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Regulation of synaptic activity by snapin-mediated endolysosomal transport and sorting

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(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.)

Editor: Andrea Leibfried

1st Editorial Decision 19 February 2015

Thank you for submitting your manuscript entitled 'Regulation of synaptic activity by endolysosomal transport and sorting'. I have now received reports of all referees, which are enclosed below.

As you will see, while the referees consider that your work is potentially interesting, referee #2 and #3 are not fully supportive of publication here at this stage. They both think that your conclusions are not sufficiently supported by the data provided. I will not list all concerns here, as the referees provide very clear and constructive reports. But you will see that both referees note that the overexpression analyses need to be better controlled and that the motility data are not convincing at this stage. Furthermore, the interaction data, which provide the basis for your conclusions, need further substantiation.
Given the interest into the topic and the constructive comments provided by the referees, I can offer to consider a revised version if you are willing to embark into a major round of revision. However, you would have to clearly substantiate your model along the lines suggested by the referees. This might demand a lot of work, and I will be happy to extend the revision time should that be useful. Please let me also know in case you have further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I am looking forward to receiving your revision.

REFEREE COMMENTS

Referee #1:

Regulation of synaptic activity by endolysosomal transport and sorting. The manuscript by Di Giovanni and Sheng addresses the fundamental question of how endosome-lysosome sorting and motor-dependent transport mechanisms regulate the function of presynaptic terminals in mammalian cells. The main focus of this paper is SNAPIN, a molecule that regulates SNARE function, binds dynein, and belongs to a sorting complex, BLOC-1. Snapin -/- neurons posses impaired neurotransmission whose mechanism is partially understood. The tripartite set of molecular interactions that SNAPIN engages offers a unique opportunity to study the interface of these mechanisms in the regulation of synaptic vesicle pools and physiology.
The authors take advantage of two mutations in SNAPIN that spare SNARE binding yet they impair the association of SNAPIN to dynein and differentially affect SNAPIN binding to the BLOC-1 subunit dysbindin. These tools as well as snapin -/- neurons allow the authors to clearly delineate how endosome retrograde transport and BLOC-1-dependent sorting influence synaptic vesicle dynamics and function. Through a comprehensive set of experiments the authors demonstrate that endosome retrograde transport by dynein regulates SV pool size and BLOC-1/AP-3-dependent sorting modulates the Ca2+-sensitivity of neurotransmitter release. The main conclusions presented by the authors are solidly supported by elegant, well-designed, and controlled experiments that span the use of electron microscopy, in vivo imaging with phluorin SV markers and genetically encoded calcium sensors targeted to SV.
Overall this is a very exciting manuscript and a fundamental contribution to a question few times formulated and experimentally tested, despite the immediate relevance of this question. Namely, how the movement and sorting activities of endosomes coordinately regulate synaptic function. While there is a handful of papers that address endosome sorting mechanisms in the nerve terminal, even fewer if one considers just the BLOC-1 complex, this is the only paper that simultaneously dissects the contribution of sorting and motor-dependent kinetics to presynaptic function.

In its present state the manuscript is very solid and in my view ready. Since, the assays and reagents are in place, I believe that with a modest effort the authors could clarify further the Ca2+-sensitivity phenotype by addressing if the synaptic levels of calcium channels are decreased in snapin -/-. Furthermore, I would like to suggest the use AP-3 instead of AP3 as the majority of AP-3 entries in PubMed are AP-3.

Referee #2:

The present paper by Di Giovanni and Sheng reports a study on Snapin, a fascinating but still enigmatic protein that has been implicated in various cell biological processes, including SNARE mediated fusion, endocytic trafficking, retrograde axonal trafficking, or Exocyst function. Mostly based on the overexpression of WT Snapin and a set of Snapin mutant variants in WT and Snapin KO neurons, the authors report a wide range of phenotypes that they interpret in the general context of endolysosomal transport and sorting.

I must admit that I had a hard time to review this paper. Many of the data and effects are really striking, but in many cases, it was impossible for me to follow the argumentation of the authors - particularly with regard to the specificity of the Snapin mutants used.

1. If I understand the authors correctly, their main conclusion is that Snapin acts via two pathways in neurons, one involving dynein binding and late endosome trafficking, and one involving BLOC-1 dependent endosomal sorting. This conclusion is based on a comparison of two mutant Snapin variants with different effects on protein-protein-interactions, one that apparently affects both main Snapin dependent functions, and one that seems to mainly affect the late endosome trafficking pathway. My concern is here that the Snapin mutants used are simply assumed to be really pathway specific, which I doubt. For example, Snapin has been found to interact with Exocyst components, and the data of the authors on Snapin KO neurons (Fig. 1C) show an accumulation of Synaptophysin

in somata, which would be compatible with perturbed Exocyst function. It is therefore possible that the Snapin mutants used in the present study affect other pathways of Snapin function that have not been tested in the present study. In essence, I find it too premature to write about 'regulation of synaptic activity by endolysosomal transport and sorting', as the authors do in their title. I have no doubt that endolysosomal transport and sorting does affect synaptic function in principle, and previous studies of the authors along with the present study show that Snapin is involved in synapse function, but I am not sure that the authors can conclude that these aspects are causally linked. Any other pathway involving Snapin might be involved, e.g. Exocyst function.

2. A second issue that I find somewhat problematic is the use of WT cells for many/most of the overexpression experiments. Why was this done? An overexpression strategy in WT background has so many problems attached. At this point, it is not possible to judge whether the approach is legit. I am missing information on the plasmids and promoters used and on the expression levels of the overexpressed proteins in the various experiments. Such information is essential, particularly when WT and mutant Snapins are compared. For example, the S50D mutation was shown by others to substantially destabilise Snapin, and it is possible that it never reaches critical concentrations in certain functional contexts. It is therefore mandatory to show that this mutant - and the others Snapin variants that were used - are expressed at comparable levels in the different experiments.

3. I do not think that the 20 AP/20 Hz stimulation can be used to assess release probability (p. 6, l. 16ff). This would require detailed electrophysiological analyses.

4. I do not understand the argument that the fact that a DIC108-268 fragment mimics the effect of Snapin L99K (Fig. 4, S2) argues against a nonspecific effect of Snapin L99K (p. 9, l. 22ff). Peptide overexpression strategies are inherently error-prone, particularly in a WT background.

5. The authors suggest that intersynaptic exchange of SVs is based on actin-dependent transport because they see no effects of Snapin KO on this process (p. 11, l. 23ff). I know of no data that would support the notion that intersynaptic SV exchange is selectively actin-based and not MT based.

6. The comigration of LAMP1 and SV cargo was observed very rarely. The authors explain this with insufficient triple-co-transfection (p. 12, l. 1ff). In general, such triple-co-transfections work very well in cultured neurons with the right combinations of plasmid DNAs. Did the authors show that the triple-co-transfection failed in most neurons?

7. I really do not think that the experiments described in the context of Fig. 6 can be used to argue that Snapin controls positional SV priming and calcium channel coupling. After all, increases in extracellular calcium concentrations would also rescue a deficit in the intrinsic calcium sensitivity of release, not only loose coupling of SVs to calcium channels. To do this properly, electrophysiological experiments with slow calcium buffers are needed.

In summary, I think the paper describes a series of striking and interesting phenotypes and effects related to Snapin function, but I think there are several shortcomings and over-interpretations that need to be rectified.

Referee #3:

In this manuscript, Di Giovanni and Sheng attempt to dissect two distinct functions for the synapse-associated protein, snapin, during synaptic vesicle release in neurons. A cohort of snapin is incorporated into BLOC-1, a protein complex that regulates synaptic vesicle protein sorting, and another cohort of snapin exists in a distinct pool that has been previously shown by the Sheng lab to support dynein-dependent retrograde transport of late endosomes and autophagosomes toward the cell body. Here the authors exploit snapin mutants with distinct binding characteristics to link each snapin function to functional readouts. The authors conclude that both activities contribute to snapin function during synaptic vesicle release.

The topic addressed by the paper is an important one, as snapin is a functionally important synapse-associated protein with many ascribed functions, and it is unclear which of these functions is

associated or not with BLOC-1. Moreover, numerous reports are emerging regarding seemingly BLOC-1-independent functions of BLOC-1 subunits (including dysbindin, discussed here), and so the topic is quite timely. Overall, the conclusions are not rock solid, but they are clearly consistent with the data. The functional and morphological data seem to be well done and the results are clear, and the functional effects of the snapin mutants on retrograde trafficking, calcium signaling and synaptic vesicle release are convincing. The calcium proximity experiments are particularly intriguing. On the other hand, there are several concerns with the manuscript in its current form. The experimental outline lacks precision, and although by the end of the paper I find myself more or less convinced of the authors' general conclusions, the conclusions drawn at each step in the paper are not fully supported by the data presented at that point. In particular, the binding data shown in Figures 3 and Suppl. Fig. 3A are incomplete and very overinterpreted, and the overstepped conclusions drawn from these data color the way that the rest of the paper is interpreted. Nevertheless, with the addition of better controls for the binding data and of analyses of binding to partners within transfected cells, exclusion of a few potential important caveats to the conclusions, and a more judicious consideration of the flow of the paper and the order in which the experiments are shown, this paper could make a very important contribution to the field. Below are specific criticisms.

Major Concerns:

1. While the morphological data in Figure 1 are nice and the quantification is appreciated, it is unclear how the authors quantified the surface area of the presynaptic terminal or the active zone length, and the images shown do not really support the quantification that synaptic vesicle number is increased in the snapin-/- mice. It is not clear what the authors mean in the Results section by "single membrane LE-like vacuoles"; such vacuolar structures typify early sorting endosomes, not late endosomes. In Panel E, what exactly do the "total" and "cytosol" fractions represent? The Figure legend should provide some indication that this is a total brain lysate, as suggested in the Materials and Methods. Is it significant that levels of SNAP25 and synaptotagmin are not increased in cko synaptosomes, despite the increased labeling for synaptic vesicle contents such as synaptophysin?

2. Given the accumulation of retrograde cargo in synapses in Figure 1 and the main argument regarding a role for snapin in retrograde trafficking throughout the paper, it escapes me why the authors address issues of vesicle release in Figures 2 and 4, rather than first nailing the effects of snapin and its mutants on retrograde transport first (Figures 3 and 5). These data flow directly from Figure 1 and build a strong argument regarding the role of different residues in retrograde trafficking, whereas the interpretations of the functional data are much more hand-wavy and rather dependent on the data yet to come in the paper (in fact, I had already written a much more negative review of this paper after reading through Figure 4 and had to rewrite the review after finishing the rest of the paper!). The authors might wish to consider how to better build the argument for a role for snapin in retrograde transport of synaptic vesicle components before describing the more tricky-to-interpret functional data, and time their conclusions appropriately to the supporting data to avoid turning off readers.

3. There are a number of concerns with Figure 3 and Suppl. Fig. S1. First, what isoform of dysbindin is used in these experiments? Is this an isoform normally associated with BLOC-1? The literature suggests that within BLOC-1, dysbindin and snapin are both labile in the absence of other subunits, and so it is not clear what the biological significance of the binary snapin/ dysbindin complex really is without the other complex components. Does dysbindin addition merely cause snapin to aggregate? Second, in Figure 3A and Suppl. Fig. S1A, it is necessary to show that the load of GST-snapin (3A) or GST-DIC (S1A) is identical in these lanes - a Coomassie- or silver-stained gel showing the GST fusion protein in each lane should be included to verify this. Third, is it clear that the L99K mutant is actually capable of folding, and does the interaction with SNAP25 simply reflect aggregation? Are there any data (such as CD-spectra) to suggest that this mutant is largely folded? Fourth, validation of the binding activities of these constructs following expression in cells (using GST-pulldowns from cell lysates) would be necessary to support the authors' conclusions regarding their activities that are shown in future figures. Fifth, as is relevant for Figures S2 and S4, does excess DIC inhibit binding of Snapin to Dysbindin? Finally, the models for function proposed in the text describing Figure 3 are presented here as fact rather than as hypothesis upon which the coming experiments are based. It would be fine to propose this model here and build the rest of the

paper as a test of the model, but the text needs to be modified to reflect the lack of fact at this point in the paper.

4. In the experiments in which snapin is transfected into neurons, to what degree are the snapin transgenes overexpressed? And are they expressed at lower levels in snapin-/- neurons? These points need to be clarified by showing representative quantitative immunoblotting.

5. The conclusions drawn from Figure 4 seem very oversimplified, particularly at this point in the paper. In this figure, the effect of overexpressing the L99K is the same as that of overexpressing wild-type snapin. Based on these data, it does not seem safe to draw conclusions regarding interactions that are disrupted by L99K, nor can one readily conclude anything regarding the role of retrograde trafficking in these responses - only that a snapin variant with improved SNAP25 and dysbindin-binding activities fails to diminish synaptic release. How can the authors exclude the possibility that overexpressed wild-type or L99K snapin competes with endogenous snapin within BLOC-1 for phosphorylation, and thus that the mimetic properties of S50D snapin averts this inhibition or favors incorporation in BLOC-1? This interpretation would not invoke any effect on retrograde transport, and needs to be addressed head on. The effect of DIC overexpression in Suppl. Fig. S2 is supportive, but might also be explained by sequestration of snapin monomers and reduced BLOC-1 formation. Analyses of BLOC-1 levels (e.g. by co-IP of snapin with dysbindin in the cell lysates) and of snapin S50 phosphorylation under the different conditions would help to allay these concerns, as would showing these functional data AFTER having shown distinctions between the wild-type and mutant snapins in Figure 5. Finally, the experiment in Figure 4D is interesting, but the interpretation of the data is extremely convoluted and a definitive conclusion cannot be appropriately drawn.

6. The motility data shown in Figure 5 are very convincing and interesting. However, from Figure 5E, the authors conclude that the low frequency of Syp/ LAMP1 colocalization in retrograde vesicles reflects low transfection efficiency; this belies the very high frequency of comigration of VGluT and LAMP1 in Figure 5F. Is it more likely that the rate by which Syp is targeted to retrograde lysosomes is much lower than VGluT? This would be consistent with data from the Faundez laboratory that AP-3 and BLOC-1 influence VGluT incorporation into synaptic vesicles, but not synaptophysin (Newell-Litwa et al., 2009).

Minor concerns:

7. In Figure S3C, please explain what the intersynaptic SV trafficking represents - is this lateral mobility of SV proteins following fusion with the plasma membrane?

8. Some of the statements in the Introduction are misleading and/or incomplete. For example, the authors state that BLOC-1 was shown by John Peter et al 2013 to promote early endosome maturation, but this was a yeast BLOC-1-like complex and it is not clear whether vertebrate BLOC-1 functions in the same manner. Di Pietro et al 2006 and Setty et al 2007 showed that BLOC-1 regulates endosomal sorting of melanosome cargoes, not lysosomal cargoes. Similarly, in neurons BLOC-1 has been shown by the Faundez group not only to bind to AP-3, as cited, but also to influence sorting of SV cargoes (Salazar et al., 2006; Newell-Litwa et al., 2009) and cargo delivery to the synapse (Larimore et al., 2011). These points should be properly cited and corrected. Also, it should be noted that SVs do not "transit through early endosomes", but rather that SV contents transit through endosomes. This is an important distinction, and as written will confuse readers.

9. In the discussion, the authors might wish to consider the differences in the effects of lysosomal transport on releasable pool size in neurons from snapin-/- animals, in which lysosomal transport of SV components is likely impeded over a long period of time, vs. the transient transfectants in which lysosomal transport is impeded only over days. This timing likely accounts for the reason why the S50D-expressing cells did not show defects in the releasable pool. The Discussion is also rather long and includes quite a bit of unnecessary reiteration of results and speculation. In particular, the last section comparing endocytic recycling vs. direct AP-2-dependent recycling is substantially off-topic.

10. The manuscript should be carefully vetted for syntax and grammar. For example, the opening sentence of the Abstract is not a sentence.

11. The title is overly broad and non-specific. A more appropriate title might be something like "Snapin regulates synaptic vesicle release via distinct activities in endolysosomal transport and sorting".

1st Revision - authors' response 11 May 2015

Referee #1:

Regulation of synaptic activity by endolysosomal transport and sorting. The manuscript by Di Giovanni and Sheng addresses the fundamental question of how endosome-lysosome sorting and motor-dependent transport mechanisms regulate the function of presynaptic terminals in mammalian cells. The main focus of this paper is SNAPIN, a molecule that regulates SNARE function, binds dynein, and belongs to a sorting complex, BLOC-1. Snapin -/- neurons posses impaired neurotransmission whose mechanism is partially understood. The tripartite set of molecular interactions that SNAPIN engages offers a unique opportunity to study the interface of these mechanisms in the regulation of synaptic vesicle pools and physiology.

The authors take advantage of two mutations in SNAPIN that spare SNARE binding yet they impair the association of SNAPIN to dynein and differentially affect SNAPIN binding to the BLOC-1 subunit dysbindin. These tools as well as snapin -/- neurons allow the authors to clearly delineate how endosome retrograde transport and BLOC-1-dependent sorting influence synaptic vesicle dynamics and function. Through a comprehensive set of experiments the authors demonstrate that endosome retrograde transport by dynein regulates SV pool size and BLOC-1/AP-3-dependent sorting modulates the Ca2+-sensitivity of neurotransmitter release. The main conclusions presented by the authors are solidly supported by elegant, well-designed, and controlled experiments that span the use of electron microscopy, in vivo imaging with phluorin SV markers and genetically encoded calcium sensors targeted to SV.

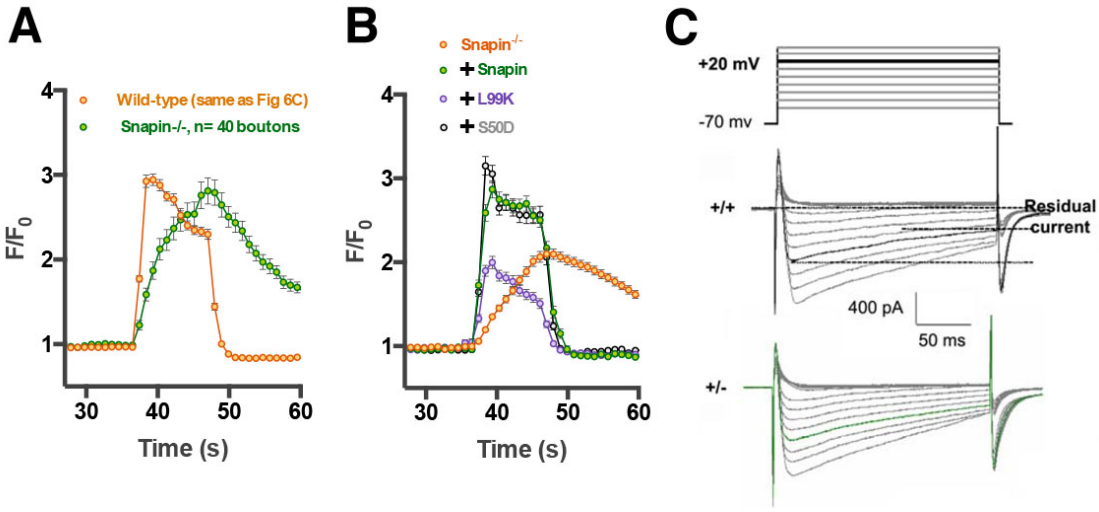
Overall this is a very exciting manuscript and a fundamental contribution to a question few times formulated and experimentally tested, despite the immediate relevance of this question. Namely, how the movement and sorting activities of endosomes coordinately regulate synaptic function. While there is a handful of papers that address endosome sorting mechanisms in the nerve terminal, even fewer if one considers just the BLOC-1 complex, this is the only paper that simultaneously dissects the contribution of sorting and motor-dependent kinetics to presynaptic function.

We are encouraged by this reviewer for such positive and enthusiastic comments on the quality of our data and the novelty of our study in addressing a fundamental question in neurobiology as how the movement and sorting activities of endosomes coordinately regulate SV pool size and the Ca^{2+}-sensitivity of SV release at presynaptic terminals.

In its present state the manuscript is very solid and in my view ready. Since, the assays and reagents are in place, I believe that with a modest effort the authors could clarify further the Ca2+-sensitivity phenotype by addressing if the synaptic levels of calcium channels are decreased in snapin -/-.

We evaluated Ca^{2+} influxes in response to 100 APs in *snapin*$^{-/-}$ boutons (n=40) using the GECO sensor. We found that the average amplitude of activity-triggered Ca^{2+} influx was similar to WT neurons, although the rising and decay time courses were delayed (see **panel A** below). In addition, neurons expressing dominant-negative mutant snapin-L99K or snapin-S50D display a similar GECO response to Ca^{2+} influx as seen in WT neurons (see revised **Fig 6C**, **6D** and **supplemental Fig S5A**). These GECO sensor measurements suggest that synaptic levels of voltage-dependent calcium channels are not significantly reduced in *snapin*$^{-/-}$ or snapin-deficient boutons. This conclusion is supported by our previous electrophysiological study in recording Ca^{2+} currents in *snapin*$^{+/-}$ *deficient* neurons: recorded Ca^{2+} currents from these synapses are similar to wild-type Ca^{2+} currents (Pan et al, 2010) (see **panel C** below).

Interestingly, when using SV-targeted synaptophysin-GECO as a calcium sensor, *snapin*$^{-/-}$ boutons displayed severely decreased GECO-synaptophysin responses to 100-AP stimulations (2.09 ± 0.06) with slowed-down kinetics. While reintroducing WT or snapin-S50D, which retains dysbindin-binding capacity, fully rescues the peak amplitude (2.87 ± 0.10 and 3.15 ± 0.11, respectively) (**Supplementary Fig S5C** and **S5D,** also shown panel B below). The dysbindin-binding defective mutant snapin-L99K failed to rescue the defective GECO-synaptophysin responses in *snapin*$^{-/-}$ boutons. Together, these data support our hypothesis that snapin regulates SV positioning with respect to Ca^{2+} entry sites via a dysbindin/BLOC-1-dependent sorting mechanism (Route 2). We include these data for the reviewer's examination.



A. Global Ca^{2+} influx in response to 100 APs in *snapin*$^{-/-}$ or WT boutons (n=40) using the GECO sensor.
B. Synaptophysin-GECO response from KO boutons following snapin rescue (Fig S4C).
C. Synaptic Ca^{2+} currents from WT and *snapin*$^{+/-}$ *deficient* neurons (Pan et al, 2010).

We add the following new statements on **page 14**:

"*We also assessed global Ca^{2+} transient at snapin*$^{-/-}$ *synapses with the cytosolic GECO sensor in response to 100 APs. The average amplitude of activity-triggered Ca^{2+} influx was similar to WT neurons, although the rising and decay time courses were delayed, suggesting that synaptic levels of voltage-dependent calcium channels are not significantly reduced in snapin-deficient boutons. This conclusion is supported by our previous electrophysiological study showing that Ca^{2+} currents from snapin-deficient synapses are similar to wild-type Ca^{2+} currents (Pan et al, 2010)".*

Furthermore, I would like to suggest the use AP-3 instead of AP3 as the majority of AP-3 entries in PubMed are AP-3.

We replaced "AP3" with "AP-3" throughout the text.

Referee #2:

The present paper by Di Giovanni and Sheng reports a study on Snapin, a fascinating but still enigmatic protein that has been implicated in various cell biological processes, including SNARE mediated fusion, endocytic trafficking, retrograde axonal trafficking, or Exocyst function. Mostly based on the overexpression of WT Snapin and a set of Snapin mutant variants in WT and Snapin KO neurons, the authors report a wide range of phenotypes that they interpret in the general context of endolysosomal transport and sorting.

I must admit that I had a hard time to review this paper. Many of the data and effects are really striking, but in many cases, it was impossible for me to follow the argumentation of the authors - particularly with regard to the specificity of the Snapin mutants used.

We appreciate the reviewer's many constructive comments on our study. We expanded our study by providing additional experimental data and reorganizing our paper. We also included new discussions to address this reviewer's concerns that strengthen and substantiate the claims presented in our manuscript. In particular, as suggested by reviewer 3, we reorganized our results into two main parts based on the logical flow of the data presentation and snapin mutants:

1. Snapin-dynein coupling to drive SVs for retrograde transport and how such a motor-driven trafficking impacts the SV pool size.

Figure 1. *Snapin*-deficient neurons display enlarged presynaptic terminals retaining various degradative organelles.
Figure 2. Snapin mutants disturb LE retrograde transport in axons.
Figure S1 (Related to **Fig 2**). Expressing the snapin-biding domain of DIC(108-268) recapitulates snapin-L99K effect on LE transport.
Figure 3. Snapin-mediated LE retrograde transport regulates total SV pool size.
Figure S2 (Related to **Fig 3**). Snapin does not affect short-distance inter-synaptic SV trafficking.
Figure 4. Altered SV exocytosis occurs at snapin-deficient presynaptic terminals**.**

2. Snapin-mediated regulation of SV positional priming via the BLOC1/AP3-dependent SV sorting pathway.

Figure 5. Snapin mutations differentially affect presynaptic activity.
Figure S3 (related to **Fig 5**). Snapin mutations discriminate its dual-role in mediating LE retrograde transport and BLOC-1 function.
Figure S4 (related to **Fig 5**). DIC (108-268) and snapin-L99K play a similar role in inhibiting SV exocytosis.
Figure 6. A snapin mutant defective in dysbindin-binding impairs SV positional priming.
Figure S5 (related to **Fig 6**). Inhibiting the snapin-dysbindin interaction impairs positional priming.
Figure 7. Deleting *snapin* affects SV molecular identity with BLOC1/AP-3 inhibition.

By using unique snapin mutants we dissected these two dynamic processes and provided new mechanistic insights into the regulation of SV pool size and synchronized fusion through snapin-mediated LE trafficking and endosomal sorting. As stated by reviewer 1 that our study made "*a fundamental contribution to a question few times formulated and experimentally tested. Namely, how the movement and sorting activities of endosomes coordinately regulate synaptic function. "This is the only paper that simultaneously dissects the contribution of sorting and motor-dependent kinetics to presynaptic function*".

1. If I understand the authors correctly, their main conclusion is that Snapin acts via two pathways in neurons, one involving dynein binding and late endosome trafficking, and one involving BLOC-1 dependent endosomal sorting. This conclusion is based on a comparison of two mutant Snapin variants with different effects on protein-protein-interactions, one that apparently affects both main Snapin dependent functions, and one that seems to mainly affect the late endosome trafficking pathway.

We apologize for insufficient clarification in our initial submission. As acknowledged by reviewer #1, the main focus of our current study is not to characterize snapin's functions. Instead, we addressed a fundamental and long-standing question by establishing how endosomal trafficking and sorting coordinately regulate presynaptic function.

To address this fundamental question, we used binding defective snapin mutants as tools. We believe that snapin mutants are ideal molecular tools in studying endosomal trafficking and sorting implicated in presynaptic regulation. Snapin binds to dysbindin, a subunit of the endosome-sorting complex BLOC-1, and acts as a dynein adaptor mediating the retrograde transport of late endosomes (LEs). In the current study, we used a *snapin* mouse model in combination with those dominant-negative snapin mutants specifically impairing LE transport or BLOC-1 endosomal sorting function. We reveal that the endolysosomal pathway exerts a bipartite regulation of synaptic activity (**Figure 7C**). First, LE transport influences the total SV pool size by shuttling SV components along the endolysosomal pathway. Second, BLOC-1/AP-3-dependent endosomal sorting determines SV composition which in turn regulate positional priming. Snapin is a key player in both processes. By balancing these two dynamic pathways, snapin coordinates releasable pool size and Ca^{2+}-sensitivity of neurotransmitter release.

However, it is noteworthy that our study does not only rely on the specificity of the mutations; we also make observations based on *snapin*$^{-/-}$ mouse model and on WT snapin expression, which has an opposite effect as the L99K and S50D mutants in both WT and *snapin*$^{-/-}$ backgrounds. The specificity of the L99K and S50D mutants is actually more relevant in the second part of the paper where we focus on BLOC-1-mediated endosomal sorting, because of their differential effects on release and positional priming (again both in the WT and *snapin*$^{-/-}$ backgrounds). These mutants allowed us to propose a role for BLOC-1/AP-3 dependent sorting in positional priming, which is consistent with alterations in the molecular identity of SVs from *snapin*$^{-/-}$ conditional KO adult mouse brains, displaying reduced BLOC-1/AP-3 cargoes contents and Rab3.

My concern is here that the Snapin mutants used are simply assumed to be really pathway specific, which I doubt. For example, Snapin has been found to interact with Exocyst components, and the data of the authors on Snapin KO neurons (Fig. 1C) show an accumulation of Synaptophysin in somata, which would be compatible with perturbed Exocyst function. It is therefore possible that the Snapin mutants used in the present study affect other pathways of Snapin function that have not been tested in the present study. In essence, I find it too premature to write about 'regulation of synaptic activity by endolysosomal transport and sorting', as the authors do in their title. I have no doubt that endolysosomal transport and sorting does affect synaptic function in principle, and pervious studies of the authors along with the present study show that Snapin is involved in synapse function, but I am not sure that the authors can conclude that these aspects are causally linked. Any other pathway involving Snapin might be involved, e.g. Exocyst function.

We are aware of two studies reporting snapin-exocyst binding. In the past, we performed various pull-downs from mouse and rat brains using GST-snapin as baits, followed by mass spectrometry. The only consistent major specific binding partners were DIC, SNAP25, dysbindin, and a few other related proteins involved in SV release at synapses under our experimental conditions. While the exocyst component Exo70 was indeed reported to interact with recombinant snapin *in vitro* (Bao et al, 2007), this study did not demonstrate any functional link between snapin and the exocyst complex. In addition, we found no evidence in the literature that cytosolic SV accumulation is associated with defects of the exocyst. PC12 cells accumulated secretory vesicles in the cytosol rather than neurites, but these cell lines are only remotely comparable to neurons and have no axons (Vega and Hsu, 2001). Using EM we have not observed such accumulation of secretory vesicles in *snapin*$^{-/-}$ neuron cell bodies.

While GFP-synaptotagmin did accumulate in the axons of *Drosophila* motor neurons after mutation of exocyst component Sec5, there was no difference in SV abundance at NMJ and no change in synaptic transmission (Murthy et al., 2003). To our knowledge, these published studies conclude that the exocyst complex is not involved in SV release or recycling (reviewed by Eauclaire and Guo, 2003). More recently, a small decrease in synapse volume with no change in SV abundance was observed at the calyx of Held after mutation of the exocyst subunit Exo70 (Shwenger and Kuner, 2010) but again no change in synaptic transmission was observed in any cases. Therefore, the

snapin$^{-/-}$ synaptic phenotypes are distinct and unlikely to be accounted for by perturbations of the exocyst complex.

Both our lab and Jens Rettig's lab previously showed that the *snapin*$^{-/-}$ mice display two main striking phenotypes: **(1)** impaired priming of large dense-core vesicles in chromaffin cells (Tian et al., 2005) and desynchronized SV release in neurons (Pan et al., 2009); and **(2)** impaired retrograde transport of LEs, resulting in accumulation of immature lysosomes and autolysosomes (Cai et al., 2010). Snapin is relatively enriched in synaptic terminal preparations, associated with SVs and late endosomes, and co-purified with the biogenesis of lysosome-related organelle complex-1 (BLOC-1) (Starcevic and Dell'Angelica, 2004), and specifically interacts with dysbindin, a subunit of BLOC-1 (Lee et al., 2012; Talbot et al., 2006). Implication of BLOC-1 in synaptic transmission was suggested in several labs. BLOC-1 interacts with the AP-3 complex, influences sorting of SV cargoes (Newell-Litwa et al., 2009; Salazar et al., 2006), mediates SV budding from early endosomes (Faundez et al., 1998), or delivers SV cargoes to the synapses (Larimore et al., 2011). BLOC-1 deficiency perturbs AP-3 levels (Newell-Litwa et al., 2010; Newell-Litwa et al., 2009), in turn affecting neurotransmitter release from hippocampal mossy fibers (Scheuber et al., 2006).

These previous works highlight the roles of snapin in regulating presynaptic function. Our current study provides new mechanistic clues as to how the movement and sorting activities of endosomes coordinately regulate presynaptic function. For instance, consistent with the de-synchronization phenotype (Pan et al, 2009), we demonstrate that snapin is involved in positional priming, an activity we related to BLOC-1-dependent endosomal sorting. We assessed positional priming by measuring Ca^{2+} transients using the SV-targeted GECO calcium sensor, thus circumventing the technical limitations encountered in electrophysiology and imaging.

Since we generated snapin conventional and conditional KO mice, we have performed various experiments in characterizing *snapin*-deficient phenotypes in neurons. We are confident that the main functions of snapin are linked to the three binding partners or pathways (SNAP25, dysbindin, and dynein), which were consistently reported by our lab and others.

We agree with this reviewer's comments that "*I have no doubt that endolysosomal transport and sorting does affect synaptic function in principle*". We are cautious not to exclude other cellular pathways in regulating presynaptic function. Instead, we revised our statement in the abstract to read "*Our study reveals a bipartite regulation of presynaptic activity by endolysosomal sorting and trafficking: LE transport regulates SV pool size and BLOC-1/AP-3-dependent sorting fine-tunes the* Ca^{2+}*-sensitivity of SV release*"**.**

Bao Y, Lopez JA, James DE, and Hunziker W (2008) Snapin interacts with the Exo70 subunit of the exocyst and modulates GLUT4 trafficking. *J Biol Chem* 283(1):324-31

EauClaire S, and Guo W (2003) Conservation and specialization. The role of the exocyst in neuronal exocytosis. *Neuron* 37:369-70

Murthy M, Garza D, Scheller RH, and Schwarz TL (2003) Mutations in the exocyst component Sec5 disrupt neuronal membrane traffic, but neurotransmitter release persists. *Neuron* 37:433-47.

Schwenger DB, and Kuner T (2010) Acute genetic perturbation of exocyst function in the rat calyx of Held impedes structural maturation, but spares synaptic transmission. *Eur J Neurosci* 32:974-84

Vega IE, and Hsu SC (2001) The exocyst complex associates with microtubules to mediate vesicle targeting and neurite outgrowth. *J Neurosci*. 21:3839-48

2. A second issue that I find somewhat problematic is the use of WT cells for many/most of the overexpression experiments. Why was this done?

The main focus of our current study is not to characterize phenotypes of snapin KO mouse or neurons. Instead, we wanted to address how the movement and sorting activities of endosomes coordinately regulate presynaptic function. To address this fundamental question, we expressed

various snapin mutants as tools in both WT and KO neurons to selectively impair dynein-driven LE trafficking or BLOC-1/dysbindin/AP-3-dependent endosomal sorting. We believe that snapin mutants are ideal molecular tools for investigations of endosomal trafficking and sorting implicated in synaptic regulation.

Snapin KO mouse embryos die most of the time early during development. Although weekly breeding of *snapin*$^{+/-}$ mice, we sometime have no homozygous *snapim*$^{-/-}$ embryos to work for culturing neurons for 6 month. Given that a detailed pHluorin study requires high neuron numbers, our KO neurons thus were only used for important rescue experiments to confirm the effects of dominant-negative mutants, as shown in **Fig 1A-1C**; **Fig 3C** and **3D**; **Supplementary Fig S2A** and **S2B**; and **Supplementary Fig S5C** and **S5D**.

In addition, our previous study demonstrated that defects in LE transport in *snapin* KO neurons lead to developmental defects in the central nervous system (Zhou et al., 2011), thus preventing us from dissecting these two dynamic pathways. A similar approach by overexpressing WT snapin and S50D mutant in WT cells, rather than using snapin KO cells, was also used in the Rettig lab (Thakur et al., 2004; Schmidt et al, 2013).

Schmidt T, Schirra C, Matti U, Stevens DR, and Rettig J (2013) Snapin accelerates exocytosis at low intracellular calcium concentration in mouse chromaffin cells. ***Cell calcium*** 54, 105-110.

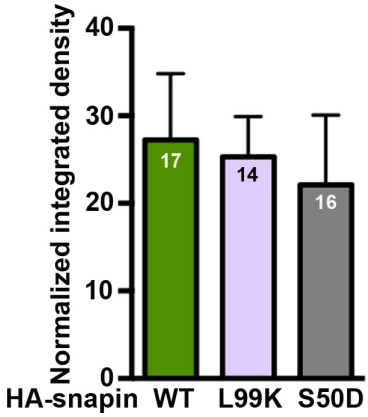
Thakur P, Stevens DR, Sheng ZH, and Rettig J (2004) Effects of PKA-mediated phosphorylation of Snapin on synaptic transmission in cultured hippocampal neurons. J Neurosci 24, 6476-6481

Zhou B, Zhu Y-B, Lin L, Cai Q, and Sheng Z-H (2011). Snapin deficiency is associated with developmental defects of the central nervous system ***Bioscience Report*** 31, 151-158.

An overexpression strategy in WT background has so many problems attached. At this point, it is not possible to judge whether the approach is legit. I am missing information on the plasmids and promoters used and on the expression levels of the overexpressed proteins in the various experiments. Such information is essential, particularly when WT and mutant Snapins are compared. For example, the S50D mutation was shown by others to substantially destabilise Snapin, and it is possible that it never reaches critical concentrations in certain functional contexts. It is therefore mandatory to show that this mutant - and the others Snapin variants that were used - are expressed at comparable levels in the different experiments.

The reviewer asked a valid question. We carefully designed and performed these expression studies. First, we used the same pcDNA vectors with CMV promoter for expression of untagged snapin and its mutants. It is very difficult to verify protein expression using cultured neuron lysates and immunoblots because transfection efficiency in neurons is relatively low and variable. Furthermore, as only transfected neurons are selected for our imaging studies, global protein levels monitored by biochemical means are rather irrelevant.

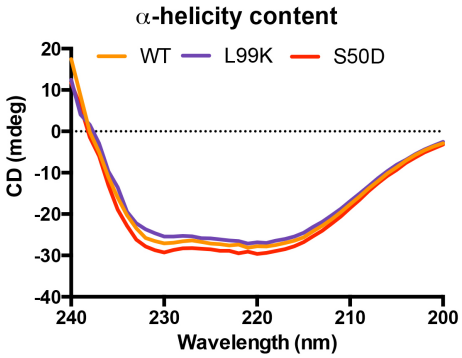
In order to minimize unspecific effects, we used untagged snapin for overexpression and rescue experiments. We have not found yet a specific snapin antibody for IC assays that we could validate in our *snapin*$^{-/-}$ mouse model. Therefore, we assessed the relative expression levels of snapin proteins by immunostaining HA-tag WT and mutant snapin in transfected cultured neurons under the same conditions. Quantitative analysis of the integrated density of HA signal is provided here for examination by the reviewer (data are means ± sd). We found that relative expression levels of these HA-tagged WT and mutant snapin in transfected neurons are similar under the same conditions.



Regarding the S50D mutation, we assume that the reviewer refers to the study by Navarro et al. (2012), which demonstrates that recombinant snapin forms oligomers in solution *in vitro* and the S50D mutant displays lower secondary structure and lesser thermal stability. However, these *in vitro* data do not imply that the S50D mutation would somehow lead to a lower snapin concentration, especially in a cellular environment.

Nevertheless they confirm that the same mutant exhibits increased association to SNARE complexes. Our imaging analysis consistently displays that S50D has similar expression when compared to other mutants or WT. Again in Rettig's studies, the same S50D mutant did not show less stability in their expression system (Thakur et al, 2004; Schmidt et al, *Cell Calcium* 2013).

To further address this reviewer's concern and test the folding capacity of the snapin mutants, as suggested by reviewer #3, we performed CD spectroscopy measurements by comparing purified His-tagged WT snapin with snapin-L99K and snapin-S50D mutants. The alpha-helix profiles suggest these two mutants have similar secondary structure folding as WT snapin (**Supplementary Fig S3E**). Thus, these snapin mutants are ideal molecular tools to assess the relative roles of endosomal trafficking and sorting in maintaining presynaptic function. We describe these new data on **page 11**.



3. I do not think that the 20 AP/20 Hz stimulation can be used to assess release probability (p. 6, l. 16ff). This would require detailed electrophysiological analyses.

This is an excellent point. We simply used the 20-AP/20-Hz stimulation to compare WT and mutant snapin in their pHluorin responses, and we did not precisely compute Pr, which indeed requires detailed electrophysiological analysis. Our imaging analysis suggests that synapses expressing snapin-S50D exhibit higher responses to 20 APs at 20Hz than WT, but remain similar to the response at 100 APs/10Hz. This altered response is a nice positive control further demonstrating that snapin-S50D indeed influences release; these data are supported by a detailed and elegant electrophysiology study in the Rettig group (Thakur et al., 2004). Nevertheless, we cautiously revised the description of 20AP/20Hz pHluorin data and removed comments that might be misleading in the text and **Fig 4** and **5** legends.

4. I do not understand the argument that the fact that a DIC108-268 fragment mimics the effect of Snapin L99K (Fig. 4, S2) argues against a nonspecific effect of Snapin L99K (p. 9, l. 22ff). Peptide overexpression strategies are inherently error-prone, particularly in a WT background.

We apologize for not clarifying this point better in the first submission. To test whether the snapin-DIC coupling-mediated trafficking impact presynaptic activity, we thought to disrupt the snapin-DIC coupling by expressing HA-snapin-L99K, a dominant-negative mutant defective in DIC-binding. Alternatively, we expressed HA-DIC (108-268), a snapin-binding domain of DIC, to competitively interfere with endogenous snapin-DIC coupling. By expressing both snapin-L99K and DIC (108-268), we verify that the presynaptic phenotypes are linked to the snapin-DIC coupling in driving LE transport. It is noteworthy that DIC (108-268) is a truncated DIC mutant, but not a peptide as the reviewer noted. We now add the rationale why we chose HA-DIC (108-268) on **page 6**:

"*Alternatively, we expressed HA-DIC (108-268), the snapin-binding domain of DIC that competitively disrupts the endogenous snapin-DIC coupling (Cai et al., 2010). Expressing DIC (108-268) in WT neurons displayed a phenotype similar to snapin-L99K expression: reduction in retrograde motility to 22.5 ± 2.0% (**Fig S1**). These data further support the notion that reduced LE retrograde motility is due to impaired snapin-DIC coupling*"**.**

5. The authors suggest that intersynaptic exchange of SVs is based on actin-dependent transport because they see no effects of Snapin KO on this process (p. 11, l. 23ff). I know of no data that would support the notion that intersynaptic SV exchange is selectively actin-based and not MT based.

This assumption is based on literature reports that intersynaptic short-range transport is based on actin filaments (Darcy et al, 2006, Staras et al., 2010). We revised this statement by citing these two papers on **page 8**:

"However, while monitoring photoconverted dendra-synaptophysin trafficking in WT and snapin$^{-/-}$ neurons, we found no significant difference in the amount of SVs transferred to neighboring boutons

*(**Supplementary Fig S2A** and **S2B**). This suggests that snapin does not influence short-range actin-based motility of SV components from one synapse to neighboring synapses, a dynamic SV local trafficking previously described (Darcy et al, 2006, Staras et al., 2010)".*

Darcy KJ, Staras K, Collinson LM and Goda Y (2006) Constitutive sharing of recycling synaptic vesicles between presynaptic boutons. Nat Neurosci. 9, 315-21.

Staras K, Branco T, Burden JJ, Pozo K, Darcy K, Marra V, Ratnayaka A, and Goda Y (2010) A vesicle superpool spans multiple presynaptic terminals in hippocampal neurons. ***Neuron*** 66, 37-44

6. The comigration of LAMP1 and SV cargo was observed very rarely. The authors explain this with insufficient triple-co-transfection (p. 12, l. 1ff). In general, such triple-co-transfections work very well in cultured neurons with the right combinations of plasmid DNAs. Did the authors show that the triple-co-transfection failed in most neurons?

The reviewer raised an important point that we did not discuss in the previous submission.
Because snapin mediates long-distance endolysosomal transport by recruiting dynein to the organelles, we asked whether a portion of recycling SV cargoes are transported through late endocytic organelles. Axonal trafficking was monitored in neurons co-expressing the SV marker Tomato-synaptophysin and the endolysosomal marker GFP-LAMP1 during 5-min dual-channel time-lapse acquisitions (revised **Fig 3G**). Co-migration of synaptophysin-labeled SV cargoes and endolysosomal markers was readily detected in 5 out of 16 neurons over-expressing snapin. The low frequency of those co-migration events is likely due to limited recycling synaptophysin moving into the endolysosomal trafficking pathway in the absence of stimulation. We now expand our discussion on this issue on **page 8**.

7. I really do not think that the experiments described in the context of Fig. 6 can be used to argue that Snapin controls positional SV priming and calcium channel coupling. After all, increases in extracellular calcium concentrations would also rescue a deficit in the intrinsic calcium sensitivity of release, not only loose coupling of SVs to calcium channels. To do this properly, electrophysiological experiments with slow calcium buffers are needed.

Our hypothesis about positional priming rested on three previous studies: (1) the Ca^{2+} sensitivity of exocytosis analyzed by Ca^{2+}-uncaging and capacitance measurements in chromaffin cells is not affected by the absence of snapin (Tian et al, 2005), and chromaffin cells share the same basic release machinery as neurons (i.e., SNARE proteins and the Ca^{2+} sensor synaptotagmin I). (2) The desynchronization of SV release in *snapin*$^{-/-}$ cortical neurons (Pan et al., 2009) is reminiscent of the perturbation of SV interaction with Ca^{2+} channels in superior cervical ganglion neurons (Mochida et al., 1996). (3) It was recently shown by the Rettig group that snapin accelerates exocytosis at low $[Ca^{2+}]_i$ in chromaffin cells (Schmidt et al., 2013).

These studies raise a mechanistic question as to how snapin regulates the Ca^{2+}-sensitivity of neurotransmitter release. In the current study, we dissected the role of snapin in regulating SV positional priming by using two different snapin mutants and by generating a SV-targeted Ca^{2+}-sensing probe, synaptophysin-GECO. Over-expressing snapin-L99K, defective in binding to both dynein DIC and the BLOC-1 subunit dysbindin, revealed a striking phenotype: SVs were exposed to a lesser amount of Ca^{2+} during trains of 100 APs (**Fig 6**). However, this phenotype was not readily observed in neurons expressing snapin-S50D, a mutant defective in DIC-binding but retaining dysbindin-binding capacity. Similarly, *snapin*$^{-/-}$ boutons displayed severely decreased GECO-synaptophysin responses to 100-AP stimulations. While reintroducing WT snapin and snapin-S50D, which retains dysbindin-binding capacity, could rescue the peak amplitude. The dysbindin-binding defective mutant snapin-L99K failed to rescue the defective GECO-synaptophysin responses in *snapin*$^{-/-}$ boutons (**Supplementary Fig S5C** and **S5D**). Together, these data support our hypothesis that snapin regulates SV positioning with respect to Ca^{2+} entry sites via a dysbindin/BLOC-1-dependent sorting mechanism (Route 2). Our findings are consistent with previous reports: *dysbindin*$^{-/-}$ hippocampal neurons exhibit slower kinetics and smaller RRP size (Chen et al., 2008) and *Drosophila dysbindin*$^{-/-}$ neuromuscular junctions display reduced Ca^{2+} sensitivity of neurotransmitter release (Dickman et al., 2012). Thus, it is likely that BLOC-1-dependent endosomal sorting and the AP-3 pathway contribute to SV positional priming.

While increasing extracellular Ca^{2+} concentrations rescues a deficit in intrinsic calcium sensitivity to some extent (obviously SVs with very low Ca^{2+} sensitivity would not be mobilized for release in any case) (**Fig 6E, 6F**), our data obtained using the SV-targeted Ca^{2+} sensor (**Fig 6C, 6D** and **Fig S5A-D**) cannot be explained by a decrease in the intrinsic Ca^{2+} -sensitivity of SVs. We demonstrate that the amount of Ca^{2+} received by SVs (assessed using synaptophysin-GECO) during stimulations is reduced when the dominant negative version of snapin is expressed and in *snapin*$^{-/-}$ cells.

Unfortunately, electrophysiological experiments using slow Ca^{2+} buffers to determine SV position with respect to Ca^{2+} channels would not only be impractical but also inappropriate. First, it would require direct patch clamping of presynaptic boutons, which is only possible in relatively large presynaptic terminals such as GABAergic basket cells or the calyx of Held that are accessible in slice preparations, not in cultured neurons. Furthermore, these studies involve heavy data modeling and only yield a range of distances where SVs might be located, which precludes precise dissection of the positional priming state of SVs in either WT or *snapin*$^{-/-}$ terminals expressing snapin and its mutants.

Finally, a new article by the Davis laboratory was published during the revision of our study, which further supports our findings that snapin and dysbindin/BLOC1 regulate positional priming. They demonstrate that the homeostatic presynaptic regulation that involves both snapin and dysbindin (Dickman et al, 2012) relies on a tightening of the SV-Ca^{2+} channels association (Muller et al, 2015). In addition, the same laboratory proposed earlier that both dysbindin and Rab3 regulate homeostatic plasticity through positional priming (Muller et al, 2011), again consistent with our findings. Both citations were added to our discussion on **page 20**.

Müller M, Pym EC, Tong A, and Davis GW (2011) Rab3-GAP controls the progression of synaptic homeostasis at a late stage of vesicle release. Neuron 69, 749-62

Müller M, Genç Ö, and Davis GW (2015) RIM-binding protein links synaptic homeostasis to the stabilization and replenishment of high release probability vesicles. Neuron 85, 1056-69

In summary, I think the paper describes a series of striking and interesting phenotypes and effects related to Snapin function, but I think there are several shortcomings and over-interpretations that need to be rectified.

We appreciate this reviewer for her/his insightful comments that strengthen and substantiate the claims presented in our manuscript. In the revision, we clarified some issues raised by this reviewer, removed some over-interpretations in the text, revised some conclusion better reflecting our data, and provided new data to address this reviewer's concerns.

Referee #3:

In this manuscript, Di Giovanni and Sheng attempt to dissect two distinct functions for the synapse-associated protein, snapin, during synaptic vesicle release in neurons. A cohort of snapin is incorporated into BLOC-1, a protein complex that regulates synaptic vesicle protein sorting, and another cohort of snapin exists in a distinct pool that has been previously shown by the Sheng lab to support dynein-dependent retrograde transport of late endosomes and autophagosomes toward the cell body. Here the authors exploit snapin mutants with distinct binding characteristics to link each snapin function to functional readouts. The authors conclude that both activities contribute to snapin function during synaptic vesicle release.

The topic addressed by the paper is an important one, as snapin is a functionally important synapse-associated protein with many ascribed functions, and it is unclear which of these functions is associated or not with BLOC-1. Moreover, numerous reports are emerging regarding seemingly BLOC-1-independent functions of BLOC-1 subunits (including dysbindin, discussed here), and so the topic is quite timely. Overall, the conclusions are not rock solid, but they are clearly consistent with the data. The functional and morphological data seem to be well done and the results are clear, and the functional effects of the snapin mutants on retrograde trafficking, calcium signaling and synaptic vesicle release are convincing. The calcium proximity experiments are particularly intriguing. On the other hand, there are several concerns with the manuscript in its current form. The experimental outline lacks precision, and although by the end of the paper I find myself more or less convinced of the authors' general conclusions, the conclusions drawn at each step in the paper are not fully supported by the data presented at that point. In particular, the binding data shown in Figures 3 and Suppl. Fig. 3A are incomplete and very overinterpreted, and the overstepped conclusions drawn from these data color the way that the rest of the paper is interpreted. Nevertheless, with the addition of better controls for the binding data and of analyses of binding to partners within transfected cells, exclusion of a few potential important caveats to the conclusions, and a more judicious consideration of the flow of the paper and the order in which the experiments are shown, this paper could make a very important contribution to the field. Below are specific criticisms.

We are encouraged that the reviewer found "*the functional and morphological data seem to be well done and the results are clear, and the functional effects of the snapin mutants on retrograde trafficking, calcium signaling and synaptic vesicle release are convincing. The calcium proximity experiments are particularly intriguing*".

We also appreciate the reviewer for many insightful comments and suggestions. We expanded our study by providing additional experimental data and reorganizing our paper. We also expanded discussion to address this reviewer's concerns that strengthen and substantiate the claims presented in our manuscript.

Major Concerns:

1. While the morphological data in Figure 1 are nice and the quantification is appreciated, it is unclear how the authors quantified the surface area of the presynaptic terminal or the active zone length, and the images shown do not really support the quantification that synaptic vesicle number is increased in the snapin-/- mice.

We apologize for not describing these measurements in more details. We did quantitative analysis based on previous descriptions (Pan et al., 2009). Briefly, the EM thin sections were stained with uranyl acetate and lead citrate (EM Facility, NINDS, NIH). The sections were examined on a JEOL (Akishima, Japan) 1200 EX electron microscope, and digital images were captured with a CCD camera system (XR-100; Advanced Microscopy Techniques, Danvers, MA). Both symmetrical and asymmetrical SV-filled presynaptic boutons were imaged at 30,000x magnification, and then analyzed by ImageJ 10.2 (NIH) with the same scaling system. Only SVs immediately adjacent to the presynaptic membrane were considered docked at the active zone (i.e., the portion of membrane apposed closely to a postsynaptic element). We do not provide measurements of the active zone (**Fig 1)** as we did not observe any difference between WT and *snapin*$^{-/-}$ boutons. For some presynaptic elements not attaching to a postsynaptic element, docked SVs were not counted in those boutons. Total SVs were counted within presynaptic boutons from the AZ to the edge of the terminal. The

surface area of the presynaptic terminal was traced by outlining synaptic terminal structure using the region selection tool (Shikorski and Stevens, 1997) and measured using ImageJ. Analyses were performed blind to the genotype of the sample. We now include this information on **page 23**.

We chose representative presynaptic terminals in **Fig 1A** based on their morphological features containing LE- or AV-like organelles in addition to SVs. Given the fact that these TEM images are just only cross-section synapses, it is not surprising to see some variability in their total number of SVs. For the reviewer's examination, we provide below the total number of SVs and the surface of three synapses pairs presented in **Fig 1A**. These values are within the average values of WT or *snapin*$^{-/-}$ group, thus these images are representative of our observations from a large number of total electron micrographs (n = 83, 92 for WT or KO respectively).

WT1 (SVs, surface) = 47 SVs, 0.39 mm^2;
WT2 (SVs, surface) = 119 SVs, 0.5 mm^2;
WT3 (SVs, surface) = 58 SVs, 0.3 mm^2
WT1-3 Avg ± S.D. (SVs, surface) = 74 ± 39 SVs, 0.4 ± 0.1 mm^2

KO1 (SVs, surface) = 120 SVs, 1.15 mm^2
KO2 (SVs, surface) = 70 SVs, 0.5 mm^2
KO3 (SVs, surface) = 148 SVs, 1.2 mm^2
KO1-3 Avg ± S.D. (SVs, surface) =113 ± 40 SVs, 0.95 ± 0.4 mm^2

It is not clear what the authors mean in the Results section by "single membrane LE-like vacuoles"; such vacuolar structures typify early sorting endosomes, not late endosomes.

We removed this statement on **page 5**.

In Panel E, what exactly do the "total" and "cytosol" fractions represent? The Figure legend should provide some indication that this is a total brain lysate, as suggested in the Materials and Methods.

We rewrote the legend of Panel E in Figure 1 on **page 33**:

"*E* *Sequential immunoblots of synaptosomal fractions (Syn), cytosolic fractions (Cytosol) and total brain lysates (Total) showing elevated endolysosomal marker LAMP-1 and autophagy marker LC3-II in synapse-enriched preparations from snapin cKO mice at P40*".

Is it significant that levels of SNAP25 and synaptotagmin are not increased in cko synaptosomes, despite the increased labeling for synaptic vesicle contents such as synaptophysin?

SNAP25 is a primarily a plasma membrane protein and its level probably doesn't reflect SV levels closely. Synaptic protein levels (i.e., synaptotagmin) are not necessarily increased in synaptosomal fractionation prepared from adult *snapin* cKO mouse brains. In contrast, EM and immunocytochemistry revealed increased SVs levels in *snapin*$^{-/-}$ presynaptic terminals *in vitro* cultured conditions. A possible explanation for this discrepancy is that aberrant, oversized and dysfunctional terminals might be eliminated *in vivo* in adult *snapin* cKO animals to optimize network activity and survival. Recent study suggests the role of microglia pruning of inappropriate synaptic terminals in vivo (Schafer et al, 2012), which would limit SV proteins increase in synaptosome preparations. We add this note in the **Fig 1** legend on **page 33.**

2. Given the accumulation of retrograde cargo in synapses in Figure 1 and the main argument regarding a role for snapin in retrograde trafficking throughout the paper, it escapes me why the authors address issues of vesicle release in Figures 2 and 4, rather than first nailing the effects of snapin and its mutants on retrograde transport first (Figures 3 and 5). These data flow directly from Figure 1 and build a strong argument regarding the role of different residues in retrograde trafficking, whereas the interpretations of the functional data are much more hand-wavy and rather dependent on the data yet to come in the paper (in fact, I had already written a much more negative review of this paper after reading through Figure 4 and had to rewrite the review after finishing the rest of the paper!). The authors might wish to consider how to better build the argument for a role for snapin in retrograde transport of synaptic vesicle components before describing the more tricky-

to-interpret functional data, and time their conclusions appropriately to the supporting data to avoid turning off readers.

We appreciate the reviewer for such a constructive suggestion. We followed the reviewer's suggestion and reorganized our results into two main parts based on the logical flow, which indeed makes the study easier to follow:

1. Snapin-dynein coupling to drive SVs for retrograde transport and how such a motor-driven trafficking impacts the SV pool size.

Figure 1. *Snapin*-deficient neurons display enlarged presynaptic terminals retaining various degradative organelles.
Figure 2. Snapin mutants disturb LE retrograde transport in axons.
Figure S1 (Related to **Fig 2**). Expressing the snapin-biding domain of DIC (108-268) recapitulates snapin-L99K effect on LE transport.
Figure 3. Snapin-mediated LE retrograde transport regulates total SV pool size.
Figure S2 (Related to **Fig 3**). Snapin does not affect short-distance inter-synaptic SV trafficking.
Figure 4. Altered SV exocytosis occurs at snapin-deficient presynaptic terminals**.**

2. Snapin-mediated regulation of SV positional priming via the BLOC1/AP3-dependent SV sorting pathway.

Figure 5. Snapin mutations differentially affect presynaptic activity.
Figure S3 (related to **Fig 5**). Snapin mutations discriminate its dual-role in mediating LE retrograde transport and BLOC-1 function.
Figure S4 (related to **Fig 5**). DIC (108-268) and snapin-L99K play the similar role in inhibiting SV exocytosis.
Figure 6. A snapin mutant defective in dysbindin-binding impairs SV positional priming.
Figure S5 (related to **Fig 6**). Inhibiting the snapin-dysbindin interaction impairs positional priming.
Figure 7. Deleting *snapin* affects SV molecular identity with BLOC1/AP-3 inhibition.

3. There are a number of concerns with Figure 3 and Suppl. Fig. S1. First, what isoform of dysbindin is used in these experiments? Is this an isoform normally associated with BLOC-1? The literature suggests that within BLOC-1, dysbindin and snapin are both labile in the absence of other subunits, and so it is not clear what the biological significance of the binary snapin/ dysbindin complex really is without the other complex components. Does dysbindin addition merely cause snapin to aggregate?

We used the dysbindin isoform reported as a component of BLOC-1. Antibodies and constructs of dysbindin were gifts from Wei Li's lab in the Chinese Academy of China. This isoform of dysbindin reported from the Li group interacts with snapin independent of other BLOC-1 components and affects the kinetics of SV release in neurons (Chen, et al., 2008; Feng et al., 2008). Our previous co-IP studies and immunoprecipitation analysis from mouse brains showed that snapin is not an exclusive member of BLOC-1 complexes (Cai et al., 2010; Zhou et al., 2012). Instead, Snapin was co-purified from LE membranes independent of other BLOC-1 components.

Chen XW, Feng YQ, Hao CJ, Guo XL, He X, Zhou ZY, Guo N, Huang HP, Xiong W, Zheng H, et al. (2008) DTNBP1, a schizophrenia susceptibility gene, affects kinetics of transmitter release. ***J Cell Biol*** 181, 791-801.

Feng YQ, Zhou ZY, He X, Wang H, Guo XL, Hao CJ, Guo Y, Zhen XC, and Li W (2008) Dysbindin deficiency in sandy mice causes reduction of snapin and displays behaviors related to schizophrenia. ***Schizophr Res*** 106, 218-228.

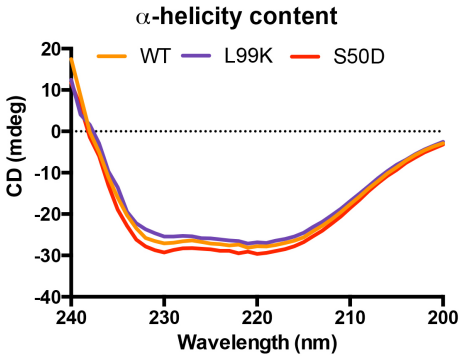
Cai Q, Lu L, Tian JH, Zhu YB, Qiao H, and Sheng ZH (2010) Snapin-regulated late endosomal transport is critical for efficient autophagy-lysosomal function in neurons. ***Neuron*** 68, 73-86

Zhou B, Cai Q, Xie Y, and Sheng ZH (2012). Snapin recruits dynein to BDNF-TrkB signaling endosomes for retrograde axonal transport and is essential for dendrite growth of cortical neurons, ***Cell Reports*** 2, 42-51.

Second, in Figure 3A and Suppl. Fig. S1A, it is necessary to show that the load of GST-snapin (3A) or GST-DIC (S1A) is identical in these lanes - a Coomassie- or silver-stained gel showing the GST fusion protein in each lane should be included to verify this.

We verified GST-protein loading in each membrane by Ponceau staining first and confirmed with anti-GST immunoblot after detecting binding proteins. At this reviewer's request, we repeated all GST pull-down assays and binding competition assays and provide sequential anti-His and anti-GST blots in the same membranes showing similar input of GST or GST-fusion proteins in all assays (**Fig 2A**, **Supplementary Fig S3A-S3D**).

Third, is it clear that the L99K mutant is actually capable of folding, and does the interaction with SNAP25 simply reflect aggregation? Are there any data (such as CD-spectra) to suggest that this mutant is largely folded?



Our two alternative GST pull-down assays by immobilizing GST-snapin-L99K, GST-snapin-S50D mutants (**Supplementary Fig S3**) or GST-SNAP25 on the beads (**Fig. S5 from Cai et al, 2010**) suggest specific binding of these proteins *in vitro*. To further test the folding capacity of snapin mutants, as suggested by the reviewer, we performed CD spectroscopy measurements and compared purified His-tagged WT snapin with snapin-L99K and snapin-S50D mutants. The alpha-helix profiles suggest these two mutants are properly folded as WT snapin (**Supplementary Fig S3E)**. We add this new data on **page 11**.

Fourth, validation of the binding activities of these constructs following expression in cells (using GST-pulldowns from cell lysates) would be necessary to support the authors' conclusions regarding their activities that are shown in future figures.

We performed this experiment as requested and included it in **Supplemental Fig S3B**. Using HA-snapin expressed in HEK cells for pull-down assay, we consistently confirmed the distinct binding capacity of these two snapin mutants: (1) the L99K mutation abolishes binding to DIC and dysbindin, but not SNAP25; (2) the S50D mutation increases binding to SNAP25, but almost abolishes binding to DIC. In addition, although the S50D mutation slightly decrease binding to dysbindin compared to WT snapin, this binding capacity still remains relatively much stronger than snapin WT for binding to SNAP25 and DIC, or snapin S50D for interaction with SNAP25.

Fifth, as is relevant for Figures S2 and S4, does excess DIC inhibit binding of Snapin to Dysbindin?

For some unknown reason we were unable to purify sufficient His-DIC to perform binding competition assays in the same controlled conditions as shown in **Supplementary Fig S3C** and **S3D**. However, those experiments demonstrate clearly that bindings of DIC and dysbindin to snapin are mutually exclusive. It is therefore very likely that excess DIC could inhibit dysbindin for binding to snapin, which is consistent with our functional studies in live neurons: over-expressing DIC(108-268) impairs both dynein-driven LE retrograde transport (**Supplementary Fig S1**) and BLOC-1-mediated positional priming (**Supplementary Fig S5A** and **S5B**), as insightfully noted by reviewer 3 in the following comments.

Finally, the models for function proposed in the text describing Figure 3 are presented here as fact rather than as hypothesis upon which the coming experiments are based. It would be fine to propose this model here and build the rest of the paper as a test of the model, but the text needs to be modified to reflect the lack of fact at this point in the paper.

We agree with the reviewer that the illustrating model in the middle of paper is based on assumptions made from the biochemical data, aimed at describing our working hypothesis. In the current revision, we removed this model to avoid confusion. Instead, we provided a description of our working hypothesis on **page 11**:

*"We propose our working hypothesis where two snapin-mediated pathways influence presynaptic activity: dynein-driven endosomal transport shuttles SV components away from terminals (**Route 1**) and BLOC-1/AP-3-dependent endosomal sorting regulates SV exocytosis at synaptic terminals (**Route 2**). While the snapin-L99K mutation impairs both Routes 1 and 2, the S50D mutation selectively impairs LE retrograde transport (Route 1). Neither mutation negatively impacts binding between snapin and SNAP25, thus avoiding direct effects on SV fusion".*

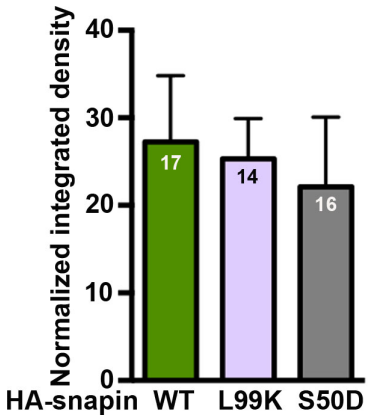
In the discussion, we conclude by proposing our model based on the experimental data on **page 17**:

*"In the current study, we used our snapin mouse model in combination with dominant-negative snapin mutants specifically impairing LE transport or BLOC-1 endosomal sorting function. We reveal that the endolysosomal pathway exerts a bipartite regulation of presynaptic activity (**Fig 7C**). First, LE transport influences the total SV pool size by shuttling SV components along the endolysosomal pathway (Route 1). Second, BLOC-1-dependent endosomal sorting determines SV composition and positional priming via AP-3-dependent recycling (Route 2). By balancing these two dynamic pathways, snapin coordinates the releasable pool size and Ca^{2+}-sensitivity of neurotransmitter release".*

4. In the experiments in which snapin is transfected into neurons, to what degree are the snapin transgenes overexpressed? And are they expressed at lower levels in snapin-/- neurons? These points need to be clarified by showing representative quantitative immunoblotting.

The reviewer asked a valid question. We carefully designed and performed these expression studies. First, we used the same pcDNA vectors with CMV promoter for expression of untagged snapin and its mutants. It is very difficult to verify protein expression using cultured neuron lysates and immunoblots because transfection efficiency in neurons is relatively low and variable. Furthermore, as only transfected neurons are selected for our imaging studies, global protein levels monitored by biochemical means are rather irrelevant

In order to minimize unspecific effects, we used untagged snapin for overexpression and rescue experiments. We have not found yet a specific snapin antibody for IC assays that we could validate in our *snapin*$^{-/-}$ mouse model. Therefore, we assessed the relative expression levels of snapin proteins by immunostaining HA-tag WT and mutant snapin in transfected cultured neurons under the same conditions. Quantitative analysis of the integrated density of HA signal is provided here for examination by the reviewer (data are means ± sd). We found that relative expression levels of these HA-tagged WT and mutant snapin in transfected neurons are similar under the same conditions.



5. The conclusions drawn from Figure 4 seem very oversimplified, particularly at this point in the paper. In this figure, the effect of overexpressing the L99K is the same as that of overexpressing wild-type snapin. Based on these data, it does not seem safe to draw conclusions regarding interactions that are disrupted by L99K, nor can one readily conclude anything regarding the role of retrograde trafficking in these responses - only that a snapin variant with improved SNAP25 and dysbindin-binding activities fails to diminish synaptic release. How can the authors exclude the possibility that overexpressed wild-type or L99K snapin competes with endogenous snapin within BLOC-1 for phosphorylation, and thus that the mimetic properties of S50D snapin averts this inhibition or favors incorporation in BLOC-1? This interpretation would not invoke any effect on retrograde transport, and needs to be addressed head on.

The reviewer raised an interesting issue on revised **Fig 5** as to whether over-expressed snapin competes with endogenous snapin in binding to BLOC-1. We think this competition is unlikely. **First**, if over-expressed WT snapin competes with endogenous snapin within BLOC-1, we would not observe any inhibitory impact because both exogenous and endogenous snapin would have the same function in neurons. **Second**, our previous studies showed that snapin is phosphorylated independently of BLOC-1 (Chheda et al, 2001) and snapin is not an exclusive member of the BLOC-1 complex (Pan et al., 2009; Cai et al., 2010; Zhou et al., 2012). Instead, snapin was immunopurified from LE membranes independently of BLOC-1 components. **Third**, although

snapin-L99K has an intact S50 phosphorylation site, its interaction with dysbindin is abolished (revised **Supplemental Fig S3A**). Thus, it is unlikely that the L99K mutant could compete with native snapin within the BLOC-1 complex for phosphorylation.

The effect of DIC overexpression in Suppl. Fig. S2 is supportive, but might also be explained by sequestration of snapin monomers and reduced BLOC-1 formation. Analyses of BLOC-1 levels (e.g. by co-IP of snapin with dysbindin in the cell lysates) and of snapin S50 phosphorylation under the different conditions would help to allay these concerns, as would showing these functional data AFTER having shown distinctions between the wild-type and mutant snapins in Figure 5.

The reviewer made an insightful comment on our data (revised **Supplemental Fig S4A** and **S4B)**. The reviewer's assumption is supported by our synaptophysin-GECO results showing SV positioning with respect to Ca^{2+} channels. Expressing snapin-L99K substantially reduced the peak amplitude of the synaptophysin-GECO response ($p<0.0001$) (**Fig 6C** and **6D**). In contrast, snapin-S50D, which retains dysbindin-binding capacity but displays reduced binding to dynein DIC, had only a minor effect on the peak amplitudes of synaptophysin-GECO responses (**Supplementary Fig S5A** and **S5B**). This suggests that the snapin-dynein coupling is unlikely required for the positional priming of SVs. To confirm this, expressing DIC (108-268) reduced synaptophysin-GECO response to the same extent as snapin-L99K, thus further supporting the biochemical competition data (**Supplementary Fig S3C**) that DIC and dysbindin compete with each other to bind snapin. Thus, as expected by this reviewer, over-expressing DIC (108-268) would impair SV positional priming by reducing snapin coupling with BLOC-1/dysbindin complexes, thus impairing AP-3-dependent sorting mechanism. We note this on **page 14**.

Finally, the experiment in Figure 4D is interesting, but the interpretation of the data is extremely convoluted and a definitive conclusion cannot be appropriately drawn.

We agree with the reviewer that we should be cautious not to draw a conclusion from these imaging results (revised **Fig 5C** and **5D**).

To provide mechanistic insights into snapin-mediated regulation of the SV cycle, we applied the V-ATPase inhibitor bafilomycin during the 1500-AP stimulation (**Fig 5C** and **5D**). When SV re-acidification is blocked by bafilomycin, the pHluorin signal mainly reflects accumulated exocytosis, thus displaying a biphasic pHluorin response: the initial rapid rise in fluorescence is followed by a slowly continued increase due to SV recruitment from the reserve pool. Surprisingly, in control neurons or neurons expressing WT snapin or snapin-S50D, the F/F_0 pHluorin response quickly reached a steady state 60 sec after stimulation onset (**Fig 5C**). Inhibiting re-acidification further revealed extensive pHluorin movements out of presynaptic boutons during prolonged stimulations, a phenotype not readily observed in the absence of bafilomycin (**Fig 5D**). The recruitment of SVs from the resting pool into the recycling pool could therefore be counterbalanced by pHluorin-labeled SV trafficking out of terminals (Route 1), thus explaining the flat appearance of the F/F_0 pHluorin response during prolonged stimulations. In contrast, in boutons expressing snapin-L99K, there was a biphasic fluorescence increase: after the initial fast rise, the F/F_0 pHluorin signal kept increasing steadily at a slower rate (**Fig 5C**). One explanation is that accumulating Ca^{2+} at presynaptic boutons during the 5-min stimulation may facilitate release of SVs with reduced Ca^{2+}-sensitivity or impaired positioning to the Ca^{2+} entry sites. Taken together, the pHluorin results suggest that over-expressing WT or snapin-L99K reduces the releasable pool size through two different mechanisms: WT snapin acts by increasing SV components trafficking into the endolysosomal pathway (Route 1) while snapin-L99K likely acts by impairing BLOC-1-dependent SV sorting from endosomes (Route 2).

We revised our description of these imaging results with more clarification on **pages 12-13**.

6. The motility data shown in Figure 5 are very convincing and interesting. However, from Figure 5E, the authors conclude that the low frequency of Syp / LAMP1 colocalization in retrograde vesicles reflects low transfection efficiency; this belies the very high frequency of comigration of VGluT and LAMP1 in Figure 5F. Is it more likely that the rate by which Syp is targeted to retrograde lysosomes is much lower than VGluT? This would be consistent with data from the Faundez laboratory that AP-3 and BLOC-1 influence VGluT incorporation into synaptic vesicles, but not synaptophysin (Newell-Litwa et al., 2009).

The reviewer raised an important point that we did not discuss in the previous submission. Because snapin mediates long-distance endolysosomal transport by recruiting dynein to the organelles, we asked whether a portion of recycling SV cargoes are transported through late endocytic organelles. Axonal trafficking was monitored in neurons co-expressing the SV marker Tomato-synaptophysin and the endolysosomal marker GFP-LAMP1 during 5-min dual-channel time-lapse acquisitions (revised **Fig 3G**). Co-migration of synaptophysin-labeled recycling SVs and endolysosomal markers was readily detected in 5 out of 16 neurons overexpressing snapin. The low frequency of those co-migration events is likely due to limited recycling synaptophysin moving into endolysosomal trafficking pathway under non-stimulation conditions. In contrast, when neurons were elicited by 600 APs at 10 Hz, more VGluT-pHluorin-labeled SV cargoes underwent recycling, thus co-trafficking with mApple-LAMP1-labeled endolysosomal organelles was robustly enhanced (**Fig 3H**).

The reviewer raises a very interesting point about the differences in synaptophysin and VGluT sorting, which we now include in our discussion on **page 8:**

"The rate of migration of VGluT-pHluorin is, however, higher than the trafficking of Tomato-synaptophysin, consistent with a previous observation that VGluT and synaptophysin are sorted through different mechanisms. AP-3- and BLOC-1 selectively influence VGluT sorting, but not synaptophysin (Newell-Litwa et al., 2009)".

Minor concerns:

7. In Figure S3C, please explain what the intersynaptic SV trafficking represents - is this lateral mobility of SV proteins following fusion with the plasma membrane?

We now clarify on **page 8** that the inter-synaptic SV trafficking is the short-range likely actin-based SV transport from one synapse to neighboring synapses, a dynamic SV local trafficking previously described (Darcy et al, 2006, Staras et al., 2010).

Darcy KJ, Staras K, Collinson LM and Goda Y (2006) Constitutive sharing of recycling synaptic vesicles between presynaptic boutons. Nat Neurosci. 9, 315-21.

Staras K, Branco T, Burden JJ, Pozo K, Darcy K, Marra V, Ratnayaka A, and Goda Y (2010) A vesicle superpool spans multiple presynaptic terminals in hippocampal neurons. ***Neuron*** 66, 37-44

8. Some of the statements in the Introduction are misleading and/or incomplete. For example, the authors state that BLOC-1 was shown by John Peter et al 2013 to promote early endosome maturation, but this was a yeast BLOC-1-like complex and it is not clear whether vertebrate BLOC-1 functions in the same manner. Di Pietro et al 2006 and Setty et al 2007 showed that BLOC-1 regulates endosomal sorting of melanosome cargoes, not lysosomal cargoes. Similarly, in neurons BLOC-1 has been shown by the Faundez group not only to bind to AP-3, as cited, but also to influence sorting of SV cargoes (Salazar et al., 2006; Newell-Litwa et al., 2009)… …and cargo delivery to the synapse (Larimore et al., 2011). These points should be properly cited and corrected. Also, it should be noted that SVs do not "transit through early endosomes", but rather that SV contents transit through endosomes. This is an important distinction, and as written will confuse readers.

We revise these statements in the introduction on **page 4**:

"BLOC-1 was shown to promote early endosome maturation in yeast (John Peter et al., 2013) and endosomal sorting of lysosome-related organelles such as melanosomes, (Di Pietro et al., 2006; Setty et al., 2007). BLOC-1 interacts with the AP-3 complex, influences sorting of SV cargoes (Newell-Litwa et al., 2009; Salazar et al., 2006), mediates SV budding from early endosomes (Faundez et al., 1998), or delivers SV cargoes to the synapses (Larimore et al., 2011). BLOC-1 deficiency perturbs AP-3 levels (Newell-Litwa et al., 2010; Newell-Litwa et al., 2009), in turn affecting neurotransmitter release from hippocampal mossy fibers (Scheuber et al., 2006). BLOC-1 is thus involved in neurotransmission, although the mechanisms remain elusive'.

9. In the discussion, the authors might wish to consider the differences in the effects of lysosomal transport on releasable pool size in neurons from snapin-/- animals, in which lysosomal transport of SV components is likely impeded over a long period of time, vs. the transient transfectants in which lysosomal transport is impeded only over days. This timing likely accounts for the reason why the S50D-expressing cells did not show defects in the releasable pool.

This is an excellent point. We added the following statement on the timing issue of phenotypes between *in vivo* mouse model versus *in vitro* cultured neurons on **page 6**.

*"Given that reduced retrograde motility was recorded during a short time-lapse imaging (5 min), LEs and SVs are expected to accumulate more robustly at synaptic terminals and SV pool size is expected to alter more significantly as observed in electron micrographs of snapin-deficient neurons (**Fig 1A** and **1B**)".*

The Discussion is also rather long and includes quite a bit of unnecessary reiteration of results and speculation. In particular, the last section comparing endocytic recycling vs. direct AP-2-dependent recycling is substantially off-topic.

At suggested by the reviewer, we deleted some off-topic discussion.

10. The manuscript should be carefully vetted for syntax and grammar. For example, the opening sentence of the Abstract is not a sentence.

We carefully proof-edited the revision.

11. The title is overly broad and non-specific. A more appropriate title might be something like "Snapin regulates synaptic vesicle release via distinct activities in endolysosomal transport and sorting".

We apologize for not clarifying better in the first submission. As acknowledged by reviewer #1, the main focus of our current study is not to characterize snapin's functions. Instead, we are addressing a fundamental and long-standing question as to how trafficking and sorting activities of endosomes coordinately regulate presynaptic function.

To address this question, we used our *snapin* mouse model in combination with dominant-negative dynein- or BLOC-1-binding-defective snapin mutants as tools to distinguish dynein-driven LE retrograde transport and BLOC-1-depednet endosomal sorting functions. We reveal that the endolysosomal pathway exerts a bipartite regulation of synaptic activity (**Figure 7C**). First, LE transport influences the total SV pool size by shuttling SV components along the endolysosomal pathway. Second, BLOC-1/AP3-dependent endosome-sorting determines SV composition and regulate positional priming. Thus, we believe that the current title better reflects the general question we addressed.

2nd Editorial Decision 27 May 2015

Thank you for submitting your revised manuscript for our consideration.
Your manuscript has now been seen once more by two of the original referees (see comments below), and I am happy to inform you that they are both broadly in favor of publication, pending satisfactory minor revision.

I would therefore like to ask you to address referee #3's remaining concerns and to provide a final version of your manuscript. Only text changes are needed, and this referee provides constructive input that I would like to ask you to take into account.

I am therefore formally returning the manuscript to you for a final round of minor revision. Once we should have received the revised version, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

REFEREE COMMENTS

Referee #2:

The authors made a serious effort to revise the paper in response to my comments. Some responses are still not entirely convincing to me (e.g. the one regarding positional priming), but I am ready to let go of this.

Referee #3:

The revised manuscript by Di Giovanni and Sheng is substantially improved relative to the original submission. The findings support the view that snapin plays a dual role in synaptic vesicle release - on the one hand by maintaining a small and healthy pool of synaptic vesicles by targeting excess vesicle contents for degradation by the lysosomal and autophagy pathways, and on the other hand through BLOC-1 by facilitating cargo recycling from endosomes into synaptic vesicles. Both activities contribute to synaptic activity. The findings are novel and of broad interest.

There remain a few vexing minor problems with the text in the revised manuscript, and the authors are encouraged to address them before publication.

1. Despite the authors' protests, the title does not accurately convey the specific message of the paper. It is admirable that the authors wish to make a large impact on the field, but the data are much more specific than the title suggests. Moreover, the title states a conclusion that was essentially proven at least 25 years ago - endolysosomal sorting and transport are both well known to play a role in regulating synaptic activity - and is more of a statement of an entire field than a summary of a single paper. The title should reflect the specific contents and contribution of this paper. Perhaps "Distinct snapin activities reveal how endolysosomal sorting and transport are coordinated to regulate synaptic activity"?

2. Snapin was recently shown to be part of a distinct complex called "BORC" that influences lysosomal positioning (Pu, Schneider et al., 2015, Dev. Cell 33: 176). BORC incorporates some BLOC-1 subunits but not others, and most notably excludes dysbindin. Might the dysbindin-independent function of snapin described here reflect BORC function? This should be considered in the introduction and the discussion.

3. The authors still have a few factual errors in the Introduction. BLOC-1 facilitates CARGO delivery to lysosome-related organelles, not endosomal sorting of the organelles themselves. The physical interaction between BLOC-1 and AP-3 was first shown by Di Pietro et al, 2006; Salazar et al showed that they cooperate functionally and present on the same organelle.

4. In rearranging the figures, the authors solved one problem (logic flow of the paper) but created another - mutants are now introduced in Fig. 2 and page 6 before they are described on page 10 and characterized in Suppl. Figure S3. The paragraph describing these mutants that starts on page 10 and goes through page 11, as well as Suppl. Figure S3 (which should be renumbered), should be moved prior to the description of the data in Figure 2 on page 6. It would also be helpful to preface all of these data with the short summary paragraph of the working model on page 11 (the paragraph after the one describing Figure S3), which explains why the authors might generate and characterize the mutants; one could say here that the authors would predict that the distinct activities might be dissected by mutants that selectively impair binding to dysbindin or dynein, and then show that they do. The dynein binding model should also be linked back to the accumulation of MVBs in snapin-/- synapses in Figure 1 to make it more clear for readers.

5. The assay in Fig. 2B needs to be explained where it is described on page 6.

6. Is Figure 3A done with wild-type or snapin-/- neurons? This should be stated both in the figure legend and the text on page 7. The assay used in panels E-H needs to be described in the text.

2nd Revision - authors' response 27 May 2015

Referee #3:

The revised manuscript by Di Giovanni and Sheng is substantially improved relative to the original submission. The findings support the view that snapin plays a dual role in synaptic vesicle release - on the one hand by maintaining a small and healthy pool of synaptic vesicles by targeting excess vesicle contents for degradation by the lysosomal and autophagy pathways, and on the other hand through BLOC-1 by facilitating cargo recycling from endosomes into synaptic vesicles. Both activities contribute to synaptic activity. The findings are novel and of broad interest.

There remain a few vexing minor problems with the text in the revised manuscript, and the authors are encouraged to address them before publication.

1. Despite the authors' protests, the title does not accurately convey the specific message of the paper. It is admirable that the authors wish to make a large impact on the field, but the data are much more specific than the title suggests. Moreover, the title states a conclusion that was essentially proven at least 25 years ago - endolysosomal sorting and transport are both well known to play a role in regulating synaptic activity - and is more of a statement of an entire field than a summary of a single paper. The title should reflect the specific contents and contribution of this paper. Perhaps "Distinct snapin activities reveal how endolysosomal sorting and transport are coordinated to regulate synaptic activity"?

We accept the reviewer's suggestion by revising the title to better reflect the specific contents of this paper. New title is:

Regulation of synaptic activity by snapin-mediated endolysosomal transport and sorting

2. Snapin was recently shown to be part of a distinct complex called "BORC" that influences lysosomal positioning (Pu, Schneider et al., 2015, Dev. Cell 33: 176). BORC incorporates some BLOC-1 subunits but not others, and most notably excludes dysbindin. Might the dysbindin-independent function of snapin described here reflect BORC function? This should be considered in the introduction and the discussion.

We cite and briefly discuss this new BORC paper on page 19 to read:

"It is noteworthy that snapin, but not dysbindin, was recently identified as a member of the newly characterized BLOC-1-related complex (BORC) in non-neuronal Hela cells (Pu et al., 2015). BORC enables microtubule-based lysosome movements toward the cell periphery. It remains to be determined whether BORC also regulates dysbindin-independent and dynein-snapin-mediated retrograde transport of LEs in axons".

3. The authors still have a few factual errors in the Introduction. BLOC-1 facilitates CARGO delivery to lysosome-related organelles, not endosomal sorting of the organelles themselves. The physical interaction between BLOC-1 and AP-3 was first shown by Di Pietro et al, 2006; Salazar et al showed that they cooperate functionally and present on the same organelle.

We correct the citation of these two papers on page 4.

4. In rearranging the figures, the authors solved one problem (logic flow of the paper) but created another - mutants are now introduced in Fig. 2 and page 6 before they are described on page 10 and characterized in Suppl. Figure S3. The paragraph describing these mutants that starts on page 10 and goes through page 11, as well as Suppl. Figure S3 (which should be renumbered), should be moved prior to the description of the data in Figure 2 on page 6.

We now revise this part of text on page 6 to logically introduce snapin mutants on dynein DIC binding for our study on dynein-driven endosomal trafficking.

"First, to identify snapin mutants that disrupt dynein-snapin coupling, we tested the effect of snapin mutations on DIC-binding by pulling down His-tagged snapin using GST-DIC. The snapin-L99K mutation abolished its interaction with DIC (**Fig 2A**). Interestingly, the snapin-S50D mutation also reduced snapin binding to DIC".
"We next verified the functional effects of these snapin mutants on axonal transport of LEs in WT cortical neurons at DIV14 co-transfected with GFP-Rab7 and a pcDNA vector alone as a control or expressing WT or mutant snapin. Control cortical neurons displayed predominant retrograde transport of LEs (**Fig 2B**)"

We feel more rationale to introduce other snapin-binding partners (dysbindin and SNAP25) on page 10 for our study on snapin-mediated SV sorting and exocytosis, which was also suggested by this reviewer during the first round of review.

It would also be helpful to preface all of these data with the short summary paragraph of the working model on page 11 (the paragraph after the one describing Figure S3), which explains why the authors might generate and characterize the mutants; one could say here that the authors would predict that the distinct activities might be dissected by mutants that selectively impair binding to dysbindin or dynein, and then show that they do. The dynein binding model should also be linked back to the accumulation of MVBs in snapin-/- synapses in Figure 1 to make it more clear for readers.

During the first submission, we actually did this way with a working model illustration. This reviewer thought that our working model is confusing because it is not supported before we present all data in the paper. We instead make a summary "two routes" hypothesis that could be dissected by snapin mutants in the text on page 11:

"We propose our working hypothesis where two snapin-mediated pathways influence presynaptic activity: dynein-driven endosomal transport shuttles SV components away from terminals (**Route 1**) and BLOC-1/AP-3-dependent endosomal sorting regulates SV exocytosis at synaptic terminals (**Route 2**). While the snapin-L99K mutation impairs both Routes 1 and 2, the S50D mutation selectively impairs LE retrograde transport (Route 1). Neither mutation negatively impacts binding between snapin and SNAP25, thus avoiding direct effects on SV fusion".

5. The assay in Fig. 2B needs to be explained where it is described on page 6.

We add the following statement on page 6 to explain Fig 2B:

"We next verified the functional effects of these snapin mutants on axonal transport of LEs in WT cortical neurons at DIV14 co-transfected with GFP-Rab7 and a pcDNA vector alone as a control or expressing WT or mutant snapin. Control cortical neurons displayed predominant retrograde transport of LEs (**Fig 2B**)".

6. Is Figure 3A done with wild-type or snapin-/- neurons? This should be stated both in the figure legend and the text on page 7. The assay used in panels E-H needs to be described in the text.

We add "WT cortical neurons" in the Fig 3A legend on page 35 and in text on page 7.

We now more clearly describe three assays in legends of Fig 3E-H on page 36.

For Panels E and F: Sample kymographs (E) and quantitative analysis (F) illustrating the dynamic trafficking of recycling SV cargoes along axons of cortical neurons co-transfected with VGluT-pHluorin-mCherry and snapin or the L99K mutant. Active axons were selected based on the pHluorin response to 100 APs, and mobile VGluT-labeled SV cargoes were tracked through mCherry during 2.5-min dual-channel recordings.

For Panel G: Sample kymographs illustrating co-trafficking of Tomato-synaptophysin with GFP-LAMP1-labeled LEs along axons from cortical neurons at DIV14. Axonal trafficking was monitored in neurons co-expressing the SV marker Tomato-synaptophysin and endolysosomal marker GFP-LAMP1 during 5-min dual-channel time-lapse acquisitions.

For Panel H: Sample kymographs illustrating co-trafficking of VGluT-pHluorin with mApple-LAMP1-labeled LEs along axons from cortical neurons at DIV14. VGlut-pHluorin fluorescence was elicited by 600 APs at 10Hz in presence of 1μM bafilomycin to prevent SV re-acidification; axonal transport was monitored during 5-min dual-channel acquisitions.

