The Journal of Physiology

# All-optical monitoring of excitation-secretion coupling demonstrates that SV2A functions downstream of evoked Ca2+ entry

Mazdak M Bradberry and Edwin R. Chapman
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*Corresponding author(s): Edwin Chapman (chapman@wisc.edu)*

*The following individual(s) involved in review of this submission have agreed to reveal their identity: Sandra Bajjalieh (Referee #2)*

## Review Timeline:


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*Reviewing Editor: Rajini Rao*


## Transaction Report:

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Dear Professor Chapman,

Re: JP-RP-2021-282601 "All-optical monitoring of excitation-secretion coupling demonstrates that SV2A functions downstream of evoked Ca2+ entry" by Mazdak M Bradberry and Edwin R. Chapman

Thank you for submitting your manuscript to The Journal of Physiology. It has been assessed by a Reviewing Editor and by 2 expert Referees and I am pleased to tell you that it is considered to be acceptable for publication following satisfactory revision.

Please advise your co-authors of this decision as soon as possible.

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If you have any queries please reply to this email and staff will be happy to assist.

Yours sincerely,

David Wyllie
Senior Editor
The Journal of Physiology

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EDITOR COMMENTS

Reviewing Editor:

The reviewers were unanimously enthusiastic about this manuscript and consider it potentially impactful to the field. Strengths of the study include addressing the pre-synaptic function of an important target of neuroactive drugs and adding to the biophysical toolkit for optical analysis of neurotransmitter release. However, both reviewers have suggestions to strengthen the work, and validate the findings further. More details on the use of animals and calibration of the calcium sensor are required. Additional experiments to confirm the lack of effect of SV2 on calcium dynamics would increase confidence in the conclusions. Please see the detailed reviews, attached.

Senior Editor:

Comments for Authors to ensure the paper complies with the Statistics Policy:
Please report exact P values.

Comments to the Author:
Your manuscript has been well-received and positively commented on by two expert referees. While not acceptable in its current form, I was to give you the opportunity to address the comments raised which will involve providing new experimental data. Should you require additional time for the experimental work to be completed then please request this - I have no issues with grant an extension to our normal 'turnaround time'.

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REFEREE COMMENTS

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This is a short but potentially impactful study that takes advantage of a novel synaptic vesicle targeted calcium indicator with red shifted fluorescence to measure presynaptic calcium signals. The authors employ this setting to examine glutamatergic transmission in SV2A knockouts via purely optical means using iGluSnFR. Overall, the results are important as they introduce a new modality to monitor neurotransmission. However, it will be important to develop the results further to fully substantiate the conclusions regarding SV2A function.

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In this paper Bradberry and Chapman report the development of an optical system to simultaneously monitor presynaptic calcium and neurotransmitter release in glutamatergic neurons.

The authors take advantage of the HaloTag system and use it to load synapses with a far-red emitting derivative of the calcium chelator BAPTA. The HaloTag protein is co-expressed with a glutamate binding protein that is trafficked to the plasma membrane and fluoresces green when glutamate is released.

They use this system to test the hypothesis that SV2 plays a role in regulating presynaptic calcium - either by affecting calcium buffering [1] or the action of calcium channels [2]. To do this they express their sensors in neurons expressing SV2A or no SV2. They report that glutamate release was compromised in synapses lacking SV2 but calcium dynamics were normal. These findings indicate that loss of SV2 does not affect calcium levels supporting the conclusion that SV2 action is limited to coupling calcium influx to exocytosis.

This is a straightforward report of a new useful addition to the biophysical toolbox. Furthermore, the findings answer a standing question about the action of SV2 in the presynapse. There are three comments/questions the authors should address.

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References cited:

1. Wan, Q.-F., et al., SV2 acts via presynaptic calcium to regulate neurotransmitter release. Neuron, 2010. 66: p. 884-895.

2. Vogl, C., et al., The synaptic vesicle glycoprotein 2A ligand levetiracetam inhibits presynaptic Ca2+ channels through an intracellular pathway. Mol Pharmacol, 2012. 82(2): p. 199-208.

3. Nowack, A., et al., SV2 regulates neurotransmitter release via multiple mechanisms. Am J Physiol Cell Physiol, 2010. 299(5): p. C960-967.

4. Lazzell, D.R., et al., SV2B regulates synaptotagmin 1 by direct interaction. J Biol Chem, 2004. 279(50): p. 52124-31.

5. Schivell, A.E., et al., Isoform-specific, calcium-regulated interaction of the synaptic vesicle proteins SV2 and synaptotagmin. Journal of Biological Chemistry, 1996. 271: p. 27770-27775.

6. Schivell, A.E., et al., SV2A and SV2C contain a unique synaptotagmin-binding site. Mol Cell Neurosci, 2005. 29(1): p. 56-64.

7. Kaempf, N., et al., Overlapping functions of stonin 2 and SV2 in sorting of the calcium sensor synaptotagmin 1 to synaptic vesicles. Proc Natl Acad Sci U S A, 2015. 112(23): p. 7297-302.

8. Yao, J., et al., Cotrafficking of SV2 and synaptotagmin at the synapse. J Neurosci, 2010. 30(16): p. 5569-78.

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END OF COMMENTS

**Confidential Review** **11-Nov-2021**

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**RESPONSE TO EDITORS AND REFEREES**
Bradberry & Chapman, JP-RP-2021-282601R1

Dear editors,

Thank you for handling our manuscript, "All-optical monitoring of excitation-secretion coupling demonstrates that SV2A functions downstream of evoked $Ca^{2+}$ entry." We have addressed each of the points raised by the editors and referees, including providing new details on calibration of the Syp-HaloTag-$JF_{646}$-BAPTA $Ca^{2+}$ sensor and the use of animals in our study. Our point-by-point responses to editor and referee comments are included below.

Please do not hesitate to contact us with any questions or matters of clarification that might arise; otherwise, we look forward to hearing from you soon.

Sincerely yours,
Mazdak Bradberry & Edwin Chapman

Reviewing Editor:

The reviewers were unanimously enthusiastic about this manuscript and consider it potentially impactful to the field. Strengths of the study include addressing the pre-synaptic function of an important target of neuroactive drugs and adding to the biophysical toolkit for optical analysis of neurotransmitter release. However, both reviewers have suggestions to strengthen the work, and validate the findings further. More details on the use of animals and calibration of the calcium sensor are required. Additional experiments to confirm the lack of effect of SV2 on calcium dynamics would increase confidence in the conclusions. Please see the detailed reviews, attached.

**We thank the Reviewing Editor for their time and assessment, and we hope that we have addressed all requirements to their satisfaction. Please see our response to Reviewer 2, below, in which we provide additional details regarding the calibration of the Syp-$JF_{646}$-BAPTA $Ca^{2+}$ sensor. We have revised the Method section to include more information about the animals used, as follows:**

(Materials and Methods) P0-P2 newborn mouse pups of either sex from $SV2B^{-/-}$, $SV2A^{+/-}$ breeders on a 129P2/OlaHsd background partially backcrossed to C57B6/J (Jackson Labs) were used.

Senior Editor:

Comments for Authors to ensure the paper complies with the Statistics Policy:
Please report exact P values.

**We have included exact P values where possible. In some cases, where an upper bound on the P value is shown (e.g. $p < 0.0001$ for Mann-Whitney tests, or $p < 0.01$ for the Aikike Information Criteria), this represents the most exact output available from our statistical software (GraphPad Prism).**

Comments to the Author:
Your manuscript has been well-received and positively commented on by two expert referees. While not acceptable in its current form, I was to give you the opportunity to address the comments raised which will involve providing new experimental data. Should you require additional time for the experimental

work to be completed then please request this - I have no issues with grant an extension to our normal 'turnaround time'.

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**We thank the reviewer for their time and favorable assessment of this work.**

1. While the authors nicely demonstrate that SV2A loss of function impact glutamate release without altering presynaptic calcium signals, these results require some further validation. For instance, does SV2A alter calcium sensitivity of release? Is there a change in release properties when release is triggered via calcium independent means? Is there a change in the size of the readily releasable pool?

**We thank the reviewer for their thoughtful consideration of the multiple factors that may influence synaptic vesicle exocytosis, and we agree that the $Ca^{2+}$ sensitivity of release is among the most important parameters in this process. A strength of the approach presented in our study is that it allows direct measurement of the $Ca^{2+}$ sensitivity of release *via* combined measurements with iGluSnFR and $JF_{646}$-BAPTA. In Figs. 2G and 2H of the manuscript, we present evidence that SV2A does not change the $Ca^{2+}$ sensitivity of release, whether $Ca^{2+}$ is measured in the extracellular medium (Fig. 2G) or in the presynaptic cytoplasm (Fig. 2H). We regret the lack of clarity in communicating these results and have modified the manuscript as follows:**

> (Results) As with the Hill parameter, values for $[Ca^{2+}]_i$ at half-maximal glutamate release were nearly identical between *Sv2a* WT and KO neurons (WT: 337 nM, 95% CI 295-523 nM; KO: 341 nM, 95% CI 294-531 nM), indicating that the reduction in glutamate release in the *Sv2a* KO can be well-approximated by linearly scaling down the $Ca^{2+}$-glutamate release curves observed for WT neurons (**Fig. 2G-H**). Our results thus demonstrate that SV2A does not change the $Ca^{2+}$ dependence of glutamate release, in accordance with prior studies (Chang and Sudhof, 2009; Custer, 2006).

**Regarding $Ca^{2+}$-independent release processes, we are presently aware of only one method to trigger release in a truly $Ca^{2+}$-independent fashion, which is the application of hypertonic solutions such as concentrated sucrose. Unfortunately, the changes in refractive index of the bath solution resulting from hypertonic sucrose application make direct measurements of sucrose-evoked glutamate release with iGluSnFR technically unfeasible in our hands. We direct the reviewer to references cited in our manuscript, Custer et al. (2006) and Chang and Sudhof (2009), for published results of SV release evoked by hypertonic sucrose application in autaptic and mass-cultured SV2A KO neurons, respectively. In autaptic culture, Custer et al. (2006) found a decrease in the sucrose-dependent RRP, while Chang and Sudhof (2009) found no sucrose RRP changes (glutamatergic or GABAergic) in mass-cultured neurons. While the cause of this discordance is unclear, we note that different results are also reported in autaptic versus mass-culture preparations of syt1 KO neurons (Liu et al. 2009). Furthermore, we emphasize the challenges in interpreting measurements of the**

**"readily releasable pool" (RRP), the definition of which can vary markedly with the measurement method used (E. Neher, *Neuron* 87(6):1131-1142, 2015). Given the availability of published studies cited above, and the combined technical and interpretative challenges in RRP studies, we feel that further experiments to examine the role of SV2 in shaping vesicle "pools" are out of the scope of the present study.**

2. The power of purely optical analysis of neurotransmission largely rests on its ability to visualize single synapse function. It is surprising that the authors do not fully take advantage of this feature but rather rely on bulk measurements. It would have been nice to see the variability and distribution of responses among synapses and document the decrease in release probability induced by SV2A deficiency.

**We share the reviewer's appreciation for the power of optical neurotransmitter measurements to map synaptic release across individual synapses. Our lab has recently demonstrated such techniques for the study of how synaptotagmin-7 mediates paired-pulse facilitation (Vevea et al., 2021). At the same time, we emphasize that optical neurotransmitter release measurements offer several other important advantages over traditional patch-clamp techniques. These include (1) more direct measurement of transmitter via an extracellular fluorescent sensor, *versus* currents resulting from the transmitter-gated opening of ion channels, which may themselves be subject to modulation and morphological changes in the postsynaptic cell; (2) a non-invasive approach that does not require whole-cell dialysis or the maintenance of critical biophysical parameters (e.g. series resistance and holding current) for extended experiments involving multiple solution changes; and (3) substantially higher throughput, as studying new synapses requires simply changing the microscopic field of view *versus* terminating the recording, finding a new cell, and obtaining a stable whole-cell recording from the new cell. Our study, which involved recording both $Ca^{2+}$ and glutamate signals from the same synapses under 5 different conditions of extracellular $Ca^{2+}$, was designed to take advantage of these latter three advantages of optical synaptic recording.**

**In these experiments, the need to repeatedly image each field of view led us to develop concerns about photobleaching, and so experiments were designed to maximize signal:noise using widely-expressed iGluSnFR and thus minimize the required illumination intensity. Localization of individual synapses in iGluSnFR experiments typically requires sparse expression of the sensor and higher illumination intensities, which were not suitable for the experiments performed here with this iGluSnFR variant. We look forward to improvements in optical neurotransmitter sensors that might better allow us to make use of all the advantages of optical approaches to studying synaptic function.**

**Finally, we emphasize that Figs. 2I-M do document the reduction in release probability in SV2A KO neurons, as evidenced by increased paired-pulse facilitation and increased $dF/F_0$(tenth/first) in SV2A KO neurons (Fig. 2I-M). How this varies on a synapse-by-synapse basis is an interesting topic that we hope to address in future studies.**

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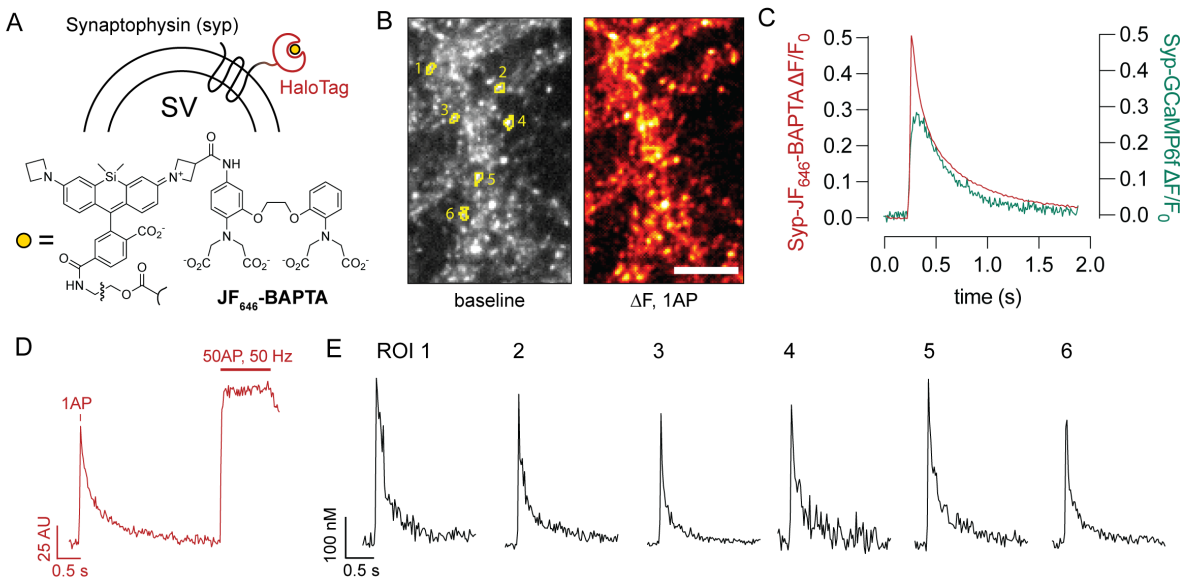
**We appreciate the reviewer's time and assessment of our work.**

1. The authors state they calibrated the calcium sensor and that the method they used is described in the Methods section. But it is not. They should add their methods and describe them (and include the data) in the results section. Also needed is more information about Figure 1D. Are these single bouton responses to a single field stimulation?

**We appreciate the reviewer's careful reading of our manuscript, and we regret the lack of clarity with which the calibration procedure was described. In this case, we used the term "calibration" to refer to a procedure by which the $Ca^{2+}$ sensor is driven to near-saturation to estimate the fluorescence intensity of a fully-occupied indicator, which allows for estimation of $[Ca^{2+}]_i$ values based on the published *in vitro* characteristics of the indicator (Marvall et al., 2000). The stimulation procedure is described in the "Glutamate and $Ca^{2+}$ imaging" section of the Methods, and we have clarified our description of the procedure for this process in the "Data analysis" section of the Methods. The edited text highlighted below:**

> (Methods) Syp-Halo-$JF_{646}$-BAPTA traces were background-subtracted, and $[Ca^{2+}]_i$ was derived using the reported *in vitro* values for the sensor's dynamic range and $Ca^{2+}$ affinity after establishing the maximal fluorescence intensity ($F_{max}$, i.e., the signal resulting from fully-occupied sensor) in each field of view (de Juan-Sanz et al., 2017; Maravall et al., 2000). The following equation was used:
>
> $$[Ca]_i = K_d \left( \frac{F/F_{max} - 1/R_f}{1 - F/F_{max}} \right)^{1/n}$$
>
> Where $K_d$ is the dissociation constant of the indicator, $F$ is the average fluorescence across the field of view in each frame, $F_{max}$ is the fluorescence achieved with delivery of a saturating stimulus, $R_f$ is the dynamic range of the indicator, and $n$ is the Hill coefficient. For $K_d$, $R_f$, and $n$, we used *in vitro* measurements ($K_D$ = 140 nM, $R_f$ = 5.5, $n$ = 1) from Deo et al. (2019). The maximum fluorescence achieved with a 50-AP, 50 Hz train was defined as 95% of the true theoretical maximum value (Maravall et al., 2000).

**For clarity, we have also included an example of this process in a revised Fig. 1, shown below. We have also edited the figure legend to clarify that the depicted responses were obtained with single field stimuli.**




**New Fig. 1. A chemogenetic sensor for presynaptic $Ca^{2+}$.** (A) $Ca^{2+}$ sensor scheme. The HaloTag protein was targeted to nerve terminals by expression as a synaptophysin fusion construct. $JF_{646}$-BAPTA bearing a HaloTag chloroalkane ligand (Deo et al., 2019) was added to the bath in AM ester form and allowed to undergo fluorogenic binding to Syp-HaloTag. (B) The reaction yields Syp-HaloTag-$JF_{646}$-BAPTA, a $Ca^{2+}$ sensor with bright resting fluorescence that matches the expected punctate distribution of synaptophysin labeling and readily reports presynaptic $Ca^{2+}$ fluxes from single action potentials. Scale bar, 10 µm. Numbered ROIs correspond to traces shown in panels (D-E). (C) Comparison of exemplary single-stimulus, full field-of-view responses between Syp-HaloTag-$JF_{646}$-BAPTA and Syp-GCaMP6f. The chemogenetic HaloTag-based approach demonstrates a substantial improvement in temporal fidelity. (D) A high-frequency stimulus train was used to obtain maximal fluorescence values for the sensor, which allows for calculation of $[Ca]_i$ (see Methods). The trace shown depicts the average fluorescence for the six labeled ROIs in panels (B) and (E). (E) Exemplary single-bouton, single-stimulus $[Ca^{2+}]_i$ traces. Among the boutons shown here, the baseline $[Ca^{2+}]_i$ was 102 ± 62 (s.d.) nM.

**Upon a closer reading of Maravall et al. (2000), we have also made a small change to our $Ca^{2+}$ imaging analysis that we feel improves the accuracy of our results. Rather than take the maximum fluorescence intensity achieved with the stimulus train as the fully-saturated intensity, we conservatively estimated that this procedure achieved 95% saturation, in accordance with the observations and discussions of Maravall et al. (2000). The resulting changes in calculated values of $[Ca^{2+}]_i$ have been made throughout the figures and text.**

2. Why 1.2mM [Ca2+]e for synaptic transmission studies?

**We likewise appreciate the reviewer's attention to the importance of extracellular $[Ca^{2+}]$ in physiologic bath solutions for synaptic transmission experiments. Two mM is commonly chosen for synaptic transmission studies in this field of study, and indeed the experiments in Fig. 1 and Fig. 2 (I-M) were carried out at 2 mM $[Ca^{2+}]_e$. We apologize for this omission, and the composition of bath solutions has been clarified in the Methods and Figure Legend as follows:**

> (Figure legend) (I) Averaged iGluSnFR signals from SV2A WT and KO neurons in response to 10-AP, 10-Hz stimulus trains. These experiments were carried out at 2 mM $[Ca^{2+}]_e$

(Methods) For the experiment shown in Fig. 1, iGluSnFR was not expressed, and the ACSF contained 2 mM $CaCl_2$ during staining, washing, and imaging. For the data shown in Fig. 2 panels I-M, no $Ca^{2+}$ sensing constructs were expressed, and coverslips were simply transferred from the incubator to the microscope and equilibrated at room temperature for at least 5 minutes before recording. In these experiments, the ACSF contained 2 mM $CaCl_2$. At least 4 minutes were allowed between stimulus trains.

**However, to ensure that our findings regarding SV2A and $Ca^{2+}$ influx were robust, we also employed a wide range of $[Ca^{2+}]_e$ in our solutions to assess the $Ca^{2+}$ dependence of glutamate release (Figure 2), and thus obtained results to support our conclusions at both sub-physiologic and supra-physiologic values of $[Ca^{2+}]_e$. In these experiments, 1.2 mM $[Ca^{2+}]_e$ was used for washing and dye application on neurons as it represented a reasonable physiologic value that was around the midpoint of all $[Ca^{2+}]_e$ values tested.**

3. This reviewer cannot understand why the authors insist that the molecular function of SV2 remains unknown. While mutational analyses suggest SV2 may perform multiple actions [3], it is well documented that SV2 binds to the calcium sensing protein synaptotagmin [4-6] and plays an essential role in trafficking synaptotagmin to synaptic vesicles [7, 8]. Furthermore, this action of SV2 is required to support normal transmitter release [8]. The apparent attempt to inflate the significance of the work is disingenuous and unnecessary. Given that the SV2s are the targets for a growing repertoire of neuroactive drugs, figuring out the full extent of SV2 action is justification enough for these studies.

**We sincerely appreciate the reviewer's assessment and apologize for the perceived attempt to inflate our work's significance. We are familiar with the works cited by the reviewer, many of which we discuss in our manuscript. We would like to point out that reference 3 (Nowack et al. 2010) directly contradicts the notion that co-trafficking of syt1 is the primary means by which SV2A supports neurotransmitter release. In that study, several SV2 mutants that did not appear to restore exocytosis did, in fact, restore syt1 expression and localization; as the authors state, "we have identified mutations that fail to rescue synaptic release probability that do not alter synaptotagmin expression or turnover" (Nowack et al., p. C965). Thus, while all authors agree that SV2A and syt1 undergo some degree of co-trafficking, the functional importance of this effect remains unclear. In our view, a co-trafficking phenomenon of uncertain functional importance does not comprise a "well-defined molecular function," especially when the sequence of the protein strongly suggests some type of molecular transport phenomenon that has yet to be defined in neuronal cells. However, we do appreciate the importance of clearly discussing the published literature, and we have thus modified our manuscript as follows:**

(Abstract) SV2A, an essential transporter-like synaptic vesicle protein, ~~has no well-defined molecular function despite being~~ is a major target for antiepileptic drugs and a receptor for clostridial neurotoxins including Botox.

(Key points summary) One of the most prescribed antiepileptic medications, levetiracetam, acts by binding a protein of ~~unknown~~ uncertain molecular function

(Introduction) The anti-epileptic drugs levetiracetam (Keppra) and brivaracetam (Briviact) bind SV2A (Klitgaard et al., 2016; Lynch et al., 2004), and it is well-established that this interaction underlies the antiepileptic action of these drugs (Kaminski et al., 2009, 2008). However, the antiepileptic mechanism of these drugs~~, as with the molecular function of SV2A,~~ is otherwise undefined at the molecular level.

(Discussion) ~~Since its discovery, the role of SV2A in synaptic transmission has remained enigmatic.~~

While SV2A is well-known to support neurotransmitter release (Chang and Sudhof, 2009; Custer, 2006), the mechanism underlying this role has remained enigmatic.

Referee #2 References cited:

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3. Nowack, A., et al., SV2 regulates neurotransmitter release via multiple mechanisms. Am J Physiol Cell Physiol, 2010. 299(5): p. C960-967.

4. Lazzell, D.R., et al., SV2B regulates synaptotagmin 1 by direct interaction. J Biol Chem, 2004. 279(50): p. 52124-31.

5. Schivell, A.E., et al., Isoform-specific, calcium-regulated interaction of the synaptic vesicle proteins SV2 and synaptotagmin. Journal of Biological Chemistry, 1996. 271: p. 27770-27775.

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7. Kaempf, N., et al., Overlapping functions of stonin 2 and SV2 in sorting of the calcium sensor synaptotagmin 1 to synaptic vesicles. Proc Natl Acad Sci U S A, 2015. 112(23): p. 7297-302.

8. Yao, J., et al., Cotrafficking of SV2 and synaptotagmin at the synapse. J Neurosci, 2010. 30(16): p. 5569-78.

Dear Professor Chapman,

Re: JP-RP-2021-282601R1 "All-optical monitoring of excitation-secretion coupling demonstrates that SV2A functions downstream of evoked Ca2+ entry" by Mazdak M Bradberry and Edwin R. Chapman

I am pleased to tell you that your paper has been accepted for publication in The Journal of Physiology, subject to any modifications to the text and/or satisfactory clarification of the Methods section that may be required by the Journal Office to conform to House rules.

NEW POLICY: In order to improve the transparency of its peer review process The Journal of Physiology publishes online as supporting information the peer review history of all articles accepted for publication. Readers will have access to decision letters, including all Editors' comments and referee reports, for each version of the manuscript and any author responses to peer review comments. Referees can decide whether or not they wish to be named on the peer review history document.

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Yours sincerely,

David Wyllie
Senior Editor
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EDITOR COMMENTS

Reviewing Editor:

Both reviewers and the handling editor have evaluated your revised manuscript and conclude that it is significantly and satisfactorily improved. If you wish, you may clarify the potential functions of SV2 as explained by Reviewer #2. Congratulations on an impactful study!

Senior Editor:

Many thanks for clarifying and editing your manuscript to address the comments raised by at its previous review. I am happy to accept your manuscript for publication. You will see that R2 has commented that you should consider re-wording the description of functions served by SV2 but I feel this could be done with a change in text at the proof stage - rather than request a further revision at this stage. Many thanks for submitting this work to The Journal of Physiology. With all best wishes for the Festive Season.

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REFEREE COMMENTS

Referee #1:

The authors have addressed my earlier questions.

Referee #2:

The revision is adequate for publication, although please point out to the authors that not all proteins that have the features of transporters act as transporters. Based on its structure, the product of Unc93b was assumed to be a transporter or channel. Then genetic studies revealed that it plays an essential role in the trafficking of a subset of toll-like receptors to endosomes. These receptors are, like synaptotagmin, type 1 membrane proteins and they exist in recycling endosomes, an analog of synaptic vesicles.

So the most accurate way to phrase the issue is that SV2s likely perform multiple functions, not all of which have been identified. The idea suggested by Nowack et al (Am J Physiol), that all of these functions are required to support neurotransmission, does not negate the importance of any single function. I'll leave it to them to decide how accurate they wish to be.

END OF COMMENTS

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**1st Confidential Review** **14-Dec-2021**

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