Small disulfide loops in peptide hormones mediate self-aggregation and secretory granule sorting

Jennifer Reck, Nicole Beuret, Erhan Demirci, Cristina Prescianotto-Baschong, and Martin Spiess

Corresponding author(s): Martin Spiess, University of Basel

Review Timeline:

Submission Date:	2021-10-27
Editorial Decision:	2021-10-27
Revision Received:	2021-12-13
Editorial Decision:	2022-01-11
Revision Received:	2022-01-12
Accepted:	2022-01-14

Scientific Editor: Eric Sawey, PhD

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Please note that the manuscript was reviewed at *Review Commons* and these reports were taken into account in the decision-making process at *Life Science Alliance.*

October 27, 2021

Re: Life Science Alliance manuscript #LSA-2021-01279

Prof. Martin Spiess
University of Basel
Biozentrum
Spitalstrasse 41
Basel, CH CH-4056
Switzerland

Dear Dr. Spiess,

Thank you for submitting your manuscript entitled "Small disulfide loops in peptide hormones mediate self-aggregation and secretory granule sorting" to Life Science Alliance. We invite you to re-submit the manuscript, revised according to your Revision Plan.

To upload the revised version of your manuscript, please log in to your account: https://lsa.msubmit.net/cgi-bin/main.plex

You will be guided to complete the submission of your revised manuscript and to fill in all necessary information. Please get in touch in case you do not know or remember your login name.

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

The typical timeframe for revisions is three months. Please note that papers are generally considered through only one revision cycle, so strong support from the referees on the revised version is needed for acceptance.

When submitting the revision, please include a letter addressing the Reviewers' comments point by point.

Thank you for this interesting contribution to Life Science Alliance. We are looking forward to receiving your revised manuscript.

Sincerely,

Eric Sawey, PhD
Executive Editor
Life Science Alliance
http://www.lsajournal.org

A. THESE ITEMS ARE REQUIRED FOR REVISIONS

-- A letter addressing the reviewers' comments point by point.

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

-- High-resolution figure, supplementary figure and video files uploaded as individual files: See our detailed guidelines for preparing your production-ready images, https://www.life-science-alliance.org/authors

-- Summary blurb (enter in submission system): A short text summarizing in a single sentence the study (max. 200 characters including spaces). This text is used in conjunction with the titles of papers, hence should be informative and complementary to the title and running title. It should describe the context and significance of the findings for a general readership; it should be written in the present tense and refer to the work in the third person. Author names should not be mentioned.

-- By submitting a revision, you attest that you are aware of our payment policies found here: https://www.life-science-alliance.org/copyright-license-fee

B. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://www.life-science-alliance.org/authors

We encourage our authors to provide original source data, particularly uncropped/-processed electrophoretic blots and spreadsheets for the main figures of the manuscript. If you would like to add source data, we would welcome one PDF/Excel-file per figure for this information. These files will be linked online as supplementary "Source Data" files.

IMPORTANT: It is Life Science Alliance policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Life Science Alliance manuscript #LSA-2021-01279

Point-by-point response

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

Apart from the default constitutive pathway for protein secretion some specialized cells (e.g., neuroendocrine cells, exocrine cells, peptidergic neurons and mast cells) exhibit additional regulated secretory pathway, where peptide hormones are stored as highly concentrated ordered manner inside electron opaque "dense core" of secretory granule for long duration until secretagogue mediated burst release. Although the general sorting receptor for packaging hormones in secretory granules is not yet identified, self-aggregation in the trans-Golgi network is a common shared property of peptide hormones and is a well-accepted potential sorting mechanism. Here the authors have hypothesized that cysteine containing small disulphide loop (CC loop), which is abundant in several hormone precursors, acts as aggregation mediator in TGN for sorting into secretory granule. They have tested the aggregation propensity of a misfolded reporter protein, NPΔ, in ER by attaching the CC loop segment of different hormones which promoted the pathological aggregation in endoplasmic reticulum (ER) of mutant provasopressin in the case of diabetes insipidus. Immunofluorescence and immunogold electron microscopy revealed accumulation of aggregates in the ER when CC loop of different hormonal origin fused NPΔ was transiently transfected in COS-1 fibroblasts and Neuro-2a neuroblastoma cells. The authors have also shown small disulphide loop mediated functional aggregation in TGN can sort a constitutively secreted protein, α1-protease inhibitor, into the secretory granule. The rerouting capacity of CC loop was tested in stably expressed AtT-20 cell line by confirming their localization with CgA-positive secretory granule as well as by studying BaCl2 mediated stimulated secretion and by testing secretory granule specific lubrol insolubility.

Major comments:

The study is highly impressive, and the results fully support the CC loop mediated hormone sorting hypothesis. However, it would be nice if the authors characterize the nature of the CC-loop mediated aggregates as hormones are reported to be stored inside secretory granules as functional amyloid (Maji et al., 2009). The mechanistic reason behind the small disulfide loop mediated aggregation was not explained in the paper. Authors may propose the probable molecular reasons behind CC loop mediated aggregation to completely justify their hypothesis.
The starting point for our analysis was the study by Maji et al. (2009) showing that granule cargo including the CC loop-containing vasopressin, oxytocin, somatostatin, and prolactin form amyloids in vitro and that granules are amyloids. Our own studies Birk et al. (2009) and Beuret et al. (2017) found fibrillar aggregation of mutant provasopressin in vitro and CCv-NPΔmyc in the ER of expressing cells. To go further, we focused in the present study on the ability of CC loops in general to aggregate in the ER and to sort by aggregation into secretory granules in vivo, rather than on the amyloid(-like) character again.

Although the hypothesis and the experimental results are highly impressive, the authors may consider adding the following experiments.

The authors replaced CC-loop by the proline/glycine repeat sequence (Pro1) as a negative control which was previously reported to abolish aggregation as well. However, the authors may completely delete the small loop forming segment, CCv, and may check the status of His-tagged fused neurophysin II (NPΔ) segment as an additional negative control.
As suggested, we used the NPΔ construct completely lacking any N-terminal extension as a further negative control. As expected, very similar levels of aggregation were measured as for Pro1-NPΔ. We have included this additional control construct in Figure 1 and 2 and we changed the text accordingly.

To find the ultrastructure authors have done immunogold assay with anti-His antibody which indicated different CC loop mediated ER aggregation. Since the amyloid-like fibril nature of pro-vasopressin mutant mediated ER aggregates was previously reported (Beuret et al., 2017), authors must check the nature of the CC loop mediated ER aggregates with amyloid specific antibody.
We have previously tested staining of ER aggregates of diabetes insipidus mutants of provasopressin with the anti-amyloid antibodies and Thioflavin – without success. Motivated by the reviewers' proposal, we again attempted staining with anti-amyloid OC antibodies and with Thioflavin, this time on CC-NPΔ ER aggregates. No signal was obtained with either method. Our attempts to stain ER aggregates might have been unsuccessful because the CC loops do not produce typical amyloids, since the ring closure prevents a perfect β conformation, or because disulfide crosslinking of the unfolded NPΔ reporter (Birk et al., 2009) hinders antibody access.

Since hormones are known to form reversible functional amyloid during their storage inside secretory granule, authors may consider characterizing the nature of the aggregates formed by CC loop fused constitutive protein in AtT-20 cell line by

immunostaining, immunoprecipitation and dot blot assay using amyloid specific antibody.

Like for ER aggregates, using anti-amyloid OC antibodies or Thioflavin, no signal could be detected in endogenous secretory granules of Neuro-2a and AtT20 cells with or without expression of CC-A1Pimyc. Maji et al. (2009) is the only study we know where Thioflavin stained granules in fluorescence microscopy. Here, sections of pituitary tissue were analyzed. It seems likely that the density and amounts of granules are much higher than in cultured AtT20 or Neuro-2a cells. We are not aware of studies where staining of granules by anti-amyloid antibodies in fixed cells was shown by immunofluorescence microscopy.

Minor comments:

In the quantification study (Figure 2C) CCc and CCr showed almost similar ER aggregates (around 40%). But authors have commented that all constructs except CCc produce statistically significant increases in cells compared to background. Authors must clarify the statement.

CCc also increased, but in a statistically not significant manner (p = 0.08). We have changed the sentence to: "It confirmed the ability of all constructs to produce an increase of cells with aggregates above background in COS-1 cells (Figure 2C), although not statistically significant for CCc (p = 0.08)."

In lubrol insolubility assay, the otherwise constitutively secreted protein A1Pimyc (negative control) showed 23% insolubility. The authors explained the observation by commenting about trapping of the protein inside granule aggregate. But CCv and CCa fused proteins showed a very slight increase (around 30%). Only CCc construct showed more than 40% insolubility. If the trapping of constitutive protein may result in 23% insolubility, all the insolubility data except CCc is not satisfactory to claim as secretory granular content of aggregated protein. The authors must explain that.

Lubrol insolubility is an empirical assay with high specificity for Golgi/post-Golgi forms, but with a relatively high background that we suggest to be due to trapping. Interpretation is based on statistical analysis of several independent experiments. It supports the conclusion of the other assays from an independent angle.

The authors have satisfactorily referenced prior studies in the field. However, authors may consider adding the following papers as they are directly connected with the hypothesis. The sorting of POMC hormone into secretory granules by disulphide loop was previously studied. (Cool et al.,1995). The N-terminal loop segment was also previously used to reroute a constitutive protein chloramphenicol acetyltransferase (Tam and Peng, 1993). S K. Maji and his coworker had previously shown that disulphide bond maintains native reversible functional amyloid structure relevant to hormone storage inside secretory granule whereas disulphide bond disruption led to rapid irreversible amyloid aggregation using cyclic somatostatin as model peptide. (Anoop et al., 2014).

Thank you for pointing out POMC, where the 13-amino acid CC loop is within a 23-amino acid disulfide loop. The recent AlphaFold structure prediction shows the inner loop to be exposed. We have now included POMC in Table I and refer to the supporting studies by Cool et al. (1995) and Tam et al. (1993) in the Introduction.
The study by Anoop et al., 2014, is already discussed in the Discussion.

Authors must check grammar and may reconstruct a few sentences where sentence construction seems complicated.
We went through the text and changed some complicated sentences to improve readability.

Reviewer #1 (Significance (Required)):

This manuscript has a significant contribution to enrich academia with fundamental research knowledge of hormone sorting mechanisms. Although constitutive and regulated secretory pathways are known for long times, the exact sorting mechanism is not yet elucidated. There is no common receptor identified yet for recruiting regulated secretary proteins inside the secretory granules.

Aggregation in the TGN is a well-accepted mechanism for sorting. However, the triggering factor for aggregation is not yet known. This study has shed light on a novel hypothesis, which has considered intramolecular disulfide bond mediated small CC loop in hormone may act as aggregation mediator. Since many regulated secretory proteins contain the short disulphide loop, the hypothesis proposed in the manuscript is interesting.

It has been confirmed that TGN is the last compartment which is common to both regulated and constitutive pathways (Kelly, 1985). There is no sorting mechanism required for the constitutive one as this is the default mechanism, whereas a regulated secretory pathway requires a specific sorting mechanism to be efficiently packaged in the secretory granules. There are two popular hypotheses about protein sorting in regulated secretory pathways. They are "sorting for entry" and "sorting for retention" (Blázquez and Kathleen, 2000). In "sorting for entry" hormones destined to go to the regulated secretory pathway start to form aggregates in the TGN specific environment excluding other proteins destined to go to the constitutive pathway. Arvan and Castle proposed the second mechanism as some hormones, like proinsulin, are initially packaged with lysosomal enzymes in immature secretory granules (ISG) (Arvan and Castle, 1998). But with time they start to aggregate and lysosomal

enzymes are removed from ISG by small constitutive-like vesicles. Although, in both the mechanisms aggregation is an essential sorting criterion the molecular events that lead to aggregation is not yet elucidated. TGN specific environmental conditions including pH (around 6.5), divalent metal ions (Zn2+, Cu2+), Glycosaminoglycans (GAGs) have potential to trigger aggregation (Dannies, Priscilla S, 2012). Though each hormone has aggregation prone regions in the amino acid sequence, there is no common amino acid sequence responsible for aggregation. The authors in this manuscript, have pointed out an interesting observation that many hormones contain small disulfide loops which are exposed due to their presence in N or C terminal or close to the processing site. Based on their observation, they hypothesized CC loop may act as aggregation driver for hormone sorting. In-cell study with CC construct from different hormones successfully rerouted a constitutively secretory protein into the regulated pathway which supported their novel hypothesis.

However, the hypothesis raises some questions to be answered regarding the molecular mechanism of CC loop mediated aggregation. Why does CC-loop promote aggregation? Does the amino acid sequence, size of the loop play a role in aggregation? The granular structure shown in the manuscript from different CC loops has different size and shape (Figure 2 and 3). What is the reason for the structural heterogeneity of the CC loop mediated dense core? Since authors have shown CC loop mediated aggregation both in functional as well as in diseased aggregation, a very important aspect to address would be the structure-function relationship of the aggregates. Since authors have rightly pointed out that not all hormones or prohormones contain CC loop, another curious question would be about the sorting mechanism of those without CC loop. The best part of the study is that it has tried to explain the well-established aggregation mediated sorting mechanism from a new perspective, which raises room for many questions to be addressed by further research.

From this study, the audience will get to know about the role of small disulphide loop in functional and diseased associated protein/peptide aggregation. The audience will also get an idea about the sorting mechanism in the regulated secretory pathway from the study. According to my expertise and knowledge where I do protein aggregation related to human diseases and hormone storage, I see this manuscript is a fantastic addition to understand the secretory granules biogenesis of hormones with storage and subsequent release.

Reference:
Maji, Samir K., et al. "Functional amyloids as natural storage of peptide hormones in pituitary secretory granules." Science 325.5938 (2009): 328-332.
Beuret, Nicole, et al. "Amyloid-like aggregation of provasopressin in diabetes insipidus and secretory granule sorting." BMC biology 15.1 (2017): 1-14.
Cool, David R., et al. "Identification of the sorting signal motif within pro-opiomelanocortin for the regulated secretory pathway." Journal of Biological Chemistry 270.15 (1995): 8723-8729.
Tam, W. W., K. I. Andreasson, and Y. Peng Loh. "The amino-terminal sequence of pro-opiomelanocortin directs intracellular targeting to the regulated secretory pathway." European journal of cell biology 62.2 (1993): 294-306.

Anoop, Arunagiri, et al. "Elucidating the Role of Disulfide Bond on Amyloid Formation and Fibril Reversibility of Somatostatin-14: RELEVANCE TO ITS STORAGE AND SECRETION." Journal of Biological Chemistry 289.24 (2014): 16884-16903.
Kelly, Regis B. "Pathways of protein secretion in eukaryotes." Science 230.4721 (1985): 25-32.
Blázquez, Mercedes, and Kathleen I. Shennan. "Basic mechanisms of secretion: sorting into the regulated secretory pathway." Biochemistry and Cell Biology 78.3 (2000): 181-191.
Arvan, Peter, and David Castle. "Sorting and storage during secretory granule biogenesis: looking backward and looking forward." Biochemical Journal 332.3 (1998): 593-610.
Dannies, Priscilla S. "Prolactin and growth hormone aggregates in secretory granules: the need to understand the structure of the aggregate." Endocrine reviews 33.2 (2012): 254-270.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Summary:

This manuscript by Reck and colleagues aim at determining the importance of short disulfide loops for the correct sorting to, and release from, secretory granules. They utilize hybrid secretory proteins where sequences encoding disulfide loop from different hormones are cloned in frame with the same secretory peptide, and assess how the presence of the disulfide loop affect the ability of the protein to aggregate in the ER and to get sorted for secretion. By immunofluorescence analysis they show that the presence of a disulfide loop increases the ability of the peptide hormone to form aggregates in the ER, and these observations are confirmed by immunogold-EM. Importantly, aggregate formation is seen both in professional secretory (N-2a) and non-secretory (COS-1) cells. Using immunofluorescence and quantitative immuoblotting, they also show that the ability to aggregate the secretory proteins coincide with increased localization to secretory granules and in increased release from cells in response to stimuli.

The results from this study are interesting and suggest that small disulfide loops may be an important part of the cargo sorting mechanism in secretory cells, and perhaps also a cause of sorting defects in certain diseases. The study is overall well conducted and worthy of publication after revision.

Major comments:

1) It is unclear to me what the relationship between the CC-loop and amyloid is. They are not involved in the formation of fibrils and amyloid, yet the authors conclude that they support the amyloid hypothesis of granule biogenesis. This must be clarified.

In this study, we do not directly provide evidence for the amyloid (or rather amyloid-like) character of aggregation and therefore the last sentence of the Discussion is not strictly correct. Our findings are consistent with the amyloid hypothesis, but they do not provide additional evidence for the amyloid nature of granules. We have now deleted the last statement of the discussion.

Maji et al. (2009) and Anoop et al. (2014) already showed that the CC loop hormones vasopressin, oxytocin, and somatostatin-14 form fibrillar aggregates in vitro, and that granules have amyloid characteristics. Our own lab showed that folding-deficient mutant forms of provasopressin formed fibrillar aggregates in vitro (Birk et al., 2009) and in the ER of expressing cells (Birk et al., 2009; Beuret et al., 2011) and that vasopressin (CCv) is responsible for this and for granule sorting (Beuret et al., 2011). In our current study unfortunately, the ER aggregates of CC-NPΔmyc (including CCv) in Neuro-2a cells were too compact to reveal fibrillarity. However, secretory granules, which contain functional amyloids, similarly do not have a fibrillar appearance. While in this study we do not provide direct evidence that CC loops produce amyloids, evidence has previously been published.

2) What is the actual function of the CC-loops? The authors show that the loops promote aggregation of cargo proteins, yet the mechanism behind this is unclear. For example, would the proteins used in this study be able to aggregate in vitro (i.e. the CC-loop enable aggregation) or do they require some co-factor/chaperone? It would also be good if the authors could clarify or explain why some CC-loops cause aggregation and others not.

Maji et al. (2009) showed for 3 different CC loops (vasopressin, oxytocin and somatostatin-14) that they aggregate in an amyloid-like manner in vitro in purified form in the absence of chaperones or other protein cofactors. Anoop et al. (2014) analyzed in vitro amyloid formation of somatostatin-14 with and without disulfide bond in more detail. The proposed function is aggregation of the hormone into secretory granules as functional amyloids, which is supported by the finding that secretory granules are positive for amyloids.

In the present study, we tested a variety of CC loops for aggregation in cells rather than in vitro. Many proteins and peptides have been shown to be able to form amyloids in vitro. The hallmark of pathological or functional amyloids is that they are still able to aggregate in living cells despite the presence of chaperones, whose function is to generally prevent aggregation.

We found all CC loops to have the ability to mediate ER aggregation and granule sorting, although to different extents. The differences are likely due to their intrinsic potency and/or the way they are presented by the reporter proteins, since we used the same rather short linkers.

3) The MS data in table 2 is very confusing, since half of the data points are missing. It is also not clear what the numbers in the table represent and if they are from a single experiment or multiple. As it is presented now, and as I interpret it, these results do not give support to the conclusion that CC loops form disulfide bonds. Since this is an important conclusion from the paper, these experiments need to be clarified, repeated or a different experimental approach used.

To be clearer, we have changed the way to present the data and included the results of three (in one case two) separate experiments in a new Figure 4 and changed the text accordingly.

4) As the authors state, it is well-known that the concentration of proteins in the ER will influence the ability to aggregate. In figure 1 and 2, the authors use transient overexpression to assess the ability of different CC-loops to induce aggregation in the ER. How were these results normalized to expression levels of the proteins? In later experiments the authors instead use stable cell lines expressing similar amounts of the different proteins. However, in these cells there is no obvious aggregation in the ER (see figure 4). It therefore becomes unclear what the role of ER aggregation for sorting to granules is.

The ER aggregation experiments were not normalized for expression levels. Plasmids were identical except for the short CC loop segments and produced similar transfection efficiencies. Stable cell lines with useful expression levels of CC-NPΔ could not be obtained, most likely because expression of mutant proteins inhibits growth.

To analyze granule sorting, we expressed CC fusion proteins with rapidly folding A1Pi as a reporter that does not accumulate in the ER. Stable cell lines were important to select clones with moderate and very similar expression levels.

5) What is the basal secretion of the different proteins, i.e. how much goes through the constitutive secretory pathway and how much goes through the regulated secretory pathway? The authors should show the resting secretion (before BaCl2 addition) for all conditions tested instead of just the change in relation to control (i.e. the way data is presented now it is not possible to tell whether BaCl2 stimulation actually cause an increased release of the peptides).

The experiment is done by comparing resting secretion (– lanes) with BaCl2 stimulated secretion (+ lanes) in Fig. 6A and C. Stimulated secretion is calculated as a ratio of resting secretion / stimulated secretion (after normalization for cell number and

supernatant loading).

6) Lastly, the importance of CC-loops for the sorting of native peptides is unclear. The authors should test the importance of these loops for aggregation, sorting and secretion of a non-hybrid hormone with naturally occurring CC-loops (and a mutated version lacking the loop). This is important, since it is so far only shown that loops can affect the secretion of non-biologically relevant hybrid hormones.
In our previous study Beuret et al. (2017), we analyzed the segments contributing to ER aggregation of folding-incompetent mutant provasopressins and to granule sorting for folding-competent mutants of provasopressins by self-aggregation at the TGN. We found separate protein segments – vasopressin (=CCv) and the glycopeptide – to contribute to aggregation in both localizations. Our study is a follow up on the finding for vasopressin, expanding to other CC loops found in peptide hormones. Our results show that CC loops in general have the ability to aggregate and contribute to granule sorting.
As exemplified by provasopressin, the CC loop may not be the only contributor. Preliminary experiments suggest the same for growth hormone. The detailed analysis of the aggregating sequences in one or more prohormone is clearly beyond the scope of our study.

Minor comments:

1) Stated that the 2x CC-loop constructs showed a positive effect in the cases of CCv and CCr, but this is not evaluated statistically.
Unpaired Student t-test results in p values of 0.065 for 2xCCv vs. CCv and 0.003 for 2xCCr vs. CCr. We modified the text accordingly.

2) Explain the abbreviation POMC
We have added the full name to the text.

3) Figure 6D. Paired Student's t-test is not appropriate for determining significance when data is not paired (unpaired t-tests used throughout the rest of the paper).
Only in the lubrol insolubility experiment did we find considerable shifts between experiments, which is why we used the paired t-test. However, using the unparied t-test produces similar results. We therefore followed the reviewer's suggestion and changed the figure to show the unpaired test as in all other figures.

Reviewer #2 (Significance (Required)):

The work in this paper builds on previous work from the same group and reinforces the notion that peptide aggregation is an important part of the sorting process that controls efficient delivery of certain proteins to nascent secretory granules, and suggest that short loops formed by disulfide bridges between closely apposed cysteine residues may be part of this sorting mechanism. The paper is of general cell biological interest, but perhaps of special interest to researches working on professional secretory cells and mechanisms of secretory protein sorting and secretion. My own research focuses on stimulus-secretion coupling pathways in secretory cells and we primarily use live cell imaging approaches to visualize different steps of secretory granule biogenesis and release.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

Summary:

Since the small disulfide loop of the nonapeptide vasopressin has been previously demonstrated to play a role the self-aggregation and secretory granule targeting of vasopressin precursor (Beuret et al., 2017), and as several other peptide hormones contain small disulfide loops, Reck and colleagues investigate in this study the requirement of small disulfide loops coming from four additional peptide hormones for the self-aggregation and secretory granule targeting of their precursors. Then, they studied the aggregation role of small disulfide loops in the ER and the TGN of two cell lines, COS1 and Neuro-2a. Using confocal and TEM, an aggregation has indeed been observed, although to different extents depending on the cell line. When fused to a constitutively secreted reporter protein, these disulfide loops induced their sorting into secretory granules, increased the stimulated secretion and Lubrol insolubility in endocrine AtT20 cells. All these results led the authors to hypothesize that small disulfide loops may act as a general device for peptide hormone aggregation and sorting, and therefore for secretory granule biogenesis.

Major comments:

The authors demonstrated the ability of small disulfide loops of peptide hormones to induce peptide precursor aggregation in ER using confocal microscopy, in COS1 and Neuro-2a cell lines, with a higher extent in COS1 cells. The authors have to moderate this conclusion and to include in their interpretation that distinct results may be due to the distinct secretory phenotype of these two cell lines: COS1 are epithelial cells, i.e. with a unique constitutive secretory pathway, while Neuro-2a as well as AtT20 cells also possess a regulated secretory pathway. Thus, the differences could be explained by the distinct molecular mechanisms involved in the formation of constitutive vesicles or secretory granules, and therefore aggregation and/or sorting processes could be distinct in the two cell types. We can also suggest to remove COS1-related results, to avoid hasty conclusions.

As suggested, we have amended the text to explain why we used COS-1 and Neuro-2a cells:
"These constructs were transiently transfected into COS-1 fibroblasts and Neuro-2a neuroblastoma cells. COS-1 cells are large and easy to analyze for aggregations by immunofluorescence microscopy, while Neuro-2a cells, although smaller and more difficult to analyze for aggregates by light microscopy, produced nice fibrillar aggregates detectable by EM for provasopressin mutants (Birk et al, 2009). Originally, Neuro-2a cells were used to study the behaviour of pathogenic diabetes insipidus mutants of provasopressin in a neuroendocrine cell type to reflect the situation in vasopressinergic neurons. For the analysis of ER aggregation, it does not matter that Neuro-2a but not COS-1 cells have a regulated secretory pathway, since the NPΔ constructs cannot reach the TGN, where regulated and constitutive pathways separate."

The data and the methods can be reproduced and the experiments are adequately replicated, using timely statistical analysis.

Minor comments:

- Figure 3: to complete TEM study, the concomitant use of an ER specific antibody would definitely demonstrate that small disulfide loop-containing aggregates are linked to ER compartment.
In our previous study Birk et al. (2009), we performed double-immunogold staining for provasopressin mutants and calreticulin to confirm aggregation in the ER. This anti-calreticulin antibody is unfortunately not commercially available anymore and other antibodies we tested were not suitable for immuno-EM. Instead, we colocalized PDI with CC-NPΔ constructs for immunofluorescence microscopy. Colocalization is so extensive that we believe EM confirmation to be unnecessary.

- Along abstract, introduction and discussion sections, the authors should avoid to conclude on the role of small disulfide loops on secretory granule biogenesis, but rather limit their conclusion on prohormone aggregation and targeting. Indeed, the present study did not highlight any direct molecular / physical link between disulfide loops and TGN membrane to drive secretory granule formation.
Granule biogenesis involves a number of processes including interaction of cargo components with the membrane and of the actomyosin complex with the forming buds, but also selfaggregation of cargo as functional amyloids. However, not to overstate the function of CC loops, we have reworded our statements in the Abstract to avoid the term "granule biogenesis".

Reviewer #3 (Significance (Required)):

- This study highlights small disulfide loops as novel signals for self-aggregating and secretory granule sorting of prohormone precursors in cells with a regulated secretory pathway. These results help to understand the molecular mechanism driving peptide hormone secretion, a physiological process which is crucial for interorgan communication and functional synchronization.
Moreover, their previous study revealed that vasopressin small disulfide loop is involved in toxic unfolded mutant aggregation in the ER (Beuret et al., 2017), which highlights the clinical potential of the work.
- Audience that might be interested in and influenced by the reported findings: cell biologists interested in cell trafficking, peptide hormone secretion
- My field of expertise: secretory granule biogenesis, hormone sorting, secretory cells, neurosecretion.

January 11, 2022

RE: Life Science Alliance Manuscript #LSA-2021-01279R

Prof. Martin Spiess
University of Basel
Biozentrum
Spitalstrasse 41
Basel, CH CH-4056
Switzerland

Dear Dr. Spiess,

Thank you for submitting your revised manuscript entitled "Small disulfide loops in peptide hormones mediate self-aggregation and secretory granule sorting". We would be happy to publish your paper in Life Science Alliance pending final revisions necessary to meet our formatting guidelines.

Along with points mentioned below, please tend to the following:
-Table should be included at the bottom of the main manuscript file or be sent as a separate file
-Table should be numbered with Arabic numerals (1, 2, 3, 4)
-there is a callout for Figure 4C in the manuscript text, but there is no such panel. Please revise
-please add callouts for Figures S1A-B and S2C to your main manuscript text

If you are planning a press release on your work, please inform us immediately to allow informing our production team and scheduling a release date.

LSA now encourages authors to provide a 30-60 second video where the study is briefly explained. We will use these videos on social media to promote the published paper and the presenting author (for examples, see https://twitter.com/LSAjournal/timelines/1437405065917124608). Corresponding or first-authors are welcome to submit the video. Please submit only one video per manuscript. The video can be emailed to contact@life-science-alliance.org

To upload the final version of your manuscript, please log in to your account: https://lsa.msubmit.net/cgi-bin/main.plex
You will be guided to complete the submission of your revised manuscript and to fill in all necessary information. Please get in touch in case you do not know or remember your login name.

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

A. FINAL FILES:

These items are required for acceptance.

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

-- High-resolution figure, supplementary figure and video files uploaded as individual files: See our detailed guidelines for preparing your production-ready images, https://www.life-science-alliance.org/authors

-- Summary blurb (enter in submission system): A short text summarizing in a single sentence the study (max. 200 characters including spaces). This text is used in conjunction with the titles of papers, hence should be informative and complementary to the title. It should describe the context and significance of the findings for a general readership; it should be written in the present tense and refer to the work in the third person. Author names should not be mentioned.

B. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://www.life-science-alliance.org/authors

We encourage our authors to provide original source data, particularly uncropped/-processed electrophoretic blots and spreadsheets for the main figures of the manuscript. If you would like to add source data, we would welcome one PDF/Excel-file per figure for this information. These files will be linked online as supplementary "Source Data" files.

Submission of a paper that does not conform to Life Science Alliance guidelines will delay the acceptance of your manuscript.

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

The license to publish form must be signed before your manuscript can be sent to production. A link to the electronic license to publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements.

Reviews, decision letters, and point-by-point responses associated with peer-review at Life Science Alliance will be published online, alongside the manuscript. If you do want to opt out of having the reviewer reports and your point-by-point responses displayed, please let us know immediately.

Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days.

Thank you for this interesting contribution, we look forward to publishing your paper in Life Science Alliance.

Sincerely,

Eric Sawey, PhD
Executive Editor
Life Science Alliance
http://www.lsajournal.org

January 14, 2022

RE: Life Science Alliance Manuscript #LSA-2021-01279RR

Prof. Martin Spiess
University of Basel
Biozentrum
Spitalstrasse 41
Basel, CH CH-4056
Switzerland

Dear Dr. Spiess,

Thank you for submitting your Research Article entitled "Small disulfide loops in peptide hormones mediate self-aggregation and secretory granule sorting". It is a pleasure to let you know that your manuscript is now accepted for publication in Life Science Alliance. Congratulations on this interesting work.

The final published version of your manuscript will be deposited by us to PubMed Central upon online publication.

Your manuscript will now progress through copyediting and proofing. It is journal policy that authors provide original data upon request.

Reviews, decision letters, and point-by-point responses associated with peer-review at Life Science Alliance will be published online, alongside the manuscript. If you do want to opt out of having the reviewer reports and your point-by-point responses displayed, please let us know immediately.

IMPORTANT: If you will be unreachable at any time, please provide us with the email address of an alternate author. Failure to respond to routine queries may lead to unavoidable delays in publication.

Scheduling details will be available from our production department. You will receive proofs shortly before the publication date. Only essential corrections can be made at the proof stage so if there are any minor final changes you wish to make to the manuscript, please let the journal office know now.

DISTRIBUTION OF MATERIALS:
Authors are required to distribute freely any materials used in experiments published in Life Science Alliance. Authors are encouraged to deposit materials used in their studies to the appropriate repositories for distribution to researchers.

You can contact the journal office with any questions, contact@life-science-alliance.org

Again, congratulations on a very nice paper. I hope you found the review process to be constructive and are pleased with how the manuscript was handled editorially. We look forward to future exciting submissions from your lab.

Sincerely,

Eric Sawey, PhD
Executive Editor
Life Science Alliance
http://www.lsajournal.org