the images within the axon and the cell body could be due to this effect.

We don’t believe that this is likely since the samples are fixed and permeabilized, removing the issue of compartment pH. We have also overstained with anti-GFP nanobody in SIM images, which would not be subject to pH-dependent fluorescence changes. We have now added an experiment showing that the effects of Retromer mutants are the same on endogenous, untagged neuroglian, which would also address this reviewer’s concern about examining tagged proteins (See data above for R1 Experiment 1).

Is the recycling of APP (or other EV cargoes) entirely through the slow (Rab11) compartment? What about the Rab4 dependent recycling compartment?

We have tested the effects of constitutively active and dominant negative Rab4 on the EV cargo Neuroglian (unfortunately all available Rab4 tools are YFP-tagged, so we could not examine Syt4 or APP). These data are included in new Figure 8F. Both Rab4<sup>CA</sup> and Rab4<sup>DN</sup> causes a significant reduction in Nrg levels, similar to Rab11, suggesting that they act in the same or overlapping recycling pathways. We also explored Rab4 levels and localization in retromer mutants, but found that the endogenous YFP-Rab4 knockin was too faint to detect at the NMJ, precluding testing if Rab4 is on the same or different compartments from Rab11. This also indicates that Rab4 is low in abundance relative to endogenous YFP-Rab7 and YFP-Rab11, which are readily detectable. Further study of the Rab4 pathway will require development of loss of function mutants or antibodies, which are beyond the scope of this study.
I'm uncomfortable with the Rab11/Vps35 double mutant. It would be preferable to be working with null alleles to allow firmer conclusions, and the Rab11 93Bi allele is not a null.

Unfortunately this is not possible as rab11 nulls are embryonic lethal (Dollar 2002). We have clarified in the Results (p13) that this is not a clean epistasis experiment, and rather shows competition between the two pathways.

The majority of the trafficking imaging is based on ectopically expressed APP-eGFP and genomically tagged Syt4-GFP. The forced expression of the APP, and the tagging of Syt4 lead to doubts about the natural trafficking of these proteins in the conditions used in the experiments. Is there an antibody against an endogenously expressed EV cargo that could be used in the Figure 8 experiments to back up the conclusions from this figure? (Anti-Arc1? See https://pubmed.ncbi.nlm.nih.gov/29328915/).

Anti-Arc, anti-syt4, and anti-App1 antibodies are difficult to work with (ie often fail to produce any staining at all), and when they do they have a high punctate nonspecific background in the muscle. They are therefore not suitable for quantifying EVs, which have a similar appearance to this background. Instead, we have addressed this concern with excellent and specific antibodies against the endogenous EV cargo neuroglian, which we characterize in new Figure S3. In new Fig 9DE, we have replaced the Syt4 data from the previous submission with a new experiment in which we quantified both Syt4 and Nrg – we obtained the same result as our previous Syt4 experiment (i.e. a balance between Rab11 and retromer activities) and found that Nrg exhibits the same behavior, indicating that this balance is relevant for multiple EV cargoes.

Reviewer #2 (Significance (Required)):

The significance of the paper is a subtle issue. My interpretation lies in my last comment for the reproducibility section. The title of the article speaks of retromer and Rab11 at pre-synaptic terminals, and I accept many of the conclusion with the caveat that much of the data rests on either ectopic expression of APP, or a genomically GFP-tagged Syt4. If the title was about APP trafficking, I feel that the level of analysis and conclusions would be excellent as another model of APP traffic in a tractable neuron, albeit with an ectopic expression, and I feel it would be a welcome addition to the APP literature. But if the paper is making the case for retromer and Rab11 generally, I would prefer to see an endogenous un-tagged marker used to back up the conclusions made. Nonetheless, the understanding of membrane traffic at the distal end of a very elongated structure is a very important one, and one that requires study in a functional synapse which this study provides. I feel to some degree that if I was to say ‘so what, how does this impinge on synapse function and growth?’ would be a bit severe, but the answer is made in the evident changes to synapse structure in Figure 4 and perhaps the authors could emphasise this more?
As stated above, we find that endogenous Nrg levels also reflect a balance between Rab11 and retromer activities, similar to Syt4 (new Fig 9DE)), making a stronger case for the balance between retromer and Rab11 generally.

We have also expanded our discussion of the physiological significance of retromer-dependent EV cargo traffic (p13-14). In particular, we reiterate that our data adds new interpretation to functional (and disease-relevant) phenotypes of retromer previously described in many papers (p17).

**Referees Cross commenting**

I would agree with reviewer #3, a re-focus on the claims of the paper allied to a trimming of the material to drill down on either the Rab11 v retromer or APP traffic.

JCB requested that we retain all elements of the story. However, we have significantly condensed and simplified description of the APP trafficking data.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

In their manuscript, the authors demonstrate that APP is secreted from drosophila motor neurons in extracellular vesicles. This route of secretion is shared by Syt4 and Tkv but not Syt1 and increased with defects in the retromer component Vps35. Vps35 defects elicit a wide range of phenotype that can be separated genetically. Vps35 defects elicit differential changes in endolysosomal vesicle pools: fewer Rab5 puncta, more Rab7 puncta, fewer but larger Rab11 puncta. Expressing DN and CA mutants of Rab5 and Rab11 change the EV pool more strongly than mutant Rab7. Also, the pool of Rab7-positive vesicles is not sensitive to Snx1 and Snx6, which control the EV branch of Vps35 signalling. Vps35 defect increases the fraction of APP in Rab11 compartment. Vps35 defect increases the fraction of Syt4 puncta (presynaptic and EV) whereas Rab11 decreases this fraction (not shown for APP).

I must admit that I am new to the area of pre-submission review. Therefore this review is a mixture of advice I would give colleagues when reading their manuscript before submission and a post submission review. The data is sound and the writing is overall good. There are several points where I think writing can be improved or things explained better. They are detailed below.

To some extent the manuscript contains two stories and the authors have not entirely decided which one they are telling. One is the story of APP secretion in extracellular vesicles. The other story is about the different pathways regulated by Vps35 in drosophila motor neurons.

We intended to tell the second story, but had to validate APP as a disease-relevant APP cargo first. To this end, we have condensed and simplified this part of the manuscript.
For the first story, Figures 4 and 5 are dispensable, but it needs to be demonstrated that the quintessential experiment (Figure 8D/E) - that the excessive secretion of EVs by the Vps35 defect can be rescued by Rab11 - also applies to APP.

See next response.

Conversely, if the story is mainly about the different facets of Vps35, one could show the new role for retromer at the example of Syt4 and in a second step demonstrate that APP is also a substrate of this pathway. The title and the very nice mechanistic work elucidating the consequences of Vps35 defect in drosophila neuromuscular junctions argue for the second story.

Hence, the key conclusion from the title is shown convincingly for Syt4, but the long introduction about APP and Figure 1 raise the expectation that the antagonism of Vps35 and Rab11 are also shown for APP and not only Syt4. I cannot say whether this experiment are realistic. It is so obvious that I almost think that the authors tried but that it was technically challenging or produced inconclusive results.

This experiment (GAL4 driving APPGFP in the Vps35 rab11 double mutant) is technically challenging as it involves combining 6 genetic elements and five chromosomes. We tried but were unable to generate the appropriate strains for this experiment. As an alternative, we have shown that Neuroglian levels follow the identical pattern to Syt4 in Vps35 Rab11 double mutant, providing a complementary dataset for balance between these pathways (Fig 9D). These data provide strong supporting evidence for the Retromer mechanism-focused story.

There are only minor issues of overinterpretation detailed below and minor points where I think methods or processing of data could be explained better.

**Minor comments:**

p.2: "By contrast, a cytosolic presynaptic protein, Complexin (Cpx), was excluded from postsynaptic APP-EGFP puncta, indicating that they arise from specific presynaptic sorting events rather than from non-specific shedding of presynaptic membrane and cytoplasm (Fig. 1B).

-> I consider this overinterpretation. In small structures such as EVs or spines, there is comparably much membrane and little cytosol, so it is natural that the puncta are positive for membrane markers and negative for the cytosolic marker.

Revise language in legend to Figure 1B: p.2: (B) Maximum intensity projection (MaxIP) of motor-neuron-derived APP-EGFP localization presynaptically and to extraneuronal puncta that exclude the presynaptic cytoplasmic protein Complexin (Cpx).

We have softened this language (p4).
Clarify language: p.2: "Thus, a large proportion of presynaptic APP is trafficked into postsynaptic EV-like structures that are associated with the neuronal membrane." -> large proportion: do you refer to the extra-neuronal vesicles only or to all of them?

Our intention is to convey that of the total APP signal at or near the NMJ, ~1/3 – 1/2 is in EVs.

associated with neuronal membrane: do you mean that the EVs remain in contact with the axon or do you mean that they contain neuronal membrane markers?

“contain” is more appropriate – the particles are separated from the presynaptic axon terminal. This sentence has been removed in editing the manuscript for length.

p.4: "Taken together, these results indicate that APP-EGFP, and particularly a CTF of this protein, are trafficked into EVs by multiple established criteria" -> I would tone it down to say "can be trafficked into EVs in S2 cells" since this experiment - while important - does not show that they are EVs in flies.

This sentence has been removed in editing the manuscript for length.

Figure 2 A-C: This is not explained in sufficient detail. What are the yellow lines in Figure 2A? How were the thresholds defined? What are the VPS mutants? Which is the postsynaptic compartment?

The presynaptic 3D volume (yellow line in the figures) was thresholded on anti-HRP signal, excluding objects < 7 µm³ (to select for the axon terminal rather than postsynaptic puncta). The postsynaptic volume is defined as a 3 µm dilation of this presynaptic volume. These definitions are found in the text, methods and Table 3. Vps mutants (and all genotypes) are described in detail in Tables 1 and 3. We have now also clarified this information in the Figure legends.

Figure 2D: Not sufficiently explained what the two planes (?) of confocal images are, what the yellow line is surrounding.

We have now referred to this in the Figure legend. “Single confocal slices of APP-EGFP expression in cell bodies in the ventral ganglion (right, outlined in yellow), and axons (left).”

Figure 2F: The alternating colors make it hard to understand. Since this is false colors anyway, I suggest to keep the same color for e.g. HRP. Also, the labels on the left of the images are hard to read, but they are the key to understanding the figure. I suggest to write them horizontally or in two lines to make the figure easier to grasp. This is one of the most important figures of the manuscript.

(Now Figure 3) We have reoriented this figure and changed the colors.
Why is there no quantification of postsynaptic Syt1? - Discuss in the text or legend. Why is there no quantification for CD8? -> either add the quantification or remove the images.

*Now Figure 3* Syt1 is not detectable postsynaptically in either control or mutant synapses. Since there are no postsynaptic puncta, our thresholding protocol picks up only haze from the vastly more abundant presynaptic signal, making quantification in the Vps35 mutant uninterpretable. This is not an issue for the bona fide EV cargoes, where the pre;post distribution is more even.

*We have removed the CD8 images.*

p.5: "We noted a significant increase in postsynaptic 50-100 nm vesicles (consistent with the expected size for endosomally-derived exosomes) in the muscle SSR immediately surrounding the neuron (Fig 3A,B), suggesting that retromer mutant NMJs release more EVs."

-> This sounds inconsistent with the earlier statement "p.5: Using structured illumination microscopy (SIM), we resolved individual presynaptic puncta and found that increased APP-EGFP levels occurred as larger and more intense puncta, rather than as a greater density of puncta (Fig S2A)" -> Discuss possible explanations.

*The earlier statement refers to postsynaptic vesicles (EVs in the muscle or postsynaptic cleft) while the latter statement refers to presynaptic puncta (endosomal compartments that are EV precursors in the donor neuron). Therefore this is not a discrepancy. We have clarified this further in the Figure Legend.*

Does the term "postsynaptic" really refer to the EVs? If so, it is somewhat misleading -> use "extrasynaptic" or "extracellular" instead?

*Some neuron-derived EVs are found extracellularly, or trapped in the infoldings of the SSR, but some are also taken up by the muscle (Korkut et al. 2009) or glia (Fuentes-Medel 2009), and therefore we feel it is more appropriate to call them postsynaptic (general) than extracellular (specific to those not yet taken up).*

Figure 5: The Vps superscripts are very hard to read, consider changing the way they are displayed, e.g. to brackets.

*Explain SIM abbreviation in legend to Figure 6.*

*We have increased the font size in the figures and added this explanation (now Fig 7).*

Quite often, there are too many references cited without specifying what they are cited for. It is good practice to give credit to everyone, but this massive amount is not helpful for the reader. An extreme example is this: "p.9: Depending on the specific cell type and cargo, loss of retromer causes differential effects on cargo flux through the endolysosomal pathway, and can result in altered cargo levels either in early
endosomes, late endosomes, or on the plasma membrane. (Chen et al., 2013; Hussain et al., 2014; Jimenez-Orgaz et al., 2017; Loo et al., 2014; Pocha et al., 2011; Steinberg et al., 2013; Strutt et al., 2019; Tian et al., 2015; Vazquez-Sanchez et al., 2018; Wang et al., 2014; Wang et al., 2013; Zhou et al., 2011). "

We have reduced the references and cited reviews rather than primary literature.

Explain why Mander's coefficients were used for Rab8 and not Pearson's, given that the latter is more common.

Mander’s coefficients are more appropriate to measure co-occurrence (how much of X is in Y structures), while Pearson’s is more appropriate for measuring correlation (how does X intensity scale with Y intensity) (see Bolte 2016 PMID 17210054, Aaron 2018 PMID 29439158). In this case we are not asking if APP is brightest in the brightest Rab11 spots, but instead what fraction of APP and Rab11 are in/on the same compartments, in control versus retromer mutants, and therefore Mander’s coefficients are more suitable.

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The translational aspect that puts the finding in the context of APP processing and hence Alzheimer's disease of course increases significance, but this story is unfortunately incomplete, so the authors need to decide whether to finish it or shift their focus on the physiology.

Again, our goal is to focus on a new function for retromer, using APP as one physiological and disease-relevant cargo. Our manuscript addresses only the trafficking, not the processing of APP.

**Referees cross commenting**

My feeling is that the three reviews each have a specific take, but they address the same issue and do not contradict each other.

I think that all agree that it is excellent data that should be published, but I think that all three would discourage the authors from moving on with the current text/figures and would rather encourage them to revise the flow of the manuscript before submission, focusing either on the translational issue of APP processing or on the more mechanistic/cell biological focus of Rab11 and retromer.
April 21, 2021

RE: JCB Manuscript #202012034R

Dr. Avital Rodal
Brandeis University
Biology
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Dear Dr. Rodal:

Thank you for submitting your revised manuscript entitled "Opposing functions for retromer and Rab11 in extracellular vesicle traffic at presynaptic terminals". 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 and Tools 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.

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 (e.g. Fig 4). 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:
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   b. Type, magnification, and numerical aperture of the objective lenses
   c. Temperature
   d. Imaging medium
   e. Fluorochromes
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   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 display items (figures and tables), however we can make an exception for your tables of reagents and methods. 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.

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).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

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

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)):

I believe that the authors have done an excellent job in responding to the reviewers' comments. The manuscript is now much better focused on one story. The new experimental data that they added supports their main conclusions. The only minor thing I found is that the year of the Inoshita reference is missing on page 5.

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

Walsh et al., provide an analysis of an endosomal trafficking itinerary at a model synapse for proteins that are commonly found as components of Extracellular Vesicles (EVs). They describe the presence of APP-EGFP in EVs, in addition to Syt4 and neuroglian, and point to a phenotype of excessive APP containing endosomes and APP positive EVs in a Vps35 retromer mutant - this is rescued via presynaptic expression of Vps35. This phenotype for Vps35 mutants is recapitulated for Sy4 and Nrg, two other EV cargoes, but not for Tkv or Syt1. The accumulation phenotype is recapitulated in mutations of other retromer components - though which component appears to split an additional phenotype of synaptic overgrowth, which is not seen in the Snx1/Snx6 double mutant though the accumulation is observed, while in the Vps27 mutant, neither synapse overgrowth or EV/endosome accumulation are observed. They then analyse the abundance and effect of manipulations in various Rab proteins, and observe evident changes in Rab5, Rab7 and Rab11 distribution and abundance, while for manipulations of the activity of these proteins, only Rab11 alterations drive evidence changes in the abundance and size of EVs. Double mutants of Rab11 and Vps35 block secretion of EVs. The data points to a balance of Rab11 and retromer activity in a pre-synaptic compartment determining the fate of endosomal cargoes to either be recycled/retained presynaptically, or secreted to the per-synaptic space. The analysis builds a narrative for endosomal cargoes destined for degradation or recycling within the synapse, or secretion. The data are strong, the supplementary data strongly develops the understanding of the reagents, mutants and alternative potential itineraries (e.g.. Vps35 mutant reduces lysosomal function to promote secretion - evidently not). The analysis of Rab4 mutants, and the addition of the Vps26 mutant to recapitulate the Vps35 data generates a robust dataset. The paper does veer between the cargoes analysed, but on balance this is reasonable.

There are a couple of points that are left hanging, but are not essential to the paper - they arise as a consequence of the analysis - the accumulation of Rab7, while the lysotracker, spin and Atg8 abundance declines, and the divergence of phenotypes (secretion vs synaptic growth) of the other retromer mutants - these are interesting, but further analysis would 'sprawl' the paper as is.

I have no comments to add to the experimental analysis, interpretation or presentation of data. My one request would be to improve the schematic cartoon in Figure 10 - I'm not that great at these myself, but if more green bars (cargo) could be added to the PM at the end of the retromer arrow (?), an indication that the Rab11 and Rab35 conditions are mutant, that retromer is composed of Vps35/26 to further map the scheme to the mutants analysed and presented. I feel it needs to tell the narrative a little better.

And please accept my apologies for the lateness of my review.