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Evolutionary Conservation of Complexins: From Choanoflagellates to Mice

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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.)

Editor: Barbara Pauly

1st Editorial Decision 09 April 2015

Thank you for the submission of your research manuscript to our editorial offices and your patience while we were conducting the peer review. We have now received the full set of reports on it.

As the reports are pasted below, I would prefer not to repeat the details of them here, but it becomes clear that while the reviewers agree in principle on the interest of the findings, they also all agree that, based on the limited analysis presented here, no strong conclusions about the evolution of complexins and the question which of their activities has evolved first can be drawn. The referees state that the second copy of Nematostella complexin (and the remaining mouse isoforms) need to be studied and that a much more extensive phylogenetic analysis, including many more species, has to be performed before any conclusions about the function and evolution of this class of proteins can be drawn. Ideally, the function of the Nematostella complexins should also be tested in Cnidarians themselves (referee 3).

Given the potential interest of your findings, I would like to give you the opportunity to address the reviewers concerns and submit a revised manuscript with the understanding that all concerns of the referees must be fully addressed and that acceptance of the manuscript would entail a second round of review.

I should also remind you that it is EMBO reports policy to allow a single round of revision only and that therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. I do realize the amount of work required to address the concerns of the reviewers in full, but feel that without a much deeper analysis, none of the referees will recommend publication of the study in EMBO reports.

I look forward to seeing a revised form of your manuscript when it is ready. Should you choose to submit your paper elsewhere, I would welcome a message to this effect.

REFEREE REPORTS:

Referee #1:

This study made good use of a simple eumetazoan system to test the early and conservative mechanism that controls neuontransmitter release by crossing organism analyses in mouse. There are four complexins in mouse (mCpx1-4, likely in rat as well) and two in jellyfish (nvCpx1 and nvCpx2). Two sets of expression experiments in double knockdown mouse neurons clearly show that the nvCpx1 can play conservative functional role, Ca2+-triggered exocytosis, in the mouse neurons but cannot rescue the unclamping of spontaneous exocytosis. The protein domains were also examined for their functional relevance. These observations led to a conclusion that the activating function of complexins is conservative across metazoans and, although no emphasis for an implication of divergent evolution for the unclamping functions, which is also interesting to understanding of the evolution of the neuronal functional system.

However, the incomplete experimental consideration dampened my enthusiasm: what are roles of nvCpx2 and mCpx3-4? Yet it was not clear in the composition whether or not the double knockdown is specific only for mCpx1-2. I am concerned with the significant similarity between the 4 mouse/rat complexin proteins that might impact the specificity of the silencing experiments. The lack of information from these uninvestigated copies lead to another possibility untested: the independent evolution of the observed exocytosis from interaction of all complexin copies in the rodents, an alternative to the authors' conclusion of conservative exocytosis in mCpx1. The nvCpx2 may be able to provide the necessary information for the experimental failure in rescuing the unclamping phenotypes.

It appears clear that the jigsaw puzzle cannot be solved by partial experiment; a complete treatment of all 2 x 4 copies of complexins in two types of divergent organisms would provide a hope for understanding the diverged functional systems while showing partial conservation. Thus, I cannot recommend this manuscript to EMBO R for publication.

Referee #2:

This looks like an interesting and valuable observation with implications on our understanding of how the complexity of the nervous systems evolved.

However, from my point of view of a molecular evolutionist, whilst Nematostella is adequately located at the tree of life with respect to this study (having neuron-like sensory cells, but not a CNS), it's impossible to derive evolutionary scenarios from comparison of two species. Lacking is a careful phylogenetioc analysis of complexins. Is Nematostella the most "ancient" species in which complexions appear? Perhaps complexin-like genes can be found in species that diverged earlier? WDo all species that reside in the tree of life between Nematostella and vertebrates have complexion genes. In short, a complexin tree to be established and compared to the tree of life. Although, this is a rather hefty task if done properly but is within the scope of a revision.

A systematic phylogenetic analysis will also reveal the relationships between the 2 Nematostella

paralogs. Are the paralogs overlapping in function, and differ only in expression? The authors state that that it complexin-1 is similar to mouse complexin-1, but what that other paralog does, resembles, and how they relate is unclear. If the Nematostella is reflecting the origins of complexion, did the duplication occurred at the onset and was retained since? Finally is is it possible that Nematostella complexin-2 has the clamping function missing in complexin-1?

Referee #3:

1. Does this manuscript report a single key finding?
YES. The manuscript reports that complexin-1 from the cnidarian Nematostella vectensis - similar to mouse complexin-1 - functions as an activator of neurotransmitter release in mouse neurons.
2. Is the reported work of significance (YES), or does it describe a confirmatory finding or one that has already been documented using other methods or in other organisms etc (NO)?
YES.
3. Is it of general interest to the molecular biology community?
YES. The data point towards an evolutionary conservation of complexin-1 function in neurotransmitter release.
4. Is the single major finding robustly documented using independent lines of experimental evidence (YES), or is it really just a preliminary report requiring significant further data to become convincing, and thus more suited to a longer¬format article (NO)?
YES.

In this manuscript, the authors describe that the genome of the cnidarian N.vectensis encodes for two Complexin genes, Complexin-1 and Complexin-2. The authors have used gene-synthesized constructs encoding Complexin-1 wildtype and several Complexin-1 mutants for plasmid construction and tested for the ability of N.vectensis Complexin-1 to rescue Complexin-1/2 function in mouse neurons. The authors show that in complexin-1/2 deficient mouse neurons, N.vectensis complexin-1 replaces mouse complexins in activating Ca2+ -triggered exocytosis, but is unable to clamp spontaneous exocytosis. The authors conclude that the activating function of complexins is conserved throughout animal evolution. In general, the article is a pleasant read and very well written. The electrophysiological recordings in mouse neurons are very convincing and clearly show that N.vectensis complexin-1 can rescue the Complexin-1/2 knockout phenotype. This is indeed a very interesting finding. On the other hand, all experiments and conclusions are based on expressing N.vectensis Complexin-1 in mouse neurons. No insights into the function of Complexin-1 in N.vectensis are provided. Thus, the major conclusions of the paper are somewhat indirect. While I understand that providing insights into the function of Complexin-1 in N.vectensis might be too much to be asked for, I think data on the function/activity of the second N.vectensis complexin (Complexin-2) are needed to draw some of the conclusion the authors have made.
Therefore, I have some major and minor comments and would strongly suggest addressing these points carefully.

Major points:
1) As mentioned above, the authors identified two Complexins in N.vectensis. It would be very important to study the function/activity of the N.vectensis Complexin-2 in mouse neurons to show that it also functions as an activator of neurotransmitter release and to rule out the possibility that N.vectensis Complexin-2 is able to clamp spontaneous exocytosis.
2) As the authors identified Complexins in cnidarians it would be very informative to expand the phylogenetic analysis and protein alignments of Complexins to other basal animals such as sponges, placozoans (animals without neurons) and ctenophores and discuss this analysis (protein domain conservation and amino acid conservation, alignments) in light of their findings. The authors actually have mentioned that Complexin(s) is (are) present in sponges in a previous publication (PMID: 23345244).
3) The strong tone of some parts of the paper (e.g., the title and some of the conclusions drawn) might be misleading for the reader. The authors should be cautious in stating "the core machinery of neurotransmitter release is shared by all animals throughout evolution". For example, how neurotransmitter release is controlled by Complexins in other basal animals (e.g. ctenophores or other cnidarians) is not known. In addition, the authors should be cautious with statements like "cnidarian exocytosis operates by fundamentally identical molecular mechanisms as mammals" as only one component of the exocytotic machinery has been tested so far (N.vectensis Complexin-1 in

this study) and all of the experiments have been performed in mouse neurons. Insights into the function of Complexin-1 in N.vectensis would answer the question regarding the evolutionary conservation of the molecular mechanism of neurotransmitter release more directly.

Minor points
4) The authors state that N.vectensis "develops only primitive neuron-like sensory cells" (Introduction, fourth paragraph). I think this is not entirely correct, N.vectensis develops sensory cells, but also many different neurons (please see for example PMID: 19170043)
5) In the Methods section information about the sequences of N.vectensis Complexin 1 and 2 (NCBI numbers?) and how alignments have been made (program?) would be useful.
6) Figure 1A would benefit from showing the domain architecture of N.vectensis Cpx1 and 2. In addition, the colour code in the alignments has to be explained and Nematostella is not spelled correctly above the alignment.

1st Revision - authors' response 10 July 2015

We thank the reviewers for their helpful critical comments. In the following, we will describe the changes instituted into the revised manuscript, provide our responses to the reviewers' questions, and describe the new data that were added. Based on the recommendation of the editor and in order to accommodate the additional data requested by the reviewers, we expanded the manuscript to a full paper. Despite its larger scope, however, we would prefer to keep the 'Results and Discussion' in a single section because that facilitates the flow of the paper. In the following, we cite the reviewers' comments in full in *italic* typeface, and list our response in **bold** typeface.

Referee #1:
This study made good use of a simple eumetazoan system to test the early and conservative mechanism that controls neuontransmitter release by crossing organism analyses in mouse. There are four complexins in mouse (mCpx1-4, likely in rat as well) and two in jellyfish (nvCpx1 and nvCpx2). Two sets of expression experiments in double knockdown mouse neurons clearly show that the nvCpx1 can play conservative functional role, Ca2+-triggered exocytosis, in the mouse neurons but cannot rescue the unclamping of spontaneous exocytosis. The protein domains were also examined for their functional relevance. These observations led to a conclusion that the activating function of complexins is conservative across metazoans and, although no emphasis for an implication of divergent evolution for the unclamping functions, which is also interesting to understanding of the evolution of the neuronal functional system.

However, the incomplete experimental consideration dampened my enthusiasm: what are roles of nvCpx2 and mCpx3-4? Yet it was not clear in the composition whether or not the double knockdown is specific only for mCpx1-2. I am concerned with the significant similarity between the 4 mouse/rat complexin proteins that might impact the specificity of the silencing experiments. The lack of information from these uninvestigated copies lead to another possibility untested: the independent evolution of the observed exocytosis from interaction of all complexin copies in the rodents, an alternative to the authors' conclusion of conservative exocytosis in mCpx1. The nvCpx2 may be able to provide the necessary information for the experimental failure in rescuing the unclamping phenotypes.

As authors, we have to confess that we are a bit confused by these comments. Our paper is not about complexin-3 and -4, and as referenced in the manuscript, hundreds of papers have been published on complexins, and scores on the double knockdown we use here as an approach which has been extensively described and validated. For example, see all the pioneering papers by the Brose lab on complexin-3 and -4. Surely the reviewer doesn't expect us to repeat all of these experiments for another paper? This is a pretty mature field, and a lot is known about the properties and expression (or lack thereof) of different rodent complexins. Moreover, we don't quite understand what the reviewer means with the statement that "*The lack of information from these uninvestigated copies lead to another possibility untested: the independent evolution of the observed exocytosis from interaction of all complexin copies in the rodents, an alternative to the authors' conclusion of conservative exocytosis in mCpx1.***" Apart from the fact that the other complexins are by no means uninvestigated, we just don't see what the reviewer**

is trying to say, for which we apologize. If he/she is suggesting the possibility that the rescue complexin does not act as a rescue but as an activator of some other site or locus, we would respond that this seems far-fetched and would contradict the results of many rescue papers with complexins performed in the past.

It appears clear that the jigsaw puzzle cannot be solved by partial experiment; a complete treatment of all 2 x 4 copies of complexins in two types of divergent organisms would provide a hope for understanding the diverged functional systems while showing partial conservation. Thus, I cannot recommend this manuscript to EMBO R for publication.

We apologize, but we again simply don't understand what the reviewer is trying to say –is it that because we don't study all complexins in the same time in the same paper, despite a huge literature on rodent complexins, he/she can't recommend our paper for publication in EMBO Reports?

Referee #2:
This looks like an interesting and valuable observation with implications on our understanding of how the complexity of the nervous systems evolved.

However, from my point of view of a molecular evolutionist, whilst Nematostella is adequately located at the tree of life with respect to this study (having neuron-like sensory cells, but not a CNS), it's impossible to derive evolutionary scenarios from comparison of two species. Lacking is a careful phylogenetioc analysis of complexins. Is Nematostella the most "ancient" species in which complexions appear? Perhaps complexin-like genes can be found in species that diverged earlier? WDo all species that reside in the tree of life between Nematostella and vertebrates have complexion genes. In short, a complexin tree to be established and compared to the tree of life. Although, this is a rather hefty task if done properly but is within the scope of a revision.

A systematic phylogenetic analysis will also reveal the relationships between the 2 Nematostella paralogs. Are the paralogs overlapping in function, and differ only in expression? The authors state that that it complexin-1 is similar to mouse complexin-1, but what that other paralog does, resembles, and how they relate is unclear. If the Nematostella is reflecting the origins of complexion, did the duplication occurred at the onset and was retained since? Finally is is it possible that Nematostella complexin-2 has the clamping function missing in complexin-1?

We have accepted the reviewer's advice, and performed two experiments that address her/his concerns. First, we have performed a complete phylogenetic analysis of complexin sequences, and recruited a bioinformatics expert for this purpose (Prof. Nick Grishin and his team, who are now co-authors on the paper). Second, we have analyzed Nematostella complexin-2.

The phylogenetic analysis of complexin sequences revealed that the reviewer is correct, and that complexins are found in evolutionarily even more ancient organisms than Nematostella (new Figs. 1 and 2). Indeed, we were somewhat shocked to discover that complexin sequences can even be detected in unicellular organisms that predate animal evolution. These data are now included in the revised paper. They actually have major implications for our thinking about complexins and about the evolution of the secretory machinery that we are eager to follow up in future.

The analysis of Nematostella complexin-2 showed that when introduced into mouse neurons, it rescues evoked release to a small extent lesser than Nematostella complexin-1, and actually causes additional uncampling instead of clamping spontaneous release (new Fig. 3). We hope this addresses the questions of this reviewer.

Referee #3:
1. Does this manuscript report a single key finding?

YES. The manuscript reports that complexin-1 from the cnidarian Nematostella vectensis - similar to mouse complexin-1 - functions as an activator of neurotransmitter release in mouse neurons.

2. Is the reported work of significance (YES), or does it describe a confirmatory finding or one that has already been documented using other methods or in other organisms etc (NO)?

YES.

3. Is it of general interest to the molecular biology community?

YES. The data point towards an evolutionary conservation of complexin-1 function in neurotransmitter release.

4. Is the single major finding robustly documented using independent lines of experimental evidence (YES), or is it really just a preliminary report requiring significant further data to become convincing, and thus more suited to a longerformat article (NO)?

YES.

In this manuscript, the authors describe that the genome of the cnidarian N.vectensis encodes for two Complexin genes, Complexin-1 and Complexin-2. The authors have used gene-synthesized constructs encoding Complexin-1 wildtype and several Complexin-1 mutants for plasmid construction and tested for the ability of N.vectensis Complexin-1 to rescue Complexin-1/2 function in mouse neurons. The authors show that in complexin-1/2 deficient mouse neurons, N.vectensis complexin-1 replaces mouse complexins in activating Ca2+ -triggered exocytosis, but is unable to clamp spontaneous exocytosis. The authors conclude that the activating function of complexins is conserved throughout animal evolution.

In general, the article is a pleasant read and very well written. The electrophysiological recordings in mouse neurons are very convincing and clearly show that N.vectensis complexin-1 can rescue the Complexin-1/2 knockout phenotype. This is indeed a very interesting finding. On the other hand, all experiments and conclusions are based on expressing N.vectensis Complexin-1 in mouse neurons. No insights into the function of Complexin-1 in N.vectensis are provided. Thus, the major conclusions of the paper are somewhat indirect. While I understand that providing insights into the function of Complexin-1 in N.vectensis might be too much to be asked for, I think data on the function/activity of the second N.vectensis complexin (Complexin-2) are needed to draw some of the conclusion the authors have made.

We very much appreciate the reviewer's enthusiasm – we also found it somewhat amazing how well a sea anemone complexin can work in a mouse neuron. We have now added functional data on the second N. vectensis complexin as suggested, and hope this will be satisfactory to the reviewer.

Therefore, I have some major and minor comments and would strongly suggest addressing these points carefully.

Major points:

1) As mentioned above, the authors identified two Complexins in N.vectensis. It would be very important to study the function/activity of the N.vectensis Complexin-2 in mouse neurons to show that it also functions as an activator of neurotransmitter release and to rule out the possibility that N.vectensis Complexin-2 is able to clamp spontaneous exocytosis.

As stated above, we have performed these experiments. N. vectensis complexin-2 is also an activator similar to complexin-1, but exhibits less potency, and also is unable to clamp spontaneous release (new Fig. 3).

2) As the authors identified Complexins in cnidarians it would be very informative to expand the phylogenetic analysis and protein alignments of Complexins to other basal animals such as sponges, placozoans (animals without neurons) and ctenophores and discuss this analysis (protein domain conservation and amino acid conservation, alignments) in light of their findings. The authors actually have mentioned that Complexin(s) is (are) present in sponges in a previous publication (PMID: 23345244).

We have performed the suggested analyses for the revised paper (new Figs. 1 and 2).

3) The strong tone of some parts of the paper (e.g., the title and some of the conclusions drawn) might be misleading for the reader. The authors should be cautious in stating "the core machinery of neurotransmitter release is shared by all animals throughout evolution". For example, how neurotransmitter release is controlled by Complexins in other basal animals (e.g. ctenophores or other cnidarians) is not known. In addition, the authors should be cautious with statements like "cnidarian exocytosis operates by fundamentally identical molecular mechanisms as mammals" as only one component of the exocytotic machinery has been tested so far (N.vectensis Complexin-1 in this study) and all of the experiments have been performed in mouse neurons. Insights into the function of Complexin-1 in N.vectensis would answer the question regarding the evolutionary conservation of the molecular mechanism of neurotransmitter release more directly.

We have changed the title of the paper and expressed our conclusions and hypotheses more cautiously.

Minor points

4) The authors state that N.vectensis "develops only primitive neuron-like sensory cells" (Introduction, fourth paragraph). I think this is not entirely correct, N.vectensis develops sensory cells, but also many different neurons (please see for example PMID: 19170043)

We have rephrased the incriminated sentence.

5) In the Methods section information about the sequences of N.vectensis Complexin 1 and 2 (NCBI numbers?) and how alignments have been made (program?) would be useful.

NCBI numbers are now included in the new sequence alignment and a new methods section on bioinformatics was added.

6) Figure 1A would benefit from showing the domain architecture of N.vectensis Cpx1 and 2. In addition, the colour code in the alignments has to be explained and Nematostella is not spelled correctly above the alignment.

We apologize for the spelling mistake. We have now included a domain map of complexins in the new Figure 1.

2nd Editorial Decision 27 July 2015

Many thanks for your patience while your revised manuscript was assessed by the original referees who have now turned in their reports on it. I am attaching them this email. I am happy to tell you that they all now support publication of the study in EMBO reports, after a few minor textural changes as evident from their reports.

I am thus writing an 'accept in principle' letter, which means that I will be happy to accept your manuscript for publication once these few minor issues/corrections have been addressed.

Formally, I would also kindly ask you to identify one, or maybe two, of the currently seven main figures that could be displayed as expanded few figures. The reason for this is that our scientific reports can only contain five main figures (plus five expanded view figures). In your case I could make an exception and allow 6 main figures, but seven is too many. Alternatively, if you want all seven figures to remain main figures, could you maybe fuse two of them together so that in the end there are no more than six main figures?

Also, all Expanded View Figures should be labeled and referenced as Figure EV1, Figure EV2 etc. in the main text of the manuscript. The legends for the EV figures should be incorporated in the main body of the text after the legends for the main figures (currently the legends for them are not part of the main manuscript file).

If all remaining corrections have been attended to, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

REFEREE REPORTS:

Referee #1:

I am glad reading the revision and response letter, which clearly explained what I did not know as an researcher in a different field. There were a lot known and published in the paralogues of the gene they investigated so I am no longer worried about any possibility of incomplete information regarding the gene family.

Referee #2:

The phylogenetic analysis performed in the context of this revision turned out to be highly informative, as it suggestions that complexins were present in unicellular organisms that emerged well before the evolution of metazoa and neuronal systems. This analysis seems to have been done in a thorough and professional manner, despite obvious caveats that are associated with such analyses (the tree is not so consistent for the non-vertebrate sequences, but the authors discuss that). The query regarding the two paralogs in Nematostella has also been clarified.

Over, I found the current version to be far more interesting and wider in scope, as reflected now in the revised summary, and I gladly recommend publication in EMBO Reports.
The only recommendation I have is for careful proof-reading of the text to eliminate typos and granitic mistakes - e.g., the article's last sentence - Nematostella complexin can functionally... indicates that their fundamental (singular to plural transition).

Referee #3:

In this revised version the authors are now showing additional data on N. vectensis complexin-2, which when introduced into mouse neurons, rescues evoked release to a small extent lesser than N. vectensis complexin-1, and actually causes additional uncampling instead of clamping spontaneous release. The authors also performed a phylogenetic analysis of complexin sequences. Both experiments are valuable additions to the manuscript.

Nevertheless, I have some comments and suggest addressing these points carefully.

1) While the analysis of Complexins in animals and close relatives is comprehensive and the authors identify complexin homologs in basal animals and in the slime mold Fonticula alba, the authors are clearly not the first to report on Complexins in non-animals. Complexins in the choanoflagellate Monosiga brevicollis and the filasterean Capsaspora owczarzaki have been found before: "Evolutionary insights into premetazoan functions of the neuronal protein homer" (PMID: 24899667). Domain architectures and accession numbers of these Complexins are also shown (supplementary information: XP_001747888.1 Complexin_ Monosiga brevicollis and XP_004364368.1 Complexin_ Capsaspora owczarzaki. Thus, some of the findings in this manuscript are not as novel as presented. I would suggest adding this citation and changing the text passages shown below.

- Results and Discussion: Surprisingly, we also discovered single complexin sequences in a few non-metazoan single-cell organisms belonging to groups such as Choanoflagellatea, Filasterea, and Nuclearia
- Abstract: Here we show that complexin sequences are conserved in some nonmetazoan unicellular

organisms as well as all metazoans...
- Introduction: Here we show that complexin sequences are not only encoded by all metazoan genomes, but are also present in the genomes of a subset of unicellular organisms that are evolutionarily older than metazoans, such as choanoflagellates
- Summary: Overall, our experiments suggest two major conclusions. First, complexins likely predate animal evolution...

2) The find of complexins in non-animals raises the interesting question about whether non-animal complexins, when introduced into mouse neurons, can rescue the described phenotype as well. The new title "Evolutionary Conservation of Complexins: From Choanoflagellates to Mice" suggests that the authors also have tested complexins from non-animals (e.g. Nuclearia, Filasterea and Choanoflagellata). Unless the authors have the data to support this I suggest changing the title to something which describes the actual results.

3) As highlighted above the authors changed the title to "Evolutionary Conservation of Complexins: From Choanoflagellates to Mice". Is there a reason why the authors choose choanoflagellates and not "From Nuclearia to Mice?" Nuclearia (and Filasterea) are more distantly related to animals than choanoflagellates and both their genome(s) encode for complexins as well.

4) The authors mention that Nematostella vectensis is a cnidarian sea anemone at the root of animal evolution (abstract). Cnidarians are the closest known sister group to bilaterians and not considered to sit at the root of animal evolution. The root of the animal tree rather belongs to sponges and ctenophores. Please change, as this is very important in light of the findings and the conclusions one can make.

5) The authors should be consisted throughout the manuscript with using either the term "animal(s)" or "metazoans(s)".

2nd Revision - authors' response 09 August 2015

Please find enclosed our re-revised manuscript entitled “Evolutionary Conservation of Complexins: From Choanoflagellates to Mice”, and the other files requested in your decision letter.

For the re-revised paper, we contracted the number of figures to 6 from 7 as you suggested, and amended the text in light of the reviewers’ additional comments as described below.

Referee #1:
I am glad reading the revision and response letter, which clearly explained what I did not know as an researcher in a different field. There were a lot known and published in the paralogues of the gene they investigated so I am no longer worried about any possibility of incomplete information regarding the gene family.

Response: Thanks – we appreciate the comments.

Referee #2:

The phylogenetic analysis performed in the context of this revision turned out to be highly informative, as it suggestions that complexins were present in unicellular organisms that emerged well before the evolution of metazoa and neuronal systems. This analysis seems to have been done in a thorough and professional manner, despite obvious caveats that are associated with such analyses (the tree is not so consistent for the non-vertebrate sequences, but the authors discuss that). The query regarding the two paralogs in Nematostella has also been clarified.

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The only recommendation I have is for careful proof-reading of the text to eliminate typos and granitic mistakes - e.g., the article's last sentence - Nematostella complexin can functionally... indicates that their fundamental (singular to plural transition).

Response: Thanks – we also appreciate this reviewer's constructive comments, and have tried to correct all of the grammatic mistakes.

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In this revised version the authors are now showing additional data on N. vectensis complexin-2, which when introduced into mouse neurons, rescues evoked release to a small extent lesser than N. vectensis complexin-1, and actually causes additional uncampling instead of clamping spontaneous release. The authors also performed a phylogenetic analysis of complexin sequences. Both experiments are valuable additions to the manuscript.

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- Abstract: Here we show that complexin sequences are conserved in some nonmetazoan unicellular organisms as well as all metazoans...

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- Summary: Overall, our experiments suggest two major conclusions. First, complexins likely predate animal evolution...

Response: We apologize for missing the Burckhard et al. paper which mentions complexin in the supplementary material but provides no sequence analysis or discussion of its domain organization. We have now cited the paper and adjusted the wording of the incriminated sentences as needed taking the information from that paper into account.

2) The find of complexins in non-animals raises the interesting question about whether non-animal complexins, when introduced into mouse neurons, can rescue the described phenotype as well. The new title "Evolutionary Conservation of Complexins: From Choanoflagellates to Mice" suggests that the authors also have tested complexins from non-animals (e.g. Nuclearia, Filasterea and Choanoflagellata). Unless the authors have the data to support this I suggest changing the title to something which describes the actual results.

Response: We respectfully disagree with the reviewer. Nowhere does the title suggest that we tested complexin from a unicellular organism in rescue experiments – we only probed their sequences, but that is the standard in the field of evolutionary biology. To the best of our knowledge, evolutionary biologists generally do not test the functional conservation of their proteins as performed in the pioneering work of Nicole King on choanoflagellates, but primarily perform sequence and expression analyses. In fact, it seems to us that the reviewer should give us some credit for performing the first test for the function of a cnidarian protein in a mammalian neuron. The title accurately describes what the paper shows, namely that there is evolutionary conservation of complexins in unicellular organisms, citing choanoflagellates as the best studied such organism.

3) As highlighted above the authors changed the title to "Evolutionary Conservation of Complexins: From Choanoflagellates to Mice". Is there a reason why the authors choose choanoflagellates and not "From Nuclearia to Mice?" Nuclearia (and Filasterea) are more distantly related to animals than choanoflagellates and both their genome(s) encode for complexins as well.

Response: See our response to point 2 above.

4) The authors mention that Nematostella vectensis is a cnidarian sea anemone at the root of animal evolution (abstract). Cnidarians are the closest known sister group to bilaterians and not considered to sit at the root of animal evolution. The root of the animal tree rather belongs to sponges and ctenophores. Please change, as this is very important in light of the findings and the conclusions one can make.

Response: Although it seems to us that there is some disagreement about this question in the literature, as non-experts we cannot tell the validity of the argument and have simply rephrased the sentence.

5) The authors should be consisted throughout the manuscript with using either the term "animal(s)" or "metazoans(s)".

Response: Not quite sure why the two terms cannot be used interchangeably, but we have now only used the term ‘metazoan’.

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I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

