Regulated resurfacing of a somatostatin receptor storage compartment fine-tunes pituitary secretion

Walaa Alshafie, Vincent Francis, Klaudia Bednarz, Yingzhou Pan, Thomas Stroh, and Peter McPherson

Corresponding Author(s): Peter McPherson, McGill University and Thomas Stroh, McGill University

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DOI: https://doi.org/10.1083/jcb.201904054
May 20, 2019

Re: JCB manuscript #201904054

Dr. Peter S McPherson
McGill University
Department of Neurology and Neurosurgery Montreal Neurological Institute McGill University
3801 rue University
Montreal, Quebec H3A 2B4
Canada

Dear Dr. McPherson,

Thank you for submitting your manuscript entitled "Signaling-activated resurfacing of an SSTR2 storage compartment fine-tunes pituitary hormone release" and we sincerely apologize for the delay in communicating our decision to you. The manuscript has been evaluated by expert reviewers, whose reports are appended below. As you will see, all of the reviewers found your work interesting and much of the data strong. However, each raised significant critiques, with two of the reviewers left unconvinced of the main conclusions that distinguish your findings from those reported previously in the literature. After an assessment of the reviewer feedback, in light of these concerns, unfortunately, our editorial decision is against publication in JCB.

We would be willing to consider a revised manuscript if you believe that you can substantially address the critiques. However, a successful outcome would require that you fully address the critiques of Reviewers #1 and #2 with additional data going beyond fluorescence imaging and that you address the question raised by Reviewer #3 regarding the possibility that receptor return to the plasma membrane is limited by ligand proteolysis as concluded previously by others. I would also ask you to more accurately communicate general aspects of the current state of knowledge that pertain to claims of novelty or conceptual advance of your study, as pointed out by Reviewer #1.

I agree with the Reviewers that it is particularly important to clearly define the SSTR2 compartment and to more convincingly distinguish it from the Golgi/TGN. Doing so is critical to the main advance claimed relative to previous work. The analogy that you make with Glut4 vesicles is interesting but can it be tested directly? The data on Rab10 depletion seem a reasonable start but these effects need to be more fully characterized. The results on TUG depletion are not publishable in their present form, even as a supplement, as they are limited to single micrographs of single cells for each condition.

Although your manuscript is intriguing, we feel that the points raised by the reviewers are more substantial than can be addressed in a typical revision period. If you wish to expedite the publication of the current data, it may be best to pursue publication at another journal.

Given interest in the topic, If you believe that you can substantially address these major concerns, we would be open to resubmission to JCB of a significantly revised and extended manuscript that fully addresses the reviewers' concerns and is subject to further peer-review. If you would like to resubmit this work to JCB, please contact the journal office to discuss an appeal of this decision or
you may submit an appeal directly through our manuscript submission system. Please note that priority and novelty would be reassessed at resubmission. We would be happy to discuss your plans for revisions if you would like feedback (in the form of a detailed point-by-point response to the reviewers' comments) to ensure you do not embark on time- and resource-consuming revisions that may not be sufficient for a successful resubmission.

Whatever decision you and your co-workers make, we hope that the attached reviewer critiques are constructive and thank you again for submitting your most interesting work for consideration. We would be happy to discuss the reviewer comments further once you've had a chance to consider the points raised in this letter. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Mark von Zastrow, MD, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

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

In this study the authors expand on previous work showing that SSTR2 recycles to a perinuclear Syntaxin 6 compartment. The authors present data that this compartment is syntaxin 6 positive and distinct from the TGN and may be related to Glut4 storage vesicles. Recycling from this compartment can be regulated by activation of surface hormone receptors. The authors present animal data correlating serum hormone levels with SSTR2 surface localization. They propose that this mechanism of SSTR2 trafficking may be part of a feedback loop regulating hormone release in the pituitary gland.

This is an interesting article in the context of SSTR2 recycling in the cells studied. But the conceptual advance seems to be overstated. First, the authors' assertion that "Our data provide the first example of regulation of a G- protein coupled receptor by signaling-mediated plasma membrane resurfacing" is not correct. The mechanisms regulating trafficking and delivery of many GPCRs, including SSTRs by PDZ domains, have been studied. There is also evidence that the surface delivery of many GPCRs, from both the endocytic recycling pathway and from perinuclear pools in the biosynthetic pathway, is regulated by signaling pathways (e.g., beta 2 adrenergic receptor via PKA, mu opioid receptors via NK1R and PKC, delta opioid receptors via ROCK/LIMK, delta opioid receptors via NGF and PTEN, CXCR4 via L-selectin, to cite some canonical and well-studied GPCRs), and sequences on receptors responsible for this regulation identified. These and other relevant results are not referenced.

Second, although a feedback loop is proposed, this feedback loop is not directly shown. Experimentally, the characterization of the SSTR2 compartment is a major component of the paper, and this needs better support. The authors present STORM data to show that SSTR2 colocalizes with syntaxin 6 distinct from the TGN. The authors acknowledge that exact colocalization cannot be shown using this technique but argue that syn6 localizes more closely to SSTR2. A more relevant negative control comparison to evaluate their hypothesis would be SSTR2 to PIST. If the
authors are arguing that SSTR2 localizes to a syn6 compartment distinct from the TGN, then the
distance of PIST (a TGN maker) from SSTR2 should be increased relative to syn6. A direct
comparison between SSTR2 and Glut4 will also help. But because this is a major point different
from previous reports, this needs electron microscopic or biochemical data.
The authors show a decrease in surface SSTR2 after SOM addition, consistent with internalization,
and a concomitant increase in the BFA-insensitive perinuclear pool after 40min of agonist
treatment. Critical controls are missing to show that this pool is endocytic and not biosynthetic in
origin - pre-treating cells with cycloheximide, blocking endocytosis, following surface-labeled
receptors. Similarly, BFA is not sufficient to show that the delivery of receptors is not biosynthetic,
especially with the long time scales that the authors use.

How the TUG siRNA (Figure S7) experiments support the authors' hypothesis that SSTR2 is in a
Glut4 like storage vesicle is unclear. For Glut4 TUG knockdown leads to increased surface
transporter in the absence of stimulated release. From the images provided it seems the major
effect of TUG knockdown is accumulation of SSTR2 in the Golgi. The authors should be clearer
about the effect they observe on TUG localization and how this is consistent with their model.

Overall the manuscript could benefit from better quantitation of the observed phenotypes. Also the
number of cells showing the observed phenotype, how the "perinuclear region" was defined, how
surface vs. cytoplasm was defined in pituitary gland sections, etc. need to be clarified.

Increase in intracellular calcium and cAMP seem sufficient to cause release of the SSTR2 internal
pool. Is PKA required for this process?

In Figure 9D do the error bars represent variation among images collected from sectioned pituitary
glands in the same animal? The major source of experimental variability is expected to be between
animals in the same GH release cycle phase, the data averaged across animals in the same phase
is a better representation.

The manuscript contains some typos that need to be corrected.

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

This study by Alshafie and colleagues uses insulin regulated exocytosis of GLUT4 as a paradigm to
investigate the mobilization of a GPCR, SSTR2 from a perinuclear compartment to the cell surface
in response to somatostatin.
The authors use microscopy to demonstrate that SSTR2 traffics from the cell surface to a
perinuclear Syntaxin6-positive compartment. They use colocalisation (or lack thereof) studies to
determine that this is not the TGN. Similarly Brefeldin-A treatment did not disrupt this compartment
(another similarity with GLUT4 trafficking).
The study is interesting, novel and will be of interest to a wide variety of cell biologists. However the
manuscript submitted requires further information in my opinion.
1. At present the study relies heavily on microscopy approaches (albeit sophisticated ones that
have been carried out and presented well) - findings from these should be corroborated using
biochemical approaches.
• Density gradient fractionation of cells treated +/- SOM should be compared to characterise the
mobilisable pool of SSTR2 (blot for it other markers).
• Endosome ablation (as in Livingstone et al 1996 Biochem J. 315: 487-495. to determine whether
the compartment overlap is similar to that reported for GLUT4.

2. The Rab10 knockout experiment is a nice addition but the cells require more characterization. For example, are other Rabs upregulated in these cells? What happens to IRAP trafficking in these cells?

3. I am confused as to why the TUG experiments are in the supplementary section - in my opinion this should either be expanded (e.g. is USP25 required - as recently published by the Bogan lab for GLUT4) and included in the main text or removed.

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

Signaling-activated resurfacing of an SSTR2 storage compartment fine-tunes pituitary hormone release

In my opinion this is an excellent study; it has been well planned, the experimental techniques are appropriate and state-of-the-art. The deciphered mechanism whereby stimulation of one GPCR promotes to exocytosis of another GPCR is both exciting and well worthy of publication. The authors have demonstrated their mechanism exists in a number of different cell lines and have concluded by providing evidence that this mechanism is also functional in vivo.

It was interesting that the SSTR2 recycling kinetics were strikingly different in the HeLa cells compared to the AtT20 cells. I wonder if the authors have given this some thought, given that it is not discussed in the manuscript. Many the recycling of many neuropeptide GPCRs is regulated by endosomal peptidases such as endothelin-converting enzyme-1 and the SSTR2 is one such example. It could be that expression levels or regulation of ECE-1 localization or activity (modified by phosphorylation) may be responsible for this difference and may be worth discussing.

I have no major issues with this study, however, the overall presentation of the study could be improved by carefully checking the manuscript and figures for typographical/formatting errors. I list a few that I have picked up:

- Abstract: There is no hyphen between G and protein
- p4: The sentence "In response to insulin receptor activation in muscle and fat cells, GLUT4 vesicles are mobilized in a Rab10-dependent manner to the cell surface where they fuse, allowing for GLUT4 dependent uptake of glucose" needs a reference.
- p5: The sentence "We compared the localization of SSTR2 to that of fluorescent transferrin (Trf), which binds the Trf receptor and following internalization marks early/recycling endosomes, and with syntaxin-6, since SSTR2 is known to traffic to a syntaxin-6-positive juxtanuclear compartment." need a reference.

Methods
Earle's buffer is frequently mis-spelt.

p15: 15.00 pm?

The number of experiments conducted is often missing from figure legends.
This is obviously a journal specific issue, but many of the merged images used red and green. This is inappropriate for color blind persons. Furthermore, in some figures the panels are mixed i.e., some red/green others yellow/magenta and labelled at the side and then within the figure.

Please check the font sizes, as on my version of the file, often the font sizes vary.

Finally, after all the excellent work, figure 8C, which portrays the mechanism is a little basic and could be greatly improved.
July 26, 2019

Dr. Mark von Zastrow, Monitoring Editor
Dr. Melina Casadio, Senior Scientific Editor
Journal of Cell Biology

Dear Drs. von Zastrow and Casadio:

Thank you for your review of our manuscript “Signaling-activated resurfacing of an SSTR2 storage compartment fine-tunes pituitary hormone release”. We were delighted to see that “… all of the reviewers found your work interesting and much of the data strong”. We were particularly happy that reviewer 2 stated “The study is interesting, novel and will be of interest to a wide variety of cell biologists” and that reviewer 3 stated “In my opinion this is an excellent study; it has been well planned, the experimental techniques are appropriate and state-of-the-art. The deciphered mechanism whereby stimulation of one GPCR promotes to exocytosis of another GPCR is both exciting and well worthy of publication”.

We were happy to learn that you are “… willing to consider a revised manuscript if you believe that you can substantially address the critiques”. We are now submitting a revised version of the manuscript in which, as outlined in detail below, we have addressed all comments raised by the reviewers, in most cases through the addition of new data. Further, we have changed the manuscript to better communicate the novelty of our findings and have cited important studies demonstrating that resurfacing of various GPCRs is regulated by signalling.

The revised manuscript is greatly improved and we thank the reviewers for their comments.

Sincerely,
Peter McPherson, PhD, FRSC
Reviewer #1

Response to comment 1) We apologize for not clearly defining the conceptual advances of our study. Specifically, the assertion that “Our data provide the first example of regulation of a G-protein coupled receptor by signalling….” is clearly wrong. We removed this line from the abstract. In addition, in the discussion section on page 14 of the revised manuscript, we have added references indicating that signalling and trafficking of GPCRs are highly intertwined with several examples, provided by the reviewer, in which signalling controls endocytic recycling of GPCRs. That said there are important conceptual advances in this study. First, we have better defined the trafficking itinerary of SSTR2 including the fact that it does not traffic to the TGN following endocytosis as previously reported. Second, we provide multiple lines of evidence including several new experiments that syntaxin-6 is not a marker of the TGN. Third, we demonstrate that SSTR2 traffics from an intracellular compartment to the surface on Glut4-like vesicles, expanding the concept of a regulated Glut4-like storage/resurfacing vesicle beyond muscle and fat. Finally, Our data provide an unexpected mechanism by which signalling-mediated plasma membrane resurfacing of SSTR2 fine-tunes pituitary hormone release.

Response to comment 2) We agree that the feedback loop is not shown directly, but all of the components of the loop are now in place in that: 1) it is already well established that activation of surface SSTR2 inhibits signalling downstream of the releasing factor receptors (including GHRH and CRF receptors) in pituitary; 2) here we confirm that SOM induces endocytosis of SSTR2 and trafficking of the receptor to a juxta-nuclear compartment where it is stored, and 3) we now show that activation of CRF receptor induces resurfacing of endocytosed SSTR2 where it is available to inhibit the release factor receptors, closing the loop. Moreover, the experiments correlating blood GH levels to the ratio of surface:intracellular SSTR2 (revised Fig. 10 and Fig. S8-10) support this feedback loop in vivo.

Response to comment 3) “Experimentally, the characterization of the SSTR2 compartment is a major component of the paper, and this needs better support. The authors present STORM data to show that SSTR2 colocalizes with syntaxin 6 distinct from the TGN. The authors acknowledge that exact colocalization cannot be shown.
using this technique but argue that syn6 localizes more closely to SSTR2. A more relevant negative control comparison to evaluate their hypothesis would be SSTR2 to PIST. If the authors are arguing that SSTR2 localizes to a syn6 compartment distinct from the TGN, then the distance of PIST (a TGN maker) from SSTR2 should be increased relative to syn6”.

Response to comment 3) We agree, this is a good idea and an important control. Thus, we performed the requested experiment, which is presented in Fig. 6 D-F of the revised manuscript. Indeed, as the reviewer predicted, the distance from SSTR2 to PIST is greater than SSTR2 to syntaxin-6. Moreover, in response to reviewer 2, comment 1, we have performed subcellular fractionation studies confirming that SSTR2 is in a syntaxin-6-positive, TGN46-negative compartment (revised Fig 5). Lastly, we used the microtubule-depolymerizing drug nocodazole to induce polarized Golgi ministacks, simplifying visualization of the Golgi complex. With nocodazole treatment, SSTR2 continues to show extensive co-localization with syntaxin-6, and the TGN markers PIST and TGN38 remain co-localized (Fig. S3 A and B and F, revised manuscript). In contrast, PIST shows markedly less co-localization with the cis-Golgi protein Giantin or with syntaxin-6 (Fig. S3 C and D and F, revised manuscript).

Reviewer #1, comment 4) "A direct comparison between SSTR2 and Glut4 will also help. But because this is a major point different from previous reports, this needs electron microscopic or biochemical data".

Response to comment 4) SSTR2 and Glut4 are each expressed in specialized systems. Thus, a direct comparison may not be relevant. We do not believe that SSTR2 is to be found in Glut4 vesicles, but instead that there is a Glut4-like compartment that is found in pituitary cells and possibly other cell types. This compartment shares molecular properties that control trafficking but carry distinct cargo depending on the cell type.

Reviewer #1, comment 5) “The authors show a decrease in surface SSTR2 after SOM addition, consistent with internalization, and a concomitant increase in the BFA-insensitive perinuclear pool after 40min of agonist treatment. Critical controls are missing to show that this pool is endocytic and not biosynthetic in origin - pre-treating cells with cycloheximide, blocking endocytosis, following surface-labeled receptors. Similarly, BFA is not sufficient to show that the delivery of receptors is not biosynthetic, especially with the long time scales that the authors use”.

Response to comment 5) We agree with the reviewer that important controls were missing, which we have added to the revised manuscript. First, in Fig. S1 A and B of the revised manuscript we have used dynasore and hypertonic sucrose to block endocytosis, demonstrating that the appearance of the juxta-nuclear pool of SSTR2 depends upon endocytosis. Second, we have performed the recycling experiments in the presence of cyclohexamide (revised Fig. S2 B). The loss of endocytosed SSTR2 from the juxta-nuclear pool and its appearance at the cell surface over the 24 h time course of the experiment is not influenced by the presence of cyclohexamide, indicating that the receptor appearing at the surface is not biosynthetic in origin but instead results from recycling.

Reviewer #1, comment 6) How the TUG siRNA (Figure S7) experiments support the authors' hypothesis that SSTR2 is in a Glut4 like storage vesicle is unclear. For Glut4 TUG knockdown leads to increased surface transporter in the absence of stimulated
release. From the images provided it seems the major effect of TUG knockdown is accumulation of SSTR2 in the Golgi. The authors should be clearer about the effect they observe on TUG localization and how this is consistent with their model.

Response to comment 6) We agree with the reviewer that the interpretation of these results were problematic. Studies on TUG will require extensive further analysis, which will be conducted in a follow up study. Thus, as suggested by reviewer 2 in comment #3, we have removed these results from the revised manuscript.

Reviewer #1, comment 7) "Overall the manuscript could benefit from better quantitation of the observed phenotypes. Also the number of cells showing the observed phenotype, how the "perinuclear region" was defined, how surface vs. cytoplasm was defined in pituitary gland sections, etc. need to be clarified".

Response to comment 7) We have added two new sub-sections to the Materials and Methods section. These are headed "Image and statistical analysis" and "Measurement of SSTR2 fluorescence in anterior pituitary cells from sections" and can be found on pages 24 and 25 of the revised manuscript. In these sections we provide details regarding quantification that address the reviewers important comments.

Reviewer #1, comment 8) "Increase in intracellular calcium and cAMP seem sufficient to cause release of the SSTR2 internal pool. Is PKA required for this process?"

Response to comment 8) We thank the reviewer for this question, which we have addressed in the revised manuscript. Specifically, we used the cell-permeable myristoylated protein kinase A inhibitor 14--22 amide (PKI) (Glass, Cheng et al. 1989), a direct inhibitor of PKA activity, in recycling experiments. PKI decreased the return of endocytosed SSTR2 to the plasma membrane indicating that PKA activity is required for CRF-induced recycling (Fig. 9 C and D of the revised manuscript).

Reviewer #1, comment 9) "In Figure 9D do the error bars represent variation among images collected from sectioned pituitary glands in the same animal? The major source of experimental variability is expected to be between animals in the same GH release cycle phase, the data averaged across animals in the same phase is a better representation".

Response to comment 9) The original figure presented variation within each animal (Fig. 9 D) and the data averaged across animals (Fig. 9 E). We agree with the reviewer and now just present the average across the animals (Fig. 10 B of the revised manuscript).

Reviewer #1, comment 10) "The manuscript contains some typos that need to be corrected".

Response to comment 10) We apologize in that upon re-reading we noticed the many typos. We have carefully edited the manuscript.

Reviewer #2

Reviewer #2, comment 1) "At present the study relies heavily on microscopy approaches (albeit sophisticated ones that have been carried out and presented well) - findings from these should be corroborated using biochemical approaches."
• Density gradient fractionation of cells treated +/- SOM should be compared to characterise the mobilisable pool of SSTR2 (blot for it other markers).

• Endosome ablation (as in Livingstone et al 1996 Biochem J. 315: 487-495. to determine whether the compartment overlap is similar to that reported for GLUT4”

Response top comment 1) We thank the reviewer for the positive comments regarding the nature and application of the microscopy studies. We do agree with the reviewer that biochemical approaches would support our conclusions. Thus, we performed two different subcellular fractionation experiments. First, we used antibody-coated magnetic beads to immunoisolate the syntaxin-6-positive compartment (revised Fig. 5). We found that the compartment is enriched in endocytosed SSTR2, but the TGN markers PIST and TGN46 are de-enriched. We also performed sucrose density gradient centrifugation experiments that allowed us to separate TGN membranes labelled with PIST from the syntaxin-6 compartment (revised Fig. S5).

Reviewer #2, comment 2) “The Rab10 knockout experiment is a nice addition but the cells require more characterization. For example, are other Rabs upregulated in these cells? What happens to IRAP trafficking in these cells?”

Response to comment 2) We agree with the reviewer that the Rab10 knockout cells required additional characterization. We thus performed immunoblots on the cells using antibodies against Rab8 and Rab13, which are the two Rabs most evolutionarily related to Rab10, and with Rab35 and Rab5, two Rabs involved in endocytic recycling. Importantly, there were no alterations in the expression of any of these Rabs when comparing knockout cells to wild-type cells (Fig. 8 B, revised manuscript). We also examined the trafficking of IRAP. While we see co-localization of IRAP with syntaxin-6 at the juxta-nuclear region, we were unable to see any changes in IRAP subcellular localization upon CRF treatment. IRAP is a cargo protein found of Glut4 vesicles. It may be that IRAP has specific functions in adipocytes downstream of insulin signalling that are not required in pituitary cells.

Reviewer #2, comment 3) “I am confused as to why the TUG experiments are in the supplementary section - in my opinion this should either be expanded (e.g. is USP25 required - as recently published by the Bogan lab for GLUT4) and included in the main text or removed”.

Response to comment 3) The TUG experiments will require extensive further study. Dr. Bogan will continue to expand upon this concept in an independent study. Therefore, we agree with the reviewer and have removed the figure from the revised manuscript.

Reviewer #3

Reviewer #3, comment 1) “It was interesting that the SSTR2 recycling kinetics were strikingly different in the HeLa cells compared to the AtT20 cells. I wonder if the authors have given this some though, given that it is not discussed in the manuscript. Many the recycling of many neuropeptide GPCRs is regulated by endosomal peptidases such as endothelin-converting enzyme-1 and the SSTR2 is one such example. It could be that expression levels or regulation of ECE-1 localization or activity (modified by phosphorylation) may be responsible for this difference and may be worth discussing". 
Response to comment 1) The reviewer raises some very interesting ideas that we had not considered. We have added a brief discussion of this issue on pg. 14 in the discussion section of the revised manuscript.

Reviewer #3, comment 2) “I have no major issues with this study, however, the overall presentation of the study could be improved by carefully checking the manuscript and figures for typographical/formatting errors. I list a few that I have picked up:

Abstract: There is no hyphen between G and protein

p4: The sentence "In response to insulin receptor activation in muscle and fat cells, GLUT4 vesicles are mobilized in a Rab10-dependent manner to the cell surface where they fuse, allowing for GLUT4 dependent uptake of glucose" needs a reference.

p5: The sentence "We compared the localization of SSTR2 to that of fluorescent transferrin (Trf), which binds the Trf receptor and following internalization marks early/recycling endosomes, and with syntaxin-6, since SSTR2 is known to traffic to a syntaxin-6-positive juxtanuclear compartment." need a reference.

Methods
Earle's buffer is frequently mis-spelt.

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The number of experiments conducted is often missing from figure legends.

This is obviously a journal specific issue, but many of the merged images used red and green. This is inappropriate for color blind persons. Furthermore, in some figures the panels are mixed i.e, some red/green others yellow/magenta and labelled at the side and then within the figure.

Please check the font sizes, as on my version of the file, often the font sizes vary.

Finally, after all the excellent work, figure 8C, which portrays the mechanism is a little basic and could be greatly improved.

Response to comment 2) We appreciate the reviewers careful read of our paper. We have addressed all of these issue in the revised manuscript.
September 6, 2019

Re: JCB manuscript #201904054R-A

Dr. Peter S McPherson
McGill University
Department of Neurology and Neurosurgery Montreal Neurological Institute McGill University
3801 rue University
Montreal, Quebec H3A 2B4
Canada

Dear Dr. McPherson,

Thank you for submitting your revised manuscript entitled "Regulated resurfacing of a somatostatin receptor storage compartment fine-tunes pituitary secretion". Your revised manuscript has been seen by two of the original reviewers. While the reviewers continue to be overall positive about the work in terms of its suitability for JCB, some important issues remain.

One of them found your revision acceptable and the other noted four points that he/she found still not satisfactorily addressed. We have discussed these points in-depth editorially and apologize for the delay in communicating our decision to you. We think that all are fair but ask you to address only two.

The primary remaining point of concern is Rev#1's point #1, the relationship of SSTR2 compartment that you compare to the previously described Glut4 compartment. I understand that this comparison may not be physiologically relevant in this cell type but do think it is reasonable, from a membrane traffic point-of-view, to determine if SSTR2 is in the same compartment or a different compartment relative to (expressed) Glut4. We do not feel that the experimental outcome would affect the editorial decision as we believe that the result is interesting either way, but addressing with data is important to establish the similarity or lack thereof relative to Glut4.

A second remaining point of concern is Rev#1's point #3, verifying fractionation behavior with TGN38 as a more widely recognized TGN marker, and also a marker used by prior work from Csaba et al (2007) to claim TGN. This is not indispensable for publication, but can you provide data on this, either from sucrose gradients or immuno-isolation?

We think that experiments requested to show functional effects on hormone release are not necessary, but Rev#1 is correct about SSTR5 also being expressed in AtT-20. So I suggest you mention that both SSTR2 and SSTR5 can inhibit L-type calcium channels necessary for regulated hormone secretion in AtT-20 cells (e.g., DOI 10.1016/0306-4522(95)00510-2).

In addition, I request that you make one wording change in the last paragraph on p4: "Here we find that SSTR2 recycles from ..." to "Here we verify that SSTR2 recycles from ...". The reason is that I think this is a main conclusion previously claimed in the Csaba et al study cited in the preceding sentence.

Our general policy is that papers are considered through only one revision cycle; however, given
that the suggested changes are relatively minor, we are open to one additional short round of revision. Please note that we will expect to make a final decision without additional reviewer input upon resubmission.

Please submit the final revision within one month, along with a cover letter that includes a point by point response to the remaining reviewer comments.

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

Sincerely,

Mark von Zastrow, MD, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

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

This is a revised manuscript that addresses the regulated recycling of SSTR2. The main conclusions are that 1) SSTR2 endocytoses into a perinuclear Syntaxin 6-containing GLUT4-like compartment, separate from the TGN, from where it recycles slowly, and 2) CRF increases SSTR2 recycling from this compartment, forming a negative feedback loop. The authors state that this could generalize a GLUT4-like homeostatic response to G protein-coupled receptor translocation.

The revision addresses some of the concerns that were pointed out in the earlier version. The authors have added several controls and corrections to strengthen the part that Syntaxin-6 compartment is distinct from a PIST/TGN compartment in AtT20 cells, and that CRF increases SSTR2 recycling. However, whether this is similar to GLUT4 and whether this is a feedback loop are not convincing.

1) The authors assert again that the SSTR2 compartment is not a GLUT4 compartment, but a GLUT4-like compartment. This is not well supported. I understand that these cells might not express GLUT4, but if the components of sorting are different, then GLUT4 and SSTR2 should be segregated into different compartments. If the components are the same, as the authors seem to suggest, then GLUT4 should be in the same compartment as SSTR2. This seems straightforward to test. The authors can also test a different transporter such as GLUT1 that might be more physiologically relevant in these cells, which could be regulated by CRF.

This is also important because of the TUG data that was removed. I understand that TUG experiments need to be pursued further and that these might be beyond this paper. But I caution the authors to be careful about ignoring data that are inconsistent with their model.

The authors refer to a previous paper for the protocol of removing surface-bound SOM, how confident are the authors that they are removing the ligand by this protocol in their experiments? Could the presence of intracellular SSTR2, interpreted as slow recycling, be simply due to small
amounts of SOM still being there?

2) Establishing the feedback loop is important because this is mentioned several times in the manuscript and seems to be a major conclusion. The part that is still missing in the feedback loop is whether SSTR2 inhibits CRF. The authors responded that "it is already well established that activation of surface SSTR2 inhibits signalling downstream of the releasing factor receptors (including GHRH and CRF receptors)", but in AtT20 cells from my read this is based on one old paper that measured ACTH release (Litvin et al., 1986). SSTR2 inhibition of ACTH release seems to be complicated and can involve SSTR5. Can the authors refer to papers that conclusively show that SSTR2 inhibits CRF release or CRFR signaling, or perform a simple experiment to test this?

3) The biochemical fractionation experiments are a great addition to the study, but for making the point that Syntaxin 6 is not in the TGN, the best comparison would be between Syntaxin 6 and an established TGN marker (TGN38).

4) The authors need to check for typos and correct references, e.g, "Wood, Park, and Brown, 1991" is referenced in two different ways, and "Bowman, S. L., et al. 2015" is not listed in the reference list.

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

The authors have address all of my concerns.
October 4, 2019

Dr. Mark von Zastrow, Monitoring Editor
Dr. Melina Casadio, Senior Scientific Editor
Journal of Cell Biology

Dear Drs. von Zastrow and Casadio:

Thank you for your review of our revised manuscript "Regulated resurfacing of a somatostatin receptor storage compartment fine-tunes pituitary secretion". We were happy to learn that one of the two remaining reviewers found the revisions acceptable. We are grateful that you and your colleagues discussed the remaining points of reviewer 1 in detail and that you have clearly indicated the important issues that remain. As outlined in detail below, we have now addressed these remaining issues through the addition of new data.

With best regards,

Peter McPherson

Comment 1) The primary remaining point of concern is Rev#1’s point #1, the relationship of SSTR2 compartment that you compare to the previously described Glut4 compartment. I understand that this comparison may not be physiologically relevant in this cell type but do think it is reasonable, from a membrane traffic point-of-view, to determine if SSTR2 is in the same compartment or a different compartment relative to (expressed) Glut4. We do not feel that the experimental outcome would affect the editorial decision as we believe that the result is interesting either way, but addressing with data is important to establish the similarity or lack thereof relative to Glut4.

Response to comment 1) Our data indicate that the SSTR2 compartment in pituitary cells is functionally analogous to the Glut4 compartment in adipocytes or muscle. Following from the comment of the reviewer, we transfected AtT20 cells with Glu4 and compared its localization to endogenous SSTR2 (revised supplemental figure 8). Interestingly, there is very good co-localization, indicating that in this Glut4 ectopic environment, the transporter localizes to the same compartment as SSTR2, further supporting the functional analogy between Glut4 storage vesicles and the Glut4-like storage vesicles containing SSTR2.

Comment 2) A second remaining point of concern is Rev#1’s point #3, verifying fractionation behavior with TGN38 as a more widely recognized TGN marker, and also a marker used by prior work from Csaba et al (2007) to claim TGN. This is not dispensable for publication, but can you provide data on this, either from sucrose gradients or immuno-isolation?

Response to comment 2) TGN38, the mouse homologue of human TGN46, was added in the sucrose fractionation experiment (revised supplemental figure 5). TGN38 is enriched in overlapping fractions with PIST. TGN46 is used in the immuno-isolation experiment (figure 5), which is performed on human HEK-293 cells.

Comment 3) We think that experiments requested to show functional effects on hormone release are not necessary, but Rev#1 is correct about SSTR5 also being expressed in AtT-20. So I suggest you mention that both SSTR2 and SSTR5 can inhibit L-type calcium channels
necessary for regulated hormone secretion in AtT-20 cells (e.g., DOI 10.1016/0306-4522(95)00510-2).

Response to comment 3) This is mentioned on page 11 of the revised manuscript.

Comment 4) In addition, I request that you make one wording change in the last paragraph on p4: “Here we find that SSTR2 recycles from ...” to “Here we verify that SSTR2 recycles from ...” The reason is that I think this is a main conclusion previously claimed in the Csaba et al study cited in the preceding sentence.

Response to comment 4) We have made the change.

Comment 5) Our general policy is that papers are considered through only one revision cycle; however, given that the suggested changes are relatively minor, we are open to one additional short round of revision. Please note that we will expect to make a final decision without additional reviewer input upon resubmission.

Response to comment 5) Thank you
October 15, 2019

RE: JCB Manuscript #201904054RR

Dr. Peter S McPherson
McGill University
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Canada

Dear Dr. McPherson,

Thank you for submitting your revised manuscript entitled "Regulated resurfacing of a somatostatin receptor storage compartment fine-tunes pituitary secretion". Thank you for your patience as we were assessing the new data and changes. We think that you have adequately addressed the concerns that we prioritized and thank you for strengthening the work through these additional revisions. We would be happy to publish your very nice study in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

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

1) eTOC summary: A 40-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.
   - Please include an eTOC statement on the title page of the final revision; it should start with "First author name(s) et al..." to match our preferred style.

2) JCB Articles are limited to 10 main and 5 supplementary figures. Each figure can span up to one entire page as long as all panels fit on the page. Given the space in the main figures, could you please try to rearrange the data to meet this limit? It would seem that there is room to add panels to many of the main figures, hence reducing the count of supplemental items.

3) Figure formatting:
   - Scale bars must be present on all microscopy images, including inset magnifications. - Please add scale bars to 4BCDE (boxed magnifications), 6BC, 8A (magnifications), S6A, S8 magnifications
   - Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please add molecular weight with unit labels on the following panels: figure 5 blots, please also include unit labels on the top blot, 8B, S5 (unit labels)

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends.
   Please indicate n/sample size/how many experiments the data are representative of: 6BCEF, 10B,
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 in the text for readers who may not have access to referenced manuscripts.

- 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 reconstructions, surface or volume rendering, gamma adjustments, etc.).

6) A summary paragraph of all supplemental material should appear at the end of the Materials and methods section.
   - Please include ~1 brief descriptive sentence per supplemental item.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

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

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).

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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.

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

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

Sincerely,

Mark von Zastrow, MD, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

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