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Acute introduction of monomeric or multimeric α-synuclein induced distinct impacts on synaptic vesicle trafficking at lamprey giant synapses.

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The following individual(s) involved in review of this submission have agreed to reveal their identity: Frederic A. Meunier (Referee #2)

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Dear Dr Morgan,

Re: JP-RP-2024-286281 "Acute introduction of monomeric or multimeric α-synuclein induced distinct impacts on synaptic vesicle trafficking at lamprey giant synapses." by Cristina Roman-Vendrell, Meral Celikag, Aurelia Hays Watson, Tim Bartels, and Jennifer R. Morgan

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If you have any queries, please reply to this email and we will be pleased to advise.

Yours sincerely,

Katalin Toth
Senior Editor
The Journal of Physiology

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EDITOR COMMENTS

Reviewing Editor:

This manuscript is focused on monomeric and multimeric alpha-synuclein on synaptic vesicle trafficking and clustering. Both reviewers found that the findings were impactful and important for the field. They found the data of high quality, rigorous and that the data supported all the conclusions. Both reviewers had expressed some concerns, in particular determining the level of alpha-synuclein overexpression. To address this concern, the authors should either directly determine alpha-synuclein levels through immunohistochemistry or provide data directly showing dextran diffusion as a proxy for alpha-synuclein concentration. In addition, Finally, the authors need to carefully revise and rewrite their manuscript to address the positive comments of the reviewers.

Please also see 'Required Items' above.

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REFEREE COMMENTS

Referee #1:

This manuscript explores the effects of monomeric and multimeric alpha-synuclein on synaptic vesicle clustering at Lamprey Giant Synapses. Previous studies have documented the impact of alpha-synuclein on vesicle clustering and synaptic transmission. By leveraging the unique properties of the lamprey giant synapse, which allows precise manipulation of the presynaptic compartment, the authors conduct an elegant and rigorous assessment of the effects of monomeric and multimeric forms of alpha-synuclein. The integration of electron microscopy and the rigorous quality control of alpha-synuclein preparations are major strengths of this study.

The manuscript is very well-written, and the experiments are thoroughly described. Overall, this is a sound study clarifying the effect of alpha synuclein on synaptic vesicles. I have the following comments to enhance the study through additional experimentation or in-depth discussions:

1. I could not find any data showing measurements of "synaptic vesicle TRAFFICKING." Is it possible that alpha-synuclein impacts the formation of synaptic vesicles?

2. Are these synapses amenable to live imaging? It would be helpful for readers to visualize synuclein within this axon/synapse. Does alpha-synuclein enter the presynaptic compartment, and is it uniquely localized there?

3. It is not clear in the manuscript when the axons were fixed for EM after alpha-synuclein injections.

Referee #2:

In this manuscript, Roman-Vendrell and colleagues explore the effects resulting from an acute injection of monomeric or multimeric forms of -Synuclein (-Syn) at the lamprey giant reticulospinal synapse. The authors report that presynaptic nerve terminals microinjected with monomeric or multimeric forms of -Syn both exhibit de-clustering of synaptic vesicles and buildup of endosome-like structures which the authors call cisternae. In addition, the authors demonstrate that only those presynaptic nerve terminals microinjected with monomeric -Syn exhibit an increase in clathrin-coated pits and the presence of structures which the authors term "fusosomes" at the synapse. The authors conclude that multimeric and monomeric forms of -Syn impair intracellular vesicle trafficking, but only monomeric -Syn additionally impaired clathrin-mediated endocytosis. The experiments are well carried out and the conclusions that the authors draw from the results presented are well-founded. The manuscript will be of interest to a broad range of researchers particularly those interested in both the physiological role of -Syn at the presynaptic nerve terminal. I have a few fairly major concerns and a few minor ones.

Major Concerns:

1. It is critical that the authors adequately quantify the level of "overexpression" that these injections generate. This could be carried out sy immunostaining of -Syn with appropriate controls to estimate the level of overexpression at the time of fixation. It is important to note that -Syn is high diffusive and these experiments will be critical to estimate the endogenous level versus overexpressed in all conditions. Further, the authors describe the co-microinjection of fluorescein dextran as a proxy to determine the concentration of -Syn that could have reached the nerve terminal; however, no such data is presented. The authors should also include images/data of the dextran dye diffusion.

2. Across all experiments, the synapses which act as controls are those from the axons injected with -Syn, but further away from the microinjection site beyond the proposed -Syn diffusion limit. Since the authors are dissecting/microinjecting the nerve, the most physical damage will happen closest to the injection site (which are the [high] and [low] -Syn conditions synapses are taken), how can the authors be sure that the effects observed are not simply due to neuronal damage from the experimental protocol or other constituents in the microinjected eluate during the purification process of -Syn? The authors should perform a control in which a purified scrambled -Syn peptide is microinjected in precisely the same conditions and injections sites.

Minor points:

3. In the methods, the authors mention a "mock control" where HEK cells without the -Syn tag (unclear what this means) underwent the same affinity purification process. What was this "mock control" for? No data of this is presented.

4. The introduction is written in such a way that it is confusing to the reader as to which experiments have been performed in previous published work, and which experiments the current manuscript performs. For example, the introduction writes: "Building upon these findings, we next wanted to determine the extent to which excess physiologic α-synuclein, and specifically the 'multimers,' impact synapses. To address this, we acutely introduced to lamprey synapses native α-synuclein isolated from the brains of neuropathologically normal human subjects.", nonetheless, these experiments were performed in a previous publication.

5. What is the phosphorylation status of the purified -Syn from HEK cells, in particular serine-129 which has recently been shown to alter SVs organisation.

6. It appears from Figure 3 (at least from the representative images chosen) that there may be a reduction in the active zone length under the [high] -Syn condition. The authors should, in addition, assess the number of docked vesicles as a density per unit area / unit length of the active zone structure. Although the total number of docked vesicles may have decreased, the density of docked vesicles may be unchanged.

7. Figure 4H. The authors find that the synaptic vesicles that remain at the [high] monomeric -Syn injected synapses are larger. Could this simply result from incorrect labelling of smaller cisternae structures as SVs? This would also explain why there appears to be an apparent reduced number of cisternae in monomeric [high] in comparison to multimeric [high]. The authors should plot a frequency distribution of (1) SV diameters (2) cisternae structure diameters. The authors should also plot a frequency distribution of diameters of all vesicle-like structures within the bouton, which could potentially show some form of multimodal distribution that can be fitted. This could help the author determine whether there was a genuine shift towards larger SV sizes or accidental classification of cisternae as SVs.

8. It also appears (at least from the representative images chosen) that the vesicle-like structures within the bouton of [high] multimeric -Syn appear to be less-spherical than in control (Figure 5 and Fig 3c). It could be interesting to measure and plot a distribution of sphericity.

9. The fusosome structures which the authors describe are quite interesting and could represent bulk endocytic structures. It might be insightful to quantify the distribution of fusosomes across the active zone structure (i.e., do they tend to form in the central region of the active zone or peripheral region of the active zone?), though this might be difficult considering the scarcity of these structures.

10. Are these fusosome structures intermediates of the cisternae structures?

END OF COMMENTS

Confidential Review **07-Jun-2024**

We sincerely thank the Editor and both Referees for their positive feedback on our manuscript and for the helpful comments. We have addressed all of the points raised in the initial submission. These changes led to the addition of new text to clarify several points, two new figures **(Figure 6, 9)**, and multiple new panels of data (images and analyses) that are included in the revised figures. The specific changes to the manuscript are described in detail below. As a consequence, we believe the manuscript is substantially approved, and we sincerely thank you for consideration of the revised manuscript for publication in the *Journal of Physiology*.

REFEREE COMMENTS

Referee #1:

This manuscript explores the effects of monomeric and multimeric alpha-synuclein on synaptic vesicle clustering at Lamprey Giant Synapses. Previous studies have documented the impact of alpha-synuclein on vesicle clustering and synaptic transmission. By leveraging the unique properties of the lamprey giant synapse, which allows precise manipulation of the presynaptic compartment, the authors conduct an elegant and rigorous assessment of the effects of monomeric and multimeric forms of alpha-synuclein. The integration of electron microscopy and the rigorous quality control of alpha-synuclein preparations are major strengths of this study.

The manuscript is very well-written, and the experiments are thoroughly described. Overall, this is a sound study clarifying the effect of alpha synuclein on synaptic vesicles. I have the following comments to enhance the study through additional experimentation or in-depth discussions:

We would like to thank Referee 1 for the helpful and constructive feedback and have addressed all of the concerns as detailed below.

1. I could not find any data showing measurements of "synaptic vesicle TRAFFICKING." Is it possible that alpha-synuclein impacts the formation of synaptic vesicles?

To address this point, we began by clarifying our definition of “synaptic vesicle trafficking” in the Introduction (p. 4, par. 2): “Initial studies performed in mammalian neurons revealed that overexpression of α-synuclein severely impaired local synaptic vesicle trafficking at presynapses, specifically exocytosis/endocytosis dynamics, as well as vesicle re-clustering after endocytosis.”

In the Discussion, we also provided a better explanation on how we determined the impacts on SV trafficking with our ultrastructural measurements (p. 21, par. 1): “For both multimeric and monomeric α-synuclein, the inhibition of local synaptic vesicle trafficking was evidenced by a loss of total synaptic vesicles at presynapses, which was partially compensated by an increase in endocytic intermediates. In addition, the loss of total synaptic membrane may be explained by the synaptic vesicle declustering/reclustering defects we observed for both α-synuclein species **(Figure 9B-C)**.”

We agree that α-synuclein may impact the formation of synaptic vesicles and have added this to the Discussion (p. 21, par. 2): “Multimeric and monomeric α-synuclein also induced a significant increase in the number of ‘cisternae’ at synapses, suggesting that α-synuclein impairs synaptic vesicle re-formation from vesicular endosomes.”

Finally, to illustrate these impacts of multimeric and monomeric α-synuclein on local synaptic vesicle trafficking at the presynapse, we have added a new figure **(Figure 9)**, which summarizes the synaptic vesicle trafficking effects caused by each molecular species of α-synuclein.

2. Are these synapses amenable to live imaging? It would be helpful for readers to visualize synuclein within this axon/synapse. Does alpha-synuclein enter the presynaptic compartment, and is it uniquely localized there?

While lamprey giant synapses are generally amenable to live imaging of calcium and FM dyes for example [see Wallace et., 2024; *Molecular Biology of the Cell* 35(1):ar10.], direct labeling of α-synuclein on either the N- or C-terminus notoriously diminishes its biological activity by altering its membrane binding or other biochemical interactions. To achieve the cleanest results, we therefore microinject unlabeled α-synuclein into lamprey giant axons, along with a fluorescent dye of similar molecular weight, followed by fixation and processing for electron microscopy. We describe this process in the Methods and Results, and we have now included an image of the injected fluorescein dextran that serves as a reasonable proxy for the extent of α-synuclein diffusion **(Figure 2C)**.

Separately, to address this question and that of Referee 2 (point 1), we performed whole mount immunofluorescence on lamprey spinal cords after axonal injection of *E.coli*-derived recombinant α-synuclein, as in (Wallace et al., 2024). Detection of injected α-synuclein with a human-specific α-synuclein antibody (MJFR1) revealed presynaptic accumulation of the protein with a small fraction remaining in the cytosol. We show an example of this in revised manuscript **(Figure 2D)**. Using the lamprey synapse model, we have now observed this distribution of injected human α-synuclein for three different variants: wild type, phosphoserine 129, and a glycosylated version (Wallace et al., 2024, and unpublished). This distribution parallels endogenous and overexpressed α-synuclein in mammalian synapse models (see Nemani et al., 2010; Scott et al., 2010; Ramalingam et al., 2024). Between the immunofluorescence results and the EM phenotypes showing strong impacts on synaptic vesicles, we are confident that the synaptic vesicles are a primary target of the injected α-synuclein.

However, this approach is not without limitations because we were simply unable to directly detect the HEK-derived α-synuclein, despite our best attempts at immunofluorescence with three different antibodies. This is likely due to differences in epitope exposure with HEK- and *E. coli*-derived α-synuclein. We address this complication in more detail in the response below and in the revised Discussion (p. 24, par. 1). Also, please see our response to Referee 2, point 1.

3. It is not clear in the manuscript when the axons were fixed for EM after alpha-synuclein injections.

We have clarified the timing of EM fixation in the revised Methods and Results sections. Methods (p. 10, par. 3): “For electron microscopy experiments, the lamprey spinal cords with α-synuclein-injected axons were fixed immediately at the end of the stimulation period in 3% glutaraldehyde, 2% paraformaldehyde in 0.1M sodium cacodylate buffer…”

Results (p. 14, par. 1): “Fixation began at the end of the stimulation period, while continuing to stimulate, and was complete within about 10 seconds, as determined by cessation of action potentials.”

Referee #2:

In this manuscript, Roman-Vendrell and colleagues explore the effects resulting from an acute injection of monomeric or multimeric forms of α-Synuclein (α-Syn) at the lamprey giant reticulospinal synapse. The authors report that presynaptic nerve terminals microinjected with monomeric or multimeric forms of α-Syn both exhibit de-clustering of synaptic vesicles and buildup of endosome-like structures which the authors call cisternae. In addition, the authors demonstrate that only those presynaptic nerve terminals microinjected with monomeric α-Syn exhibit an increase in clathrin-coated pits and the presence of structures which the authors term "fusosomes" at the synapse. The authors conclude that multimeric and monomeric forms of α-Syn impair intracellular vesicle trafficking, but only monomeric α-Syn additionally impaired clathrin-mediated endocytosis. The experiments are well carried out and the conclusions that the authors draw from the results presented are well-founded. The manuscript will be of interest to a broad range of researchers particularly those interested in both the physiological role of α-Syn at the presynaptic nerve terminal. I have a few fairly major concerns and a few minor ones.

We would like to thank Referee 2 for the helpful and constructive feedback, and we have addressed these concerns as detailed below.

Major Concerns:

1. It is critical that the authors adequately quantify the level of "overexpression" that these injections generate. This could be carried out by immunostaining of α-Syn with appropriate controls to estimate the level of overexpression at the time of fixation. It is important to note that α-Syn is high diffusive and these experiments will be critical to estimate the endogenous level versus overexpressed in all conditions. Further, the authors describe the co-microinjection of fluorescein dextran as a proxy to determine the concentration of α-Syn that could have reached the nerve terminal; however, no such data is presented. The authors should also include images/data of the dextran dye diffusion.

As the reviewer indicated, we typically co-inject a fluorophore-conjugated dextran as a proxy for the injected α-synuclein protein (10 kDa for monomeric α-synuclein, 70 kDa for multimeric α-synuclein). As requested, we now show an example of a dye-injected axon in the revised manuscript **(Figure 2C)**. This allows us to determine the maximum distance the α-synuclein could have diffused in our preparations and ensures that the microinjection pipets did not clog.

While the co-injected dextran is a useful method for estimating the α-synuclein concentration that enters the axon, it is not a perfect proxy, as we recently demonstrated that recombinant *E. coli*-derived α-synuclein accumulates preferentially on presynaptic vesicle clusters upon injection into lamprey giant axons (Wallace et al., 2024; *Molecular Biology of the Cell;* doi: 10.1091). We now show an example of this in the revised manuscript **(Figure 2D)**.

Anticipating the question about endogenous vs. exogenous α-synuclein at synapses, we have been trying to perform immunofluorescence against the injected HEK-derived α-synuclein for quite some time now, including during the revision period. It simply hasn't worked. We tried 3 different α-synuclein antibodies (MJFR1, clone 2F12, Abcam ab53726), and none of them

resulted in a detectable immunofluorescence signal for HEK-derived α-synuclein. This is likely due to the differences in epitope exposure. So, despite our best efforts, we cannot provide measurements of endogenous vs. overexpressed (injected) α-synuclein with the tools available at this time. We acknowledge this limitation in the revised Discussion (p. 24): "Moreover, our attempts to accurately quantify the in-situ concentration of the microinjected HEK-derived monomeric and multimeric α-synuclein via immunofluorescence have been more challenging compared to *E. coli* derived material, possibly due to a difference in epitope exposure between the folded and unfolded species. We are thereby limited in a detailed examination of their levels within axons and/or synapses that could provide additional insights into their biological activities. However, we know that recombinant wild type α-synuclein and phosphoserine 129 α-synuclein preferentially accumulate on presynaptic vesicle clusters **(Figure 2C)** (Wallace *et al.*, 2024). Furthermore, the EM phenotypes reported here for both multimeric and monomeric α-synuclein indicate strong impacts on the synaptic vesicle clusters and synaptic vesicle morphologies themselves, indicating that the synaptic vesicles are a primary target of the injected α-synuclein (**Figures 3-6)**. Nonetheless, understanding any differences in the behavior of monomeric versus multimeric human α-synuclein should remain a goal for future experiments, as this could reveal important and novel biological activities of α-synuclein depending on the cellular context from which it was derived."

2. Across all experiments, the synapses which act as controls are those from the axons injected with α-Syn, but further away from the microinjection site beyond the proposed α-Syn diffusion limit. Since the authors are dissecting/microinjecting the nerve, the most physical damage will happen closest to the injection site (which are the [high] and [low] α-Syn conditions synapses are taken), how can the authors be sure that the effects observed are not simply due to neuronal damage from the experimental protocol or other constituents in the microinjected eluate during the purification process of α-Syn? The authors should perform a control in which a purified scrambled α-Syn peptide is microinjected in precisely the same conditions and injections sites.

Although generating a scrambled α-synuclein is a great idea, it is also costly, would potentially introduce different contaminations (given its synthetic background), and the necessary experiments would take at least 6-8 more months. Therefore, to address these points, we leaned on results published in our prior studies, which collectively rule out injection damage, as well as any potential effects of the SEC eluate constituents. We added a new paragraph to the Discussion (p. 21-22, par. 3), which reads: "The synaptic phenotypes we report here are specific for α-synuclein since injection of other similar-sized inert proteins (e.g. GST) or synaptic chaperones (e.g. Hsc70) had little to no impact on synaptic morphology under the same conditions (Busch *et al.*, 2014; Banks *et al.*, 2020). Moreover, with low-frequency stimulation (5 Hz), or in the absence of stimulation, excess human α-synuclein has little to no effect on synaptic morphology, indicating an activity-dependent effect while also ruling out artifacts due to microinjection damage (Busch *et al.*, 2014; Wallace *et al.*, 2024). Finally, we have previously showed that injection of SEC samples immunodepleted of α-synuclein had no effect on synapses, ruling out any effects of the eluate constituents (Roman-Vendrell *et al.*, 2021). Thus, the synaptic phenotypes reported here are highly specific for excess α-synuclein at stimulated synapses and are in line with results from our prior studies, as will be discussed in more detail below."

Minor points:

3. In the methods, the authors mention a "mock control" where HEK cells without the α-

Syn tag (unclear what this means) underwent the same affinity purification process. What was this "mock control" for? No data of this is presented.

We have now clarified in the Methods how the mock controls were used (p. 8, par. 1):
“The mock controls, containing only potential contaminants of the affinity step, were further used for determination of purity via LC-MS/MS and background subtraction in CD spectroscopy as in (de Boni et al. 2022).”

4. The introduction is written in such a way that it is confusing to the reader as to which experiments have been performed in previous published work, and which experiments the current manuscript performs. For example, the introduction writes: "Building upon these findings, we next wanted to determine the extent to which excess physiologic α-synuclein, and specifically the 'multimers,' impact synapses. To address this, we acutely introduced to lamprey synapses native α-synuclein isolated from the brains of neuropathologically normal human subjects.", nonetheless, these experiments were performed in a previous publication.

We agree with the reviewer that this was confusing and have therefore reorganized and revised the last 2 paragraphs of the Introduction (p. 5-6), so that it is clear which findings were from the published work versus the new study.

5. What is the phosphorylation status of the purified α-Syn from HEK cells, in particular serine-129 which has recently been shown to alter SVs organisation.

We performed additional biochemical characterization with a phosphoserine 129-specific α-synuclein antibody and showed that the HEK-derived α-synuclein is not phosphorylated at serine 129. These data are now shown as a new panel in **Figure 1B** with the following text revision (p. 13, par. 1): “Phosphorylation of α-synuclein at serine 129 (pS129) is a post-translational modification that occurs in very low levels in neurons under physiological conditions and builds up in pathological conditions, and this modification modulates α-synuclein functions at synapses (Parra-Rivas *et al.*, 2023; Ramalingam *et al.*, 2023; Wallace *et al.*, 2024). α-Synuclein purified from HEK cells was not detectably phosphorylated at serine 129 **(Figure 1B, right)**.”

6. It appears from Figure 3 (at least from the representative images chosen) that there may be a reduction in the active zone length under the [high] α-Syn condition. The authors should, in addition, assess the number of docked vesicles as a density per unit area / unit length of the active zone structure. Although the total number of docked vesicles may have decreased, the density of docked vesicles may be unchanged.

We performed additional analyses of the density of docked SVs and report that only the multimeric α-synuclein decreases both the number and density of docked SVs at the active zone while monomeric α-synuclein had no effect. The active zone lengths were unchanged across experimental conditions. These new data are reported in the Results section and in a new **Figure 6** of the revised manuscript.

7. Figure 4H. The authors find that the synaptic vesicles that remain at the [high] monomeric α-Syn injected synapses are larger. Could this simply result from incorrect labelling of smaller cisternae structures as SVs? This would also explain why there

appears to be an apparent reduced number of cisternae in monomeric [high] in comparison to multimeric [high]. The authors should plot a frequency distribution of (1) SV diameters (2) cisternae structure diameters. The authors should also plot a frequency distribution of diameters of all vesicle-like structures within the bouton, which could potentially show some form of multimodal distribution that can be fitted. This could help the author determine whether there was a genuine shift towards larger SV sizes or accidental classification of cisternae as SVs.

We have performed additional analyses on the frequency distributions of SV diameters and cisternae perimeters. These new data are shown in **Figure 3I and 3O** for multimeric α-synuclein and **Figure 4I and 4O** for monomeric α-synuclein. As can be seen in **Figure 4I**, there was a shift in the peak synaptic vesicle diameter at the higher concentrations of monomeric α-synuclein, which was largely due to an increase in synaptic vesicles with diameters between 60-90 nm. We classify cisternae as any vesicular structures >100 nm, which in the vast majority of cases are large and non-spherical. In order to visualize these cisternae structures better, we added new 3D reconstructions in **Figures 3L and 4L**. Given that the cisternae are large and non-uniformly shaped (unlike synaptic vesicles which are uniform in size and shape), it is not possible to combine them with synaptic vesicles. While we cannot be absolutely certain that none of the cisternae were misclassified as synaptic vesicles, the shape of the synaptic vesicle diameter frequency distribution and paucity of vesicles >90 nm **(see Figure 3I, 4I)** would suggest that this was not a confounding issue.

8. It also appears (at least from the representative images chosen) that the vesicle-like structures within the bouton of [high] multimeric α-Syn appear to be less-spherical than in control (Figure 5 and Fig 3c). It could be interesting to measure and plot a distribution of sphericity.

The reviewer is correct that the synaptic vesicles are less spherical with α-synuclein than in controls. We performed an analysis to determine the eccentricity of SVs as a measure of their circularity which we are using as a proxy for sphericity. Sphericity requires a volumetric measurement we cannot obtain in our micrographs. By drawing two perpendicular lines from one edge of the SV to the other we can represent the major and minor axis of an ellipse to the best of our ability. The analyses revealed that both multimeric and monomeric α-synuclein increased eccentricity of the synaptic vesicles, indicating that they were less spherical and more elliptical. These data are shown in the revised manuscript in **Figures 3J** and **Figure 4J**.

9. The fusosome structures which the authors describe are quite interesting and could represent bulk endocytic structures. It might be insightful to quantify the distribution of fusosomes across the active zone structure (i.e., do they tend to form in the central region of the active zone or peripheral region of the active zone?), though this might be difficult considering the scarcity of these structures.

We quantified the distribution of fusosomes across the active zone structure, as suggested, and now show that they are evenly distributed within the central and peripheral regions of active zones **(Figure 8C)**.

10. Are these fusosome structures intermediates of the cisternae structures?

We cannot be sure of the identity of the fusosomes, as this would require molecular markers to determine unequivocally, which we hope to develop in the future. To address this point, we added a statement to the Discussion (p. 21, par. 2): “Monomeric α-synuclein also induced fusosomes at the active zone **(Figure 9C)**. While the identity of these structures remains to be determined, they could be recycling or bulk endosomes that are aberrantly fusing with the active zone or alternatively a consequence of multivesicular fusion events.”

Dear Dr Morgan,

Re: JP-RP-2024-286281R1 "Acute introduction of monomeric or multimeric α-synuclein induced distinct impacts on synaptic vesicle trafficking at lamprey giant synapses." by Cristina Roman-Vendrell, Jaqulin N. Wallace, Aurelia Hays Watson, Meral Celikag, Tim Bartels, and Jennifer R. Morgan

We are pleased to tell you that your paper has been accepted for publication in The Journal of Physiology.

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All queries at proof stage should be sent to: TJP@wiley.com.

Following copyediting and typesetting the accepted version will be published online.

Yours sincerely,

Katalin Toth
Senior Editor
The Journal of Physiology

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Reviewing Editor:

The authors have done an excellent job of responding to the previous critiques. There are no further concerns.

REFEREE COMMENTS

Referee #1:

The authors have properly addressed my concerns. Congratulations on this well conducted study!

Referee #2:

The authors have tried their best to answer my queries and criticisms and their revised version is, in my opinion, a great fit for J Physiol.

1st Confidential Review **27-Sep-2024**