A novel dual Ca2+ sensor system regulates Ca2+-dependent neurotransmitter release

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Re: JCB manuscript #202008121

Dr. Zhitao Hu
University of Queensland
The University of Queensland
Upland Road 79
St Lucia, QLD 4072
Australia

Dear Dr. Hu,

Thank you for submitting your manuscript entitled "A novel dual Ca2+ sensor system regulates Ca2+-dependent neurotransmitter release in C. elegans". The manuscript was assessed by three expert reviewers, whose comments are appended to this letter. We invite you to submit a revision that addresses the reviewers' key concerns, as outlined here.

As you will see, the reviewers have voiced considerable enthusiasm for the paper, but reviewers #1 and #2 have raised a number of concerns, including issues of interpretation and the need for additional experiments, that will need to be addressed before the paper would be deemed appropriate for publication in JCB.

We hope, in particular, that you will be able to address each of reviewer #2's comments in full, including new data to support the main conclusions of the study. In addition, it will be important to explicitly address point 3 regarding discussion of your findings with syt3 in context of other dual C2 domain proteins. With respect to reviewer #1's comments, we agree with the reviewer that experimental examination of the importance of the SNT-1 and -3 C2A/B domains would increase the impact of the study and so we would encourage you to do the suggested chimera experiments. However, we acknowledge that this is not strictly needed to support the main conclusions of the paper so we will not require such experiments for resubmission. We hope that you will be able to address each of reviewer #1's other points, though.

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

GENERAL GUIDELINES:

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

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.
***IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.***

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

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

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

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

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

Sincerely,

Vann Bennett, PhD  
Monitoring Editor  
Journal of Cell Biology

Tim Spencer, PhD  
Executive Editor  
Journal of Cell Biology

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

Li et al identified a C. elegans synaptotagmin isoform (SNT-3) that is capable of acting as a Ca2+ sensor for neurotransmitter release at NMJs. Unlike other C. elegans synaptotagmin isoforms (SNTs), SNT-3 does not have N-terminal transmembrane region, but the C2A/B domain is capable of binding to Ca2+ as well as membranes. The SNT-3 deficiency caused a mild defect in locomotions of the worms. However, the SNT-3 deficiency did not generate any significant defects in evoked and spontaneous neurotransmitter release at NMJs. SNT-3 acts as a Ca2+ sensor at NMJs only in the absence of SNT-1. The authors concluded that C. elegans utilizes both SNT-3
and SNT-1 for the regulation of neurotransmitter release at NMJs. I am overall supportive of eventual publication of this manuscript. However, I think there could be some improvements that do not necessarily require new experiments. My comments below are only recommendations/suggestions.

Major points:
1. The rescue efficacy of SNT-3 is much weaker than that of SNT-1 for neurotransmitter release at SNT-3/SNT-1 deficient NMJs (Figure 1).
   The authors tested the importance of the transmembrane region of SNT-1 by demonstrating that SNT-1 without N-terminal transmembrane region only partially rescues the SNT-1 deficiency at NMJs (Figure 9). I suggest that the authors test SNT-3 mutant with the N-terminal transmembrane region from SNT-1 to demonstrate the importance of the C2A/B domains from SNT-3 vs. SNT-1.

2. Figure 3
   A and B: Did the authors employ the C2A domain or the C2B domain for ITC experiments? If not, did the authors perform the ITC experiment using the C2A/B domain (is that possible)?
   C: In general, synaptotagmin binds to acidic membranes in a Ca2+-dependent manner. However, the results show that SNT-3 interacts with neutral membranes (PC/PE) in a Ca2+-dependent manner, and that the interaction between SNT-3 and the acidic membrane (Folch) is inhibited by Ca2+. Overall, it is very difficult to conclude because the quality of the results.

Minor points:
1. Figure 1, S1
   Among SNT isoforms, only SNT-3 does not have N-terminal transmembrane region. I am not sure if SNT-3 belongs to the C. elegans synaptotagmin group (SNT). As an exception, mouse synaptotagmin 17 does not have the N-terminal transmembrane region, but at least its N-terminal is palmitoylated and anchored in the membrane. Based on the domain structure, SNT-3 is closer to rabphilin or DOC (double C2 protein) rather than synaptotagmin.

2. The authors suggested that SNT-3 regulates Ca2+-dependent neurotransmitter release at NMJs when SNT-1 is inhibited. 
   Can the authors find any physiological conditions that inhibit SNT-1? The results suggest that SNT-3 is dispensable in the presence of SNT-1 (instead of the SNT-1/-3 dual system) at least for neurotransmitter release at NMJs.

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

This manuscript by Li, Hu and colleagues tackles a problem that has interested synaptic physiologists throughout the molecular age: redundancy among Ca2+ sensors for exocytosis. While synaptotagmin-1 (and syt-2) have long been known as the predominant fast Ca2+ sensors for synaptic exocytosis from flies to humans, many species demonstrate Ca2+-sensitive synaptic transmission that persists in the absence of this protein. Despite extensive research, the "other" Ca2+ sensor-which in mammals demonstrates slow kinetics and a linear Ca2+ response (Kochubey & Schneggenburger, Neuron 2011)-has not yet been conclusively identified, though candidates include proteins in the Doc2 family.

Using a combination of electrophysiology, fluorescence imaging, and biochemistry, Li et al seek to
show that, at the C. elegans NMJ, this second sensor - which lacks a transmembrane domain - is snt-3. This manuscript represents a thorough study of Ca2+-secretion coupling at the worm NMJ and raises some interesting ideas, particularly regarding the role of vesicle volume in shaping quantal size, and the role of tandem C2 domain proteins in sculpting synaptic vesicles. The authors' work is largely solid, but there are serious issues with manuscript, particularly regarding the authors' interpretation of their results and their discussion of existing literature. If these concerns are addressed, the manuscript should be suitable for publication in JCB, but we emphasize that these revisions are substantial and include performing additional experiments.

Major concerns

1. The idea of snt-3 as a "slow" sensor is not consistent with the data. When snt-3 is the primary Ca2+ sensor (i.e., in snt-1 mutants), the 10-90% rise time is unaltered, and the decay tau is actually reduced, suggesting that the sensor itself is at least as fast as snt-1. The increased latency prior to the response when snt-3 is the primary sensor suggests that an element of the excitation-secretion pathway upstream of Ca2+ sensing may be responsible, e.g., altered conduction machinery in the motor neurons. Such changes cannot be ruled out without experimental evidence. Alternatively, more time might be required for a suitable Ca2+ signal to present itself to the sensor (this reviewer is not aware of studies that define the kinetics of Ca2+ flux in these terminals under this stimulation paradigm). In this case, snt-3 may not be intrinsically slow, but rather have a substantially lower affinity for Ca2+ in the presence of membranes as compared to snt-1, or the sensor may be substantially farther away from Ca2+ channels than snt-1. While the authors suggest that this final situation is the case, there are issues with this interpretation as well: in unc-13 mutants (unc-13S) showing weaker Ca2+-secretion coupling, this latency is not observed, and the 10-90% rise time is affected. Regardless, nothing in this study suggests that the intrinsic kinetics of snt-3 are slower than those of snt-1; if anything, the opposite may be true. More substantial biochemical assays would be great help in clarifying matters here.

2. The liposome-binding assays in Fig. 3C are almost impossible to interpret for several reasons. There is no evidence of replication of these results, and no substantial Ca2+-dependent binding activity is observed in any of the conditions tested. The hallmark of syt-1’s biochemical action is Ca2+-dependent binding to anionic phospholipids (e.g., liposomes containing phosphatidylserine), and the authors fail to demonstrate even this basic positive control. Some demonstration of Ca2+-dependent phospholipid binding, how it changes in mutant snt-3 variants, and the use of snt-1 as a positive control are essential if this paper is to be accepted for publication. Additional experiments such as a Ca2+ dose-response for phospholipid binding would strongly bolster the authors' comparisons of this sensor with syt1. If the authors wish to comment on the intrinsic kinetics of the Ca2+ sensor, time-resolved measurements biochemical experiments should be performed.

3. The manuscript fails to mention - at any point - the established role for Doc2 proteins in supporting spontaneous and asynchronous release at mammalian synapses, despite the authors' citation of a paper on the subject (Diez-Arazola et al. J. Neurosci 2020). This is a well-defined case of a tandem C2 domain Ca2+ sensor without a transmembrane domain playing an important supporting role in synaptic transmission. Doc2’s absence from the synaptotagmin family is no reason for this glaring omission; the statement, "Thus far, our findings in relation to SNT-3 provide the first indication that a synaptotagmin can trigger SV release in the absence of a TM domain," is highly misleading. The authors must contextualize the role of snt-3 with a discussion of Doc2 for this paper to be suitable for publication. Moreover, the manuscript really requires some kind of phylogenetic analysis to determine whether snt-3 shares a common molecular ancestor with other TMD-less tandem C2 domain Ca2+ sensors such as Doc2 or rabphilin. Or, is snt-3 most closely to
one of the 17 synaptotagmin isoforms, and if so, which one?

4. The data in Fig. 6 are very difficult to interpret in terms of a difference between snt-1 and snt-3, as different channelrhodopsin variants—which may have different abundances, gating properties, and Ca2+ permeabilities—are used for the two mutants. The authors must compare the short-term plasticity profiles for these two optogenetic actuators in the WT and at least one mutant for these data to be meaningful.

Minor concerns:

1. The authors also fail to cite a couple of other important works in the field - namely Kochubey and Schneggenburger Neuron 2011, and the other manuscript by Chen, Jonas et al. in Cell Reports 2017 describing the various roles of syt7 at central mammalian synapses.

2. The authors suggest that, in snt-3 mutants, the fact that "the distally docked SV pool significantly increased" suggests that "release of the distal docked SV pool is impeded." While the authors do go on to show that snt-3 release depends on the shorter unc13 variant, the presence of more docked vesicles at a distal pool does not imply an impediment to release. In general, docked vesicles are more likely to be released.

3. It is interesting that snt-1 and snt-3 have opposing effects on terminal size, but that the compound mutant has an exacerbated snt-3 (smaller vesicle) phenotype. This matches the mini phenotype and suggests that vesicle size sets an upper limit on the amount of transmitter released per fusion event. However, the statement "priming is partially suppressed by the simultaneous loss of snt-1 and snt-3" is misleading, as snt-3 loss does not appear to cause an additional priming deficit over that of snt-1.

4. The authors should include a graph that directly compares the docking distribution (vesicles vs: distance from plasma membrane) between WT and snt-3 mutants and comment on any observed differences.

5. Important methodological details are left out: how was optogenetic stimulation performed? Which snt-3 line was used for the data in Fig. 6? Why not use electrical stimulation for short-term plasticity? What temperature were the worms kept at for locomotion experiments?

6. In Fig. 7, the authors should include a graphical legend for the snt-1 mutants (as for snt-3 in fig. 8), as their terminology contrasts with that used by others in the field (i.e., "C2AB" often indicates the absence of a TMD). This legend could be expanded to replace the one shown in Fig. 9.

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

The authors have written the most carefully performed and most thorough manuscript that I have reviewed in the last few years. I suggest that the manuscript be published in its present form. There is certainly a growing field of research in relation to synaptotagmins, in both mammals and other animals, and this manuscript is not the first one to introduce syt-3 as an important molecule. However, the current manuscript is, as I mentioned, of such a high quality, that I recommend its rapid publication.
Overview:

We are pleased that the reviewers found our results to be of interest. Their reviews provided many helpful suggestions, which we believe have greatly improved the manuscript. In response to these comments, we have added several new experiments. 1) We made the phylogenetic tree which shows that SNT-3 is not a homolog of rabphilin-3A or Doc2. 2) We collected more evoked EPSCs rescued by neuronal SNT-3, and the new results demonstrate that the used SNT-3 cDNA is almost fully functional in neurotransmission. 3) We measured membrane binding of SNT-1, SNT-3, and their Ca²⁺ binding mutations using a co-sedimentation assay. Our results showed that wild-type SNT-1 and SNT-3 exhibited very clear Ca²⁺-dependent liposome binding, but the DN mutations in their C2 domains abolished the Ca²⁺-dependent liposome binding. 4) We constructed the chimeric protein by fusing the N terminal of SNT-1 to the N terminal of SNT-3 and found that this fusion protein cannot rescue synaptic transmission in snt-1 mutants. However, because we don’t have evidence showing that this chimera is properly localized on synaptic vesicles, we did not add those results in the revised manuscript.

We have reorganized the manuscript incorporating the new results into the figures and text. Full responses to all reviewer comments are detailed below. We hope that the reviewers agree that these changes address their major concerns and that our manuscript is now acceptable for publication in the Journal of Cell Biology.

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

Li et al identified a C. elegans synaptotagmin isoform (SNT-3) that is capable of acting as a Ca²⁺ sensor for neurotransmitter release at NMJs. Unlike other C. elegans synaptotagmin isoforms (SNTs), SNT-3 does not have N-terminal transmembrane region, but the C2A/B domain is capable of binding to Ca²⁺ as well as membranes. The SNT-3 deficiency caused a mild defect in locomotion of the worms. However, the SNT-3 deficiency did not generate any significant defects in evoked and spontaneous neurotransmitter release at NMJs. SNT-3 acts as a Ca²⁺ sensor at NMJs only in the absence of SNT-1. The authors concluded that C. elegans utilizes both SNT-3 and SNT-1 for the regulation of neurotransmitter release at NMJs. I am overall supportive of eventual publication of this manuscript. However, I think there could be some improvements that do not necessarily require new experiments. My comments below are only recommendations/suggestions.

Major points:

1. The rescue efficacy of SNT-3 is much weaker than that of SNT-1 for neurotransmitter release at SNT-3/SNT-1 deficient NMJs (Figure 1).

   We have repeated the overexpression rescue of SNT-3 in the snt-1; snt-3 double mutants by focusing on transgenic worms with obviously better movement, and found that the rescue was significantly improved, with the evoked EPSCs being restored to almost the same level as in the snt-1 mutants. These new results demonstrate that SNT-3 can fully rescue the evoked EPSCs in the double mutants. The SNT-3 overexpression data in the revised manuscript includes all recordings (Figure 1D, E).

Page 6:

“Neuronal expression of snt-3 cDNA in snt-1; snt-3 double mutants restored the evoked EPSCs to 90% of the level in snt-1 mutants” (Was the rescue significantly different from snt-1 alone? If not, can you say so in this sentence?)

The authors tested the importance of the transmembrane region of SNT-1 by demonstrating
that SNT-1 without N-terminal transmembrane region only partially rescues the SNT-1 deficiency at NMJs (Figure 9). I suggest that the authors test SNT-3 mutant with the N-terminal transmembrane region from SNT-1 to demonstrate the importance of the C2A/B domains from SNT-3 vs. SNT-1.

We actually have done this experiment by fusing the N terminal sequence of SNT-1 to SNT-3 (N\textsuperscript{SNT-1}-SNT-3). The idea was to test whether SNT-3 can replace SNT-1 when targeted to synaptic vesicles. We found that the N\textsuperscript{SNT-1}-SNT-3 chimera failed to rescue the locomotion and evoked EPSC defects in the \textit{snt-1} mutants. Moreover, expression of this chimeric protein in \textit{snt-1; snt-3} double mutants restored evoked EPSC to the same level as the \textit{snt-1} mutants, with a comparable latency. These results indicate that the N\textsuperscript{SNT-1}-SNT-3 chimera cannot function as SNT-1 in synaptic transmission, but instead retains SNT-3 functionality. However, the reason we did not add these results to the manuscript is the lack of evidence that this chimera is localized on synaptic vesicles. We did not observe an accumulation of N\textsuperscript{SNT-1}-SNT-3::mApple fluorescence in the cell bodies in \textit{unc-104} (kinesin) mutants, indicating that this chimeric protein may not be able to properly localize on SVs.
Figure legend:
**Tethering SNT-3 on SVs does not alter its function.**

(A) SV-associated SNT-3 was generated by adding the N-terminal sequence of SNT-1 (0-155aa) to the N terminal of SNT-3.

(B) Representative locomotory trajectories and quantification of the average locomotion speed for the indicated genotypes, including wild type, snt-1, and N*1-SNT-3 rescue in snt-1 mutants. Data are mean ± SEM (***, p < 0.001 when compared to wild type; n.s., non-significant; one-way ANOVA).

(D-G) Representative traces of evoked EPSCs (E) and mEPSCs (H), and quantification of the EPSC amplitude, charge transfer, latency (F) and mEPSC frequency and amplitude (H) in snt-1 mutants, and N*1-SNT-3 rescue worms. Data are mean ± SEM (**, p < 0.01, ***, p < 0.001 when compared to wild type; n.s., non-significant; one-way ANOVA).

(H, J) Example traces of evoked EPSCs and quantification of the EPSC amplitude, charge transfer, and latency from the indicated genotypes and transgenes. Data are mean ± SEM (**, p < 0.01, ***, p < 0.001 when compared to wild type; n.s., non-significant; one-way ANOVA).

2. Figure 3

A and B: Did the authors employ the C2A domain or the C2B domain for ITC experiments? If not, did the authors perform the ITC experiment using the C2A/B domain (is that possible)?

Yes, we performed the ITC experiment by using the C2AB domain of SNT-3.

C: In general, synaptotagmin binds to acidic membranes in a Ca2+-dependent manner. However, the results show that SNT-3 interacts with neutral membranes (PC/PE) in a Ca2+-dependent manner, and that the interaction between SNT-3 and the acidic membrane (Folch) is inhibited by Ca2+. Overall, it is very difficult to conclude because the quality of the results.

To confirm whether SNT-3 binds to the plasma membrane, we performed a liposome co-sedimentation assay. Our new results showed that both SNT-1 and SNT-3 exhibited clear Ca2+-dependent liposome binding in several independent experiments. These results have been incorporated into the revised manuscript as follows (Figure 3).

Page 10:

“To determine whether SNT-3 C2 domains possess Ca2+-dependent membrane binding activity, we next performed a liposome co-sedimentation assay. The cytoplasmic domain (C2AB) of SNT-1 was used as a positive control. Our results showed that wild type C2AB of either SNT-1 or SNT-3 co-sedimented with liposomes (25%PS/75%PC) in the presence of 1 mM Ca2+. However, in the absence of Ca2+ (1mM EGTA), no significant levels of C2AB-membrane binding were detected (Figure 3C, D). Mutations that disrupt the Ca2+ binding sites in the C2 domain of SNT-1 or SNT-3 (SNT-1 C2AB D3,4N, SNT-3 C2AB D3,4N) abolished the Ca2+-dependent interactions with liposomes (Figure 3C, D). Together, these results demonstrate that SNT-3, similar to SNT-1, also has a Ca2+-dependent phospholipid binding activity."

Page 41:

**Figure 3. SNT-3 binds Ca2+ and membranes.**

(A-B) Ca2+ binding properties of SNT-3 was measured by isothermal titration calorimetry (ITC). (A) raw data; and (B) integrated and normalized data fitted with a 1:1 binding model. The average binding affinity (Kd) was calculated by carrying out 3 independent titrations. (C-D) Ca2+-dependent membrane interactions of wild type and mutant versions of SNT-1 C2AB and SNT-3 C2AB were determined using liposome co-sedimentation experiments. Representative images of SDS-PAGE gels were shown in (C). “-1 Ca2+” indicates 1 mM Ca2+, and “-1 Ca2+” indicates 1 mM EGTA. The percentage of protein in pellets were quantified using ImageJ and were plotted in (D). Co-sedimentation experiments were independently repeated 5 times for SNT-1 C2AB and 4 times for SNT-3 C2AB variants.”

Minor points:

1. Figure 1, S1
Among SNT isoforms, only SNT-3 does not have N-terminal transmembrane region. I am not sure if SNT-3 belongs to the C. elegans synaptotagmin group (SNT). As an exception, mouse synaptotagmin 17 does not have the N-terminal transmembrane region, but at least its N-terminal is palmitoylated and anchored in the membrane. Based on the domain structure, SNT-3 is closer to rabphilin or DOC (double C2 protein) rather than synaptotagmin. We did consider whether SNT-3 is a homolog of other C2 domain proteins such as rabphilin-3A and Doc2, based on the fact that SNT-3 is structurally different from all other synaptotagmin Ca\(^{2+}\) sensors which have an N terminal TM domain. However, several pieces of evidence do not support this notion. First of all, a phylogenetic analysis revealed that SNT-3 is closest to SNT-1 and the mouse Syt1/2, but not the mouse rabphilin-3A or Doc2 (Figure S11). Second, both rabphilin-3A and Doc2 have functional domains in their N termini, a Zn\(^{2+}\) finger domain in rabphilin-3A which binds to Rab-3, and a MID motif in Doc2 that binds to Munc13. In contrast the SNT-3 N terminal, consisting of only 11 aa, does not appear to be functional. Third, the worm RBF-1, encoded by the rbf-1 gene, appears to be the homolog of the mouse rabphilin-3A, displaying high sequence similarity (45% in N terminal Zn\(^{2+}\) domain and 48% in the C2 domain) (Staunton et al., 2001). Our unpublished results show that synaptic transmission is not altered in either rbf-1 mutants, or the snt-1;rbf-1 double mutants, suggesting that RBF-1 does not function as a Ca\(^{2+}\) sensor at C. elegans NMJs. Moreover, rabphilin-3A and RBF-1 are known to be localized on synaptic vesicles (Staunton et al., 2001), whereas SNT-3, based on our results, is not on synaptic vesicles (Figure 2). Fourth, Doc2 has been shown to be required for baseline transmission, including asynchronous release in cultured hippocampal neurons (Yao et al., 2011), and spontaneous release in cultured cortical neurons (Pang et al., 2011; Courtney et al., 2018). In contrast, the snt-3 single mutant worms do not exhibit any synaptic transmission defect, suggesting that SNT-3 is functionally different from Doc2. Finally, our findings that cytoplasmic SNT-1 can still trigger spontaneous and fast evoked neurotransmitter release indicate that a TM domain is not required for a synaptotagmin to act as a Ca\(^{2+}\) sensor. Together these observations support the classification of SNT-3 as a member of the synaptotagmin protein family. This information has been added to the discussion as follows:

Page 28-29:
With the exception of mouse synaptotagmin 17, SNT-3 is the only synaptotagmin homolog lacking a TM domain. We, therefore, considered the possibility that SNT-3 could be a homolog of other double C2 domain containing proteins such as rabphilin-3A and Doc2, which both lack an N terminal TM domain. However, several pieces of evidence do not support this notion. First, a phylogenetic analysis revealed that SNT-3 is closest to SNT-1 and the mouse Syt1/2, but not the mouse rabphilin-3A or Doc2 (Figure S11). Second, both rabphilin-3A and Doc2 have functional domains in their N termini, a Zn\(^{2+}\) finger domain in rabphilin-3A which binds to Rab-3, and a MID motif in Doc2 that binds to Munc13. In contrast the SNT-3 N terminal, consisting of only 11 aa, does not appear to be functional. Third, the worm RBF-1, encoded by the rbf-1 gene, appears to be the homolog of the mouse rabphilin-3A, displaying high sequence similarity (45% in N terminal Zn\(^{2+}\) domain and 48% in the C2 domain) (Staunton et al., 2001). Our unpublished results show that synaptic transmission is not altered in either rbf-1 mutants, or the snt-1;rbf-1 double mutants, suggesting that RBF-1 does not function as a Ca\(^{2+}\) sensor at C. elegans NMJs. Moreover, rabphilin-3A and RBF-1 are known to be localized on synaptic vesicles (Staunton et al., 2001), whereas SNT-3, based on our results, is not on synaptic vesicles (Figure 2). Fourth, Doc2 has been shown to be required for baseline transmission, including asynchronous release in cultured hippocampal neurons (Yao et al., 2011), and spontaneous release in cultured cortical neurons (Pang et al., 2011; Courtney et al., 2018). In contrast, the snt-3 single mutant worms do not exhibit any synaptic transmission defect, suggesting that SNT-3 is functionally different from Doc2. Finally, our findings that cytoplasmic SNT-1 can still trigger spontaneous and fast evoked neurotransmitter release indicate that a TM domain is not required for a synaptotagmin to act as a Ca\(^{2+}\) sensor. Together these observations support the classification of SNT-3 as a member of the synaptotagmin protein family.

2. The authors suggested that SNT-3 regulates Ca\(^{2+}\)-dependent neurotransmitter release at NMJs when SNT-1 is inhibited.
Can the authors find any physiological conditions that inhibit SNT-1? The results suggest that SNT-3 is dispensable in the presence of SNT-1 (instead of the SNT-1/-3 dual system) at least for neurotransmitter release at NMJs.

Thus far, we don’t have effective ways to inhibit SNT-1’s function under physiological conditions. We even tried to optically inactivate SNT-1 by tagging miniSOG to SNT-1, and we did find decreased synaptic transmission after the illumination under which SNT-1 is supposed to be inactivated. However, due to the limitation of this technique, the observed changes in synaptic transmission may not be induced specifically by SNT-1.

Although our data do not support that SNT-3 is required for synaptic transmission, it does have a minor role in regulating animal behaviour. The locomotion speed was significantly decreased by 30% in the two snt-3 mutants, and this phenotype was restored by neuronal expression of SNT-3 cDNA (Figure 1). It should be noted that we only examined the baseline transmission in snt-3 mutants, and we cannot exclude the possibility that SNT-3 is required for activity-dependent changes in synaptic transmission and plasticity. A similar case has been made for Syt7, as this protein is not required for baseline synaptic transmission, but mediates asynchronous release in Syt1 KO neurons. Moreover, knockout of Syt7 strongly suppresses synaptic facilitation. In the future, we will investigate potential roles of SNT-3 in other cellular pathways.

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

This manuscript by Li, Hu and colleagues tackles a problem that has interested synaptic physiologists throughout the molecular age: redundancy among Ca2+ sensors for exocytosis. While synaptotagmin-1 (and syt-2) have long been known as the predominant fast Ca2+ sensors for synaptic exocytosis from flies to humans, many species demonstrate Ca2+-sensitive synaptic transmission that persists in the absence of this protein. Despite extensive research, the "other" Ca2+ sensor-which in mammals demonstrates slow kinetics and a linear Ca2+ response (Kochubey & Schneggenburger, Neuron 2011)-has not yet been conclusively identified, though candidates include proteins in the Doc2 family.

Using a combination of electrophysiology, fluorescence imaging, and biochemistry, Li et al seek to show that, at the C. elegans NMJ, this second sensor - which lacks a transmembrane domain - is snt-3. This manuscript represents a thorough study of Ca2+-secretion coupling at the worm NMJ and raises some interesting ideas, particularly regarding the role of vesicle volume in shaping quantal size, and the role of tandem C2 domain proteins in sculpting synaptic vesicles. The authors' work is largely solid, but there are serious issues with manuscript, particularly regarding the authors' interpretation of their results and their discussion of existing literature. If these concerns are addressed, the manuscript should be suitable for publication in JCB, but we emphasize that these revisions are substantial and include performing additional experiments.

Major concerns

1. The idea of snt-3 as a "slow" sensor is not consistent with the data. When snt-3 is the primary Ca2+ sensor (i.e., in snt-1 mutants), the 10-90% rise time is unaltered, and the decay tau is actually reduced, suggesting that the sensor itself is at least as fast as snt-1. The increased latency prior to the response when snt-3 is the primary sensor suggests that an element of the excitation-secretion pathway upstream of Ca2+ sensing may be responsible, e.g., altered conduction machinery in the motor neurons. Such changes cannot be ruled out.
without experimental evidence. Alternatively, more time might be required for a suitable Ca\(^{2+}\) signal to present itself to the sensor (this reviewer is not aware of studies that define the kinetics of Ca\(^{2+}\) flux in these terminals under this stimulation paradigm). In this case, snt-3 may not be intrinsically slow, but rather have a substantially lower affinity for Ca\(^{2+}\) in the presence of membranes as compared to snt-1, or the sensor may be substantially farther away from Ca\(^{2+}\) channels than snt-1. While the authors suggest that this final situation is the case, there are issues with this interpretation as well: in unc-13 mutants (unc-13S) showing weaker Ca\(^{2+}\)-secretion coupling, this latency is not observed, and the 10-90% rise time is affected. Regardless, nothing in this study suggests that the intrinsic kinetics of snt-3 are slower than those of snt-1; if anything, the opposite may be true. More substantial biochemical assays would be great help in clarifying matters here.

We agree that referring to SNT-3 as a slow Ca\(^{2+}\) sensor is misleading due to the faster decay of the evoked EPSCs in the snt-1 mutants in which SNT-3 is the primary Ca\(^{2+}\) sensor. More accurately, it acts as the second Ca\(^{2+}\) sensor that mediates delayed neurotransmitter release according to the significantly increased synaptic latency. We have refreshed our statements throughout the revised manuscript. In some places, we used “primary and secondary Ca\(^{2+}\) sensors” to replace “fast and slow Ca\(^{2+}\) sensors” to avoid misleading statements.

**Page 2:**
“Here we report a new Ca\(^{2+}\) sensor, SNT-3, which triggers delayed Ca\(^{2+}\)-dependent neurotransmitter release.”

**Page 4:**
“Here we provide evidence that SNT-3 acts as an additional Ca\(^{2+}\) sensor that triggers delayed evoked neurotransmitter release at the worm NMJ.”

**Page 5:**
**Results**
**SNT-3 is required for delayed evoked neurotransmitter release**
Numerous studies have shown that Syt1 functions as a Ca\(^{2+}\) sensor that triggers fast…..”

**Page 7:**
“Collectively, these results demonstrate that SNT-3 triggers a delayed component of evoked neurotransmitter release, revealed in the absence of the fast Ca\(^{2+}\) sensor SNT-1, likely by acting as a second Ca\(^{2+}\) sensor.”

**Page 13:**
“The prolonged latency in the evoked EPSCs when SNT-3 is the primary Ca\(^{2+}\) sensor (i.e., in snt-1 mutants) indicates that SNT-3 mediates delayed neurotransmitter release.”

**Page 22:**
“Although we cannot conclude that SNT-3 is a slow Ca\(^{2+}\) sensor like Syt7, due to the fast decay of the evoked EPSCs mediated by SNT-3, these two Ca\(^{2+}\) sensors share many similarities including localization and function.”

**Page 27:**
“In contrast, we show that SNT-3 is cytoplasmic but mediates delayed neurotransmitter release. Together these results indicate that the release kinetics mediated by distinct Ca\(^{2+}\) sensors appears to be determined by their intrinsically different C2 domains rather than differences in their membrane association.”

**Page 45:**
**Figure 7. SNT-3 triggers a delayed release when SNT-1 binding Ca\(^{2+}\) is impaired.**

(A) Representative images showing the SNT-3::mApple distribution in cholinergic axons in wild type and snt-1 mutant background. Scale bar, 5µm.”

**Page 23:**
“Whereas, the evoked EPSCs are unaltered in Syt7 knockout mice or snt-3 mutant worms (Maximov et al., 2008; Bacaj et al., 2013; Weber et al., 2014), the effects of Syt7 or SNT-3 knockout on baseline evoked transmitter release is only observed in the absence of Syt1 or SNT-1 (Figure 1), suggesting a common mechanism by which the primary Ca\(^{2+}\) sensor (Syt1 and SNT-1) suppresses the secondary Ca\(^{2+}\) sensor (Syt7 and SNT-3) under physiological conditions.”

It should be noted that synaptic delay has been observed in many species lacking Syt1, indicating that the underlying mechanisms are likely to be conserved. We agree with the reviewer that the delay may not be caused by differential Ca\(^{2+}\) sensing abilities of SNT-1 and SNT-3. Instead, it is likely to be determined by the differential spatial location of these two Ca\(^{2+}\) sensors in the synapse and their differential coupling to Ca\(^{2+}\) entry. This is supported by
two pieces of evidence. First, SNT-3 exhibits a diffuse distribution in axons compared to SNT-1 which displays a punctate distribution (Figure 2E1-E6). Second, SNT-3 is specifically required for the fusion of UNC-13S-mediated synaptic vesicles which are further away from Ca\(^{2+}\) entry, but not for the fusion of UNC-13L-mediated synaptic vesicles which are close to Ca\(^{2+}\) entry (Figure 5D, E) (Hu et al., 2013; Bohme et al., 2016), indicating that SNT-3 acts in the distal synaptic region where it takes longer for Ca\(^{2+}\) to arrive, thereby producing a significant delay when SNT-3 is the primary Ca\(^{2+}\) sensor. It should be noted that synaptic delay was also observed in UNC-13S-mediated slow neurotransmitter release. We have added the quantification into the revised manuscript (see Figure 5). However, we cannot rule out other possibilities for the differential roles of SNT-1 and SNT-3. For example, both Ca\(^{2+}\) sensors are believed to bind to SNARE complex when active, but we do not know whether the SNARE complexes they bind to are in similar or different states (tight or loose). We also do not know whether the two Ca\(^{2+}\) sensors have differential kinetics when binding to the plasma membrane. Addressing these questions is beyond the scope of the present study.

2. The liposome-binding assays in Fig. 3C are almost impossible to interpret for several reasons. There is no evidence of replication of these results, and no substantial Ca\(^{2+}\)-dependent binding activity is observed in any of the conditions tested. The hallmark of syt-1’s biochemical action is Ca\(^{2+}\)-dependent binding to anionic phospholipids (e.g., liposomes containing phosphatidylinerine), and the authors fail to demonstrate even this basic positive control. Some demonstration of Ca\(^{2+}\)-dependent phospholipid binding, how it changes in mutant snt-3 variants, and the use of snt-1 as a positive control are essential if this paper is to be accepted for publication. Additional experiments such as a Ca\(^{2+}\) dose-response for phospholipid binding would strongly bolster the authors’ comparisons of this sensor with syt1. If the authors wish to comment on the intrinsic kinetics of the Ca\(^{2+}\) sensor, time-resolved measurements biochemical experiments should be performed.

We agree that a standard liposome binding assay is necessary. Therefore, we performed co-sedimentation experiments on SNT-1, SNT-3 and their Ca\(^{2+}\) binding mutants. The liposomes used in these experiments were 25\%DOPS/75\%DOPC. Our results showed that both wild type SNT-1 and SNT-3 exhibited PS/PC liposome binding properties in a Ca\(^{2+}\)-dependent manner, whereas the D/N mutations blocked the Ca\(^{2+}\)-dependent liposome binding.

Experiments on SNT-1 were repeated 5 times and those on SNT-3 were done 4 times. We have incorporated representative and quantified results into the revised manuscript in Figure 3C,D and the results section as follows.

Page 11:

“Figure 3. SNT-3 binds Ca\(^{2+}\) and membranes.

(A-B) Ca\(^{2+}\) binding properties of SNT-3 were measured by isothermal titration calorimetry (ITC). (A) raw data; and (B) integrated and normalized data fitted with a 1:1 binding model. The average binding affinity (Ka) was calculated by carrying out 3 independent titrations. (C-D) Ca\(^{2+}\)-dependent membrane interactions of wild type and mutant versions of SNT-1 C2AB and SNT-3 C2AB were determined using liposome co-sedimentation experiments. Representative images of SDS-PAGE gels were shown in (C). “+ Ca\(^{2+}\)” indicates 1 mM Ca\(^{2+}\), and “- Ca\(^{2+}\)” indicates 1 mM EGTA. The percentage of protein in pellets were quantified using ImageJ and were plotted in (D). Co-sedimentation experiments were independently repeated 5 times for SNT-1 C2AB and 4 times for SNT-3 C2AB variants.”

Page 10:

“To determine whether SNT-3 C2 domains possess Ca\(^{2+}\)-dependent membrane binding activity, we next performed a liposome co-sedimentation assay. The cytoplasmic domain (C2AB) of SNT-1 was used as a positive control. Our results showed that wild type C2AB of either SNT-1 or SNT-3 co-sedimented with liposomes (25\%PS/75\%PC) in the presence of 1 mM Ca\(^{2+}\). However, in the absence of Ca\(^{2+}\) (1mM EGTA), no significant levels of C2AB-membrane binding were detected (Figure 3C, D). Mutations that disrupt the Ca\(^{2+}\) binding sites in the C2 domain of SNT-1 or SNT-3 (SNT-1 C2AB D3,4N, SNT-3 C2AB D3,4N) abolished
the Ca\(^{2+}\)-dependent interactions with liposomes (Figure 3C, D). Together, these results demonstrate that SNT-3, similar to SNT-1, also has a Ca\(^{2+}\)-dependent phospholipid binding activity.”

Unfortunately, we could not conduct experiments to determine the Ca\(^{2+}\) dose-response for phospholipid binding due to the lack of corresponding equipment. It will take a long time to order this equipment and finish the experiments during the current pandemic. We hope the reviewer understands. We hope to perform those experiments in our next synaptotagmin story.

3. The manuscript fails to mention - at any point - the established role for Doc2 proteins in supporting spontaneous and asynchronous release at mammalian synapses, despite the authors' citation of a paper on the subject (Diez-Arazola et al. J. Neurosci 2020). This is a well-defined case of a tandem C2 domain Ca\(^{2+}\) sensor without a transmembrane domain playing an important supporting role in synaptic transmission. Doc2's absence from the synaptotagmin family is no reason for this glaring omission; the statement, "Thus far, our findings in relation to SNT-3 provide the first indication that a synaptotagmin can trigger SV release in the absence of a TM domain," is highly misleading. The authors must contextualize the role of snt-3 with a discussion of Doc2 for this paper to be suitable for publication. Moreover, the manuscript really requires some kind of phylogenetic analysis to determine whether snt-3 shares a common molecular ancestor with other TMD-less tandem C2 domain Ca\(^{2+}\) sensors such as Doc2 or rabphilin. Or, is snt-3 most closely to one of the 17 synaptotagmin isoforms, and if so, which one?

We have now extended our analysis to take these suggestions to ascertain whether SNT-3 is a homolog of other C2 domain proteins such as rabphilin-3A and Doc2, based on the fact that SNT-3 is structurally different from all other synaptotagmin Ca\(^{2+}\) sensors, which have an N terminal TM domain. However, several pieces of evidence do not support this notion. First of all, we conducted a phylogenetic analysis, which revealed that SNT-3 is closest to SNT-1 and mouse Syt1/2, but not mouse rabphilin-3A or Doc2 (Figure S11). Second, both rabphilin-3A and Doc2 have functional domains in their N termini, a Zn\(^{2+}\) finger domain in rabphilin-3A which binds to Rab-3, and a MID motif in Doc2 that binds to Munc13. In contrast the SNT-3 N terminal, consisting of only 11aa, does not appear to be functional. Third, the worm RBF-1, encoded by the rbf-1 gene, appears to be the homolog of the mouse rabphilin-3A, displaying high sequence similarity (45% in N terminal Zn\(^{2+}\) domain and 48% in the C2 domain) (Staunton et al., 2001). Our unpublished results show that synaptic transmission is not altered in either rbf-1 mutants, or the snt-1;rbf-1 double mutant, suggesting that RBF-1 does not function as a Ca\(^{2+}\) sensor at C. elegans NMJs. Moreover, rabphilin-3A and RBF-1 are known to be localized on synaptic vesicles (Staunton et al., 2001), whereas SNT-3, based on our results, is not on synaptic vesicles (Figure 2). Fourth, Doc2 has been shown to be required for baseline transmission, including asynchronous release in cultured hippocampal neurons (Yao et al., 2011), and spontaneous release in cultured cortical neurons (Pang et al., 2011; Courtney et al., 2018). In contrast, the snt-3 single mutant worms do not exhibit any synaptic transmission defect, suggesting that SNT-3 is functionally different from Doc2. Finally, our findings that cytoplasmic SNT-1 can still trigger spontaneous and fast evoked neurotransmitter release indicate that a TM domain is not required for a synaptotagmin to act as a Ca\(^{2+}\) sensor. Together these observations support the classification of SNT-3 as a member of the synaptotagmin protein family. This information has been added to the discussion as follows:

Page 28:

With the exception of mouse synaptotagmin 17, SNT-3 is the only synaptotagmin homolog lacking a TM domain. We, therefore, considered the possibility that SNT-3 could be a homolog of other double C2 domain
containing proteins such as rabphilin-3A and Doc2, which both lack an N terminal TM domain. However, several pieces of evidence do not support this notion. First, a phylogenetic analysis revealed that SNT-3 is closest to SNT-1 and the mouse Syt1/2, but not mouse rabphilin-3A or Doc2 (Figure S11). Second, both rabphilin-3A and Doc2 have functional domains in their N termini, a Zn2+ finger domain in rabphilin-3A which binds to Rab-3, and a MID motif in Doc2 that binds to Munc13. In contrast the SNT-3 N terminal, consisting of only 11aa, does not appear to be functional. Third, the worm RBF-1, encoded by the rbf-1 gene, appears to be the homolog of the mouse rabphilin-3A, displaying high sequence similarity (45% in N terminal Zn2+ domain and 48% in the C2 domain) (Staunton et al., 2001). Our unpublished results show that synaptic transmission is not altered in either rbf-1 mutants, or the snt-1;rbf-1 double mutant, suggesting that RBF-1 does not function as a Ca2+ sensor at C. elegans NMJs. Moreover, rabphilin-3A and RBF-1 are known to be localized on synaptic vesicles (Staunton et al., 2001), whereas SNT-3, based on our results, is not on synaptic vesicles (Figure 2).

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4. The data in Fig. 6 are very difficult to interpret in terms of a difference between snt-1 and snt-3, as different channelrhodopsin variants—which may have different abundances, gating properties, and Ca2+ permeabilities—are used for the two mutants. The authors must compare the short-term plasticity profiles for these two optogenetic actuators in the WT and at least one mutant for these data to be meaningful.

We agree that it is better to use the same optogenetic strain (either ChR2 or its variant ChIEF) to compare the role of SNT-1 and SNT-3 in synaptic depression. In our current optical line ZxIs6, ChR2 was integrated into the same chromosome as the snt-3 allele, making the crosses challenging. But we finally generated the ZxIs6;snt-3 double mutants after several attempts (suggesting that the map distance of the inserted ChR2 and the snt-3 locus are quite close). We therefore measured synaptic depression in the newly generated ZxIs6;snt-3 line. Our results demonstrated that the depression τ and the replenishment rate in snt-3 mutants were indistinguishable from the control animals, consistent with our results observed in the ChIEF;snt-3 line. These results confirmed that SNT-3 is not involved in the regulation of synaptic depression. It should be noted that the new collected train EPSCs exhibited slight difference in synaptic depression with those recorded long time ago (likely due to changes in the LED light intensity). We have incorporated the new results into the revised manuscript (Figure 6).

Minor concerns:

1. The authors also fail to cite a couple of other important works in the field - namely Kochubey and Schneggenburger Neuron 2011, and the other manuscript by Chen, Jonas et al. in Cell Reports 2017 describing the various roles of syt7 at central mammalian synapses. These two papers have been added.

Page 7:
“Thus far, our findings in relation to SNT-3 suggest that a TM domain may not be required for a synaptotagmin to trigger SV release”

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2. The authors suggest that, in snt-3 mutants, the fact that "the distally docked SV pool significantly increased" suggests that "release of the distal docked SV pool is impeded." While the authors do go on to show that snt-3 release depends on the shorter unc13 variant, the presence of more docked vesicles at a distal pool does not imply an impediment to release. In general, docked vesicles are more likely to be released.

While there is a general correlation between SV docking observed by HPF EM and vesicle priming, as discussed in the manuscript we speculate “…. that SNT-3 is required for full zipperung of the SNARE complex in the priming process, with some vesicles appearing docked but not fusion competent in snt-3 mutants.” As the reviewer points out we do go on to demonstrate that SNT-3 is required for UNC-13S-mediated SV fusion. We also discuss the fact that “ Previous studies have identified distinct docked vesicle pools at these synapses. Specifically, SVs proximal to the dense projection (DP) (<90nm) are docked by interactions between vesicle-associated RAB-3 and DP localized UNC-10(RIM-Rab-3 interacting molecule), require the long isoform of UNC-13 to become primed and exhibit release kinetics tightly coupled to Ca\(^{2+}\)-entry (Weimer et al., 2006; Hammarlund et al., 2007; Gracheva et al., 2008). More distally docked SVs (90nm-300nm) require the short UNC-13MR isoform and exhibit slower release kinetics with loose Ca\(^{2+}\)-coupling (Hu et al., 2013).” Thus, the accumulation of distally docked SV’s in snt-3 mutants, observed in the present study, is consistent with the conclusion that SNT-3 is required for release of UNC-13S primed SV’s and in the absence of SNT-3, vesicles accumulate distally as they lack SNT-3-dependent fusion.

3. It is interesting that snt-1 and snt-3 have opposing effects on terminal size, but that the compound mutant has an exacerbated snt-3 (smaller vesicle) phenotype. Actually snt-1 mutants have normal terminal area (see Figure 4I), so there’s no “opposing effects” of SNT-1 and SNT-3 on terminal size, just a smaller terminal area in the absence of SNT-3 (i.e. in the snt-3 single and snt-1;snt-3 double mutants).

This matches the mini phenotype and suggests that vesicle size sets an upper limit on the amount of transmitter released per fusion event.

It is true that snt-3 mutants have smaller SVs and are able to suppress the increased SV diameter in snt-1;snt-3 double mutants. We currently have no explanation for the effects of SNT-3 on SV diameter, but we speculate in the manuscript that SNT-3 may play a role in SV recycling (this could be during endocytosis or SV sorting). The larger of SV diameter of snt-1 mutants on the other hand has been previously attributed to well-characterized endocytic defects possibly through interactions with the complex AP2, resulting in fewer and abnormally large SVs. Given that release is more severe in the snt-1;snt-3 double mutants, the endocytic defects associated with SV turnover are likely to be reduced, thereby opposing the formation of abnormal SVs due to loss of SNT-1-dependent endocytosis.

However, the statement "priming is partially suppressed by the simultaneous loss of snt-1 and snt-3" is misleading, as snt-3 loss does not appear to cause an additional priming deficit over that of snt-1.

We agree that the quantal content is not significantly reduced in snt-1;snt-3 double relative to snt-1. We apologize for this misstatement. This section summary has been changed to the following.

Page 12:
“These results indicate that SV priming is partially suppressed by the loss of SNT-1 but not SNT-3. Given that evoked release is completely eliminated, these data suggest that both SNT-1 and SNT-3 have additional functions downstream of priming, in Ca\(^{2+}\)-triggered release.”
4. The authors should include a graph that directly compares the docking distribution (vesicles vs: distance from plasma membrane) between WT and snt-3 mutants and comment on any observed differences.

We define docked SVs as 0nm from the plasma membrane (this information is now added to the Materials and Methods section). The distribution of this pool relative to the dense projection is plotted for WT and snt-3 in Supplemental Figure 6A. The percentages of total docked SVs further subdivided into proximal and distal docked SV pools are shown in figure 4B-D. If you are asking about SVs that appear physically tethered by observable filaments that are not in full contact with the PM, we see no significant difference between these in the various mutants relative to the WT (data not shown).

Page 36:
“A docked synaptic vesicle was defined as a synaptic vesicle whose membrane was morphologically contacting the plasma membrane (distance to plasma membrane =0nm).”

5. Important methodological details are left out: how was optogenetic stimulation performed? Which snt-3 line was used for the data in Fig. 6? Why not use electrical stimulation for short-term plasticity? What temperature were the worms kept at for locomotion experiments?

We used a single wavelength LED (470nm, Thorlabs) to provide optogenetic stimulation. This information has been added to the “Materials and Methods” section in the revised manuscript (see page 36). Electrical stimulation uses a loose patch microelectrode to deliver high-voltage pulses that causes rapid deterioration of the ventral nerve cord, and is unsuitable for long duration train stimulations required for this analysis.

Page 36:
“The pipette containing hypertonic sucrose was placed at the end of the patched muscle cell. A single wavelength LED (470nm, Thorlabs) was used to provide optogenetic stimulation. The intensity was adjusted to the maximal level.”

The tm5776 allele was used in Figure 6. We have labelled in the revised manuscript.

Page 45:
“Synaptic depression and SV replenishment were investigated by applying a train of light stimuli (1Hz and 5Hz) onto the ventral nerve cord of control animals (zxIs 6) and snt-1(md290), snt-3(tm5776) mutants with expression of ChR2 in their cholinergeric motor neurons.”

For locomotion experiments, worms were kept at room temperature (22°C)

Page 32:
“Worm movement recordings (under room temperature 22°C) were started 10 min after the worms were transferred.”

Page 36:
“All recordings were performed at room temperature (22°C).”

6. In Fig. 7, the authors should include a graphical legend for the snt-1 mutants (as for snt-3 in fig. 8), as their terminology contrasts with that used by others in the field (i.e., "C2AB" often indicates the absence of a TMD). This legend could be expanded to replace the one shown in Fig. 9.

As suggested by the reviewer, a graphical legend has been added to Figure 7. Moreover, we have used SNT-1FL and SNT-3FL to replace SNT-1C2AB and SNT-3C2AB in Figure 7, Figure 8, and Figure 9, to keep consistency with other papers.
Reviewer #3 (Comments to the Authors (Required)):
The authors have written the most carefully performed and most thorough manuscript that I have reviewed in the last few years. I suggest that the manuscript be published in its present form. There is certainly a growing field of research in relation to synaptotagmins, in both mammals and other animals, and this manuscript is not the first one to introduce syt-3 as an important molecule. However, the current manuscript is, as I mentioned, of such a high quality, that I recommend its rapid publication.
We really appreciate these comments!
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Wen H, Linhoff MW, McGinley MJ, Li GL, Corson GM, Mandel G, Brehm P (2010) Distinct roles for two synaptotagmin isoforms in synchronous and asynchronous transmitter release at zebrafish neuromuscular junction. Proceedings of the National Academy of Sciences of the United States of America 107:13906-13911.

Yao J, Gaffaney JD, Kwon SE, Chapman ER (2011) Doc2 is a Ca2+ sensor required for asynchronous neurotransmitter release. Cell 147:666-677.
January 6, 2021

RE: JCB Manuscript #202008121R

Dr. Zhitao Hu
University of Queensland
The University of Queensland
Upland Road 79
St Lucia, QLD 4072
Australia

Dear Professor Hu:

I am pleased to inform you that your revised manuscript entitled "A novel dual Ca2+ sensor system regulates Ca2+-dependent neurotransmitter release in C. elegans" has been positively evaluated by two of the original reviewers whose comments are attached. Based on these reviews we are happy to accept your paper for publication pending final revisions necessary to meet our formatting guidelines (see details below).

Please note that while a few minor issues remain (as indicated by reviewer #2), we feel that these can be readily dealt with during final editing via additions to the text and discussion. Please be sure to provide a final point-by-point rebuttal which documents how each of reviewer #2’s issues have been addressed along with your final revised manuscript.

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

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

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

1) Text limits: Character count for Articles is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends. In this case, you are somewhat over this limit but we should be able to give you the extras space. However, please do your best to be as concise as possible when revising (though we do realize that you will need to add somewhat to this total in order to address reviewer #2's final concerns).

2) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis, including cropped gels. Please provide molecular weight/size markers for the gel in figure 3C.

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and...
methods. On that note, we noticed that while you mention the ANOVA tests in your statistics section, you do not describe the Student's t tests that you run in figures 5, 6, and 7. Please add all statistical tests and information to this section of the methods.

For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, since you used parametric tests in your study (e.g. t-tests, ANOVA, etc.), you should have first determined whether the data was normally distributed before selecting that test. In the stats section of the methods, please indicate how you tested for normality. If you did not test for normality, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

4) Title: The title should be less than 100 characters including spaces and should be concise but accessible to a general readership. While your current title is technically accurate, since the abstract explicitly mentions the species used (C. elegans), we think that you can remove this from the title (e.g. the new title would be "A novel dual Ca2+ sensor system regulates Ca2+-dependent neurotransmitter release").

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

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

7) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
   a. Make and model of microscope
   b. Type, magnification, and numerical aperture of the objective lenses
   c. Temperature
   d. Imaging medium
   e. Fluorochromes
   f. Camera make and model
   g. Acquisition software
   h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

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

9) Supplemental materials: There are normally strict limits on the allowable amount of supplemental data. Articles may usually have up to 5 supplemental figures. At the moment, you currently have 11 such figures. While we will be able to give you the extra space in this case, please do not add to this total.
   In addition, supplemental figures should be numbered consecutively and without reference to the
main figure they are related to (e.g. Supplemental figures 1-11, without mention of the main figures). Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

10) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person. It should begin with "First author name(s) et al..." to match our preferred style.

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

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

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

B. FINAL FILES:

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

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

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

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

**It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.**

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Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days. If complications arising from measures taken to
prevent the spread of COVID-19 will prevent you from meeting this deadline (e.g. if you cannot retrieve necessary files from your laboratory, etc.), please let us know and we can work with you to determine a suitable revision period.

Thank you for submitting your interesting work to the Journal of Cell Biology, and I am looking forward to its publication.

Sincerely yours,

Vann Bennett
For the Journal of Cell Biology

Tim Spencer, PhD
Executive Editor
Journal of Cell Biology

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

The authors have addressed my earlier questions. I do not see any major issues that preclude publication.

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

This much-improved manuscript by Li et al is an excellent and thorough study of Ca2+-secretion coupling at the C elegans body wall NMJ, well-suited for publication in JCB. In addition to the quality of this work, I appreciate the author's diligence and careful attention to the comments of all reviewers. The following points are meant as questions or suggestions for the authors' consideration:

1. The statement "SV fusion mediated by SNT-3 has lower Ca2+ sensitivity" (p. 13) is confusing and potentially misleading. In these experiments, SV fusion mediated by SNT-3 was more "sensitive" to a reduction in external Ca2+. To establish the "Ca2+ sensitivity of SV fusion" in snt-1 KO terminals, a proper Ca2+ dose-response is needed, as it is unclear at what external Ca2+ concentrations this response saturates. Likewise, the intrinsic Ca2+ sensitivity of SNT-1 and SNT-3 may be identical, and the observed differences may be caused by different cytoplasmic Ca2+ concentrations during stimulation (e.g. due to differences in SV-CaV spatial coupling, as the authors suggest). The authors should clarify their assessment here.

2. Discussion, p. 23: the authors suggest that SNT-1 takes functional priority over SNT-3 because it rapidly buffers Ca2+ ions, preventing them from reaching SNT-3. I am skeptical of this argument, particularly because the authors do not make any quantitative argument about the abundance and binding kinetics of other Ca2+ buffers, and how they compare to SNT-1, in these nerve terminals. My suspicion is that SNT-1 contributes only slightly to Ca2+ buffering on the relevant spatial and temporal scales here. An alternative explanation would be that, in WT worms, all primed SNT-3 enabled vesicles also contain SNT-1 and thus are unaffected by loss of SNT-3. In the absence of SNT-1, a subset of these vesicles - which may now reside at other locations in the nerve terminal...
due to trafficking/priming defects, etc - now relies on SNT-3 for exocytosis. The trend observed in Fig. 7B - which might reach significance with more data collected, given the large variance in the data - supports this interpretation.

3. I suggest that the authors temper the claim that "the RRP is normal in syt1 or syt2 knockouts in the mouse CNS" (discussion p. 24). This is a controversial issue, and this phenotype may depend on e.g. the culture method used (autapses vs mass culture, e.g. doi: 10.1523/JNEUROSCI.1341-09.2009), or the method of measuring the RRP.

4. Discussion, p. 24, same paragraph: it is unclear to me what the evidence is that "SV priming requires both Ca2+ sensors." The RRP and EPSC are unchanged in the absence of SNT-3; how can it thus be required? The authors may consider stating instead that e.g. "both Ca2+ sensors may contribute to SV priming."

5. Finally, the authors should comment on the findings in Fig. 4 where loss of SNT-3 in a SNT-1 KO background rescues SV number, but results in a loss in priming. The former would be opposing actions of these two isoforms while the latter suggests redundant functions. Can you the authors posit a mechanistic model that takes these divergent function into account.
We are glad that all reviewers have agreed to accept our manuscript. Below are our responses to the additional comments of reviewer 2.

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

This much-improved manuscript by Li et al is an excellent and thorough study of Ca2+-secretion coupling at the C elegans body wall NMJ, well-suited for publication in JCB. In addition to the quality of this work, I appreciate the author's diligence and careful attention to the comments of all reviewers. The following points are meant as questions or suggestions for the authors' consideration:

1. The statement "SV fusion mediated by SNT-3 has lower Ca2+ sensitivity" (p. 13) is confusing and potentially misleading. In these experiments, SV fusion mediated by SNT-3 was more "sensitive" to a reduction in external Ca2+. To establish the "Ca2+ sensitivity of SV fusion" in snt-1 KO terminals, a proper Ca2+ dose-response is needed, as it is unclear at what external Ca2+ concentrations this response saturates. Likewise, the intrinsic Ca2+ sensitivity of SNT-1 and SNT-3 may be identical, and the observed differences may be caused by different cytoplasmic Ca2+ concentrations during stimulation (e.g. due to differences in SV-CaV spatial coupling, as the authors suggest). The authors should clarify their assessment here.

We agree that Ca2+ sensitivity should be assessed in more Ca2+ concentrations, although our results indicate that there is a potential decrease in Ca2+ sensitivity. We have rephrased our statement to emphasize the fact that SVs mediated by SNT-3 have a loose coupling with Ca2+ entry.

Page 14: "SVs mediated by SNT-3 have loose coupling to Ca2+ entry
...We next examined whether the SV release mediated by the two Ca2+ sensors exhibits differential synaptic properties such as Ca2+ dependence...However, the evoked EPSCs in snt-1 mutants displayed a more severe reduction in 0.5mM Ca2+, the reduction in EPSC amplitude and charge transfer dropping by ~85% (Figure 5A-C), indicating that SV fusion mediated by SNT-3 was more sensitive to a reduction in external Ca2+ with a decreased Ca2+ dependence.

The more reduction in lower Ca2+ and prolonged latency of the evoked EPSCs, together with the relatively diffuse axonal expression of SNT-3::mApple (Figure 2), suggest that the SV release mediated by SNT-3 might occur further away from Ca2+ channels."

2. Discussion, p. 23: the authors suggest that SNT-1 takes functional priority over SNT-3 because it rapidly buffers Ca2+ ions, preventing them from reaching SNT-3. I am skeptical of this argument, particularly because the authors do not make any quantitative argument about the abundance and binding kinetics of other Ca2+ buffers, and how they compare to SNT-1, in these nerve terminals. My suspicion is that SNT-1 contributes only slightly to Ca2+ buffering on the relevant spatial and temporal scales here. An alternative explanation would be that, in WT worms, all primed SNT-3 enabled vesicles also contain SNT-1 and thus are unaffected by loss of SNT-3. In the absence of SNT-1, a subset of these vesicles - which may now reside at other locations in the nerve terminal due to trafficking/priming defects, etc - now relies on SNT-3 for exocytosis. The trend observed in Fig. 7B - which might reach significance with more data collected, given the large variance in the data - supports this interpretation.

We raised the possibility that SNT-1 rapidly buffers Ca2+ ions based on the fact that this Ca2+ sensor is more concentrated around Ca2+ entry with a tight coupling. Indeed, we have preliminary data showing that Ca2+ entry is increased in snt-1 mutants in AWC neuron in worm (from our collaborator’s lab). This indicates that SNT-1 has a strong Ca2+ buffering function in the NMJ synapses in worm. One of our projects is focusing on Ca2+ imaging in
the AWC neuron to examine more detailed mechanisms by which the two Ca\(^{2+}\) sensors are coordinated. However, we cannot rule out the possibility suggested by the reviewer. To test this, we will need to make a mApple/GFP knockin line to compare expression level and localization of endogenous snt-3 in wild type and snt-1 mutants. This will be investigated in our next synaptotagmin story.

3. I suggest that the authors temper the claim that "the RRP is normal in syt1 or syt2 knockouts in the mouse CNS" (discussion p. 24). This is a controversial issue, and this phenotype may depend on e.g. the culture method used (autapses vs mass culture, e.g. doi: 10.1523/JNEUROSCI.1341-09.2009), or the method of measuring the RRP.

We have rephrased our statement. The suggested paper was cited.

Page 24:
“The RRP is unaltered or decreased in Syt1 or Syt2 knockout neurons in cultured hippocampal or cortical neurons (Pang et al., 2006; Liu et al., 2009; Bacaj et al., 2015), and it is almost abolished by the loss of Syt1 in the fly NMJ (Yoshihara et al., 2010).”

4. Discussion, p. 24, same paragraph: it is unclear to me what the evidence is that "SV priming requires both Ca\(^{2+}\) sensors." The RRP and EPSC are unchanged in the absence of SNT-3; how can it thus be required? The authors may consider stating instead that e.g. "both Ca\(^{2+}\) sensors may contribute to SV priming."

We have rephrased our statement.

Page 24:
“Our findings therefore support the notion that both Ca\(^{2+}\) sensors may contribute to SV priming (i.e., Syt1/7, SNT-1/3).”

5. Finally, the authors should comment on the findings in Fig. 4 where loss of SNT-3 in a SNT-1 KO background rescues SV number, but results in a loss in priming. The former would be opposing actions of these two isoforms while the latter suggests redundant functions. Can you the authors posit a mechanistic model that takes these divergent function into account.

We actually have addressed this question in the revised manuscript.

Page 11:
“In the snt-1;snt-3 double mutants, SV numbers were restored to wildtype levels, possibly accumulating due to the lack of release (Figure 4F).”

Bacaj T, Wu D, Burre J, Malenka RC, Liu X, Sudhof TC (2015) Synaptotagmin-1 and -7 Are Redundantly Essential for Maintaining the Capacity of the Readily-Releasable Pool of Synaptic Vesicles. PLoS biology 13:e1002267.

Liu H, Dean C, Arthur CP, Dong M, Chapman ER (2009) Autapses and networks of hippocampal neurons exhibit distinct synaptic transmission phenotypes in the absence of synaptotagmin I. The Journal of neuroscience : the official journal of the Society for Neuroscience 29:7395-7403.

Pang ZP, Sun J, Rizo J, Maximov A, Sudhof TC (2006) Genetic analysis of synaptotagmin 2 in spontaneous and Ca\(^{2+}\)-triggered neurotransmitter release. The EMBO journal 25:2039-2050.

Yoshihara M, Guan Z, Littleton JT (2010) Differential regulation of synchronous versus asynchronous neurotransmitter release by the C2 domains of synaptotagmin 1. Proceedings of the National Academy of Sciences of the United States of America 107:14869-14874.