

Neuronal segmentation in cephalopod arms

Corresponding Author: Ms Cassady Olson

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This contribution documents convincingly the segmented nature of the octopus peripheral nervous system in the arm cords. Moreover, it reveals a remarkable high resolution of the segmentation far beyond that suggested by the serial order of the suckers. The report is well written and largely clear, save for minor points detailed below.

Certainly, a significant contribution to both literature and the language is the word "suckerotomy" introduced in line 21 of the Summary and later in line 108 of the text. However, its meaning is not quite clear in the Summary. It would be clearer if a more precise descriptor better than "spatial topography" were added to the Summary, or even replaced the new word.

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The description of washes in the histological procedures (e.g., 3x15 min) is laboratory jargon that could be clarified the first use by saying 3x15 min (3 times for 15 minutes each).

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The transcripts cloned for in situ hybridization might be clearly identified. Otherwise, unless one is completely up on the literature, the abbreviations are meaningless. For instance, SYT1 as synaptotagmin.

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Some of the stain text labels blend with the background image, for instance in Fig 3(e). In these cases, it may help to flip the order of the identified stains, such as for Fig 3(e) switching the positions of the F-actin and SMI-31 label texts so they are more readable. Also, it would be helpful to clarify in certain figure legends, such as for Figures 2 and 3, what the arrowheads are specifically identifying.

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Page 34, Extended Data Figure 3: In (h), the labeling may be clearer here if dots are used for denoting Motor neuron territories, instead of diagonal lines.

Line 86 – "banching"?

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

Comments to Authors

Olson et al. examine the neuroanatomy of the adult arms in two representatives of the coleoid cephalopods, Octopus (octopods) and Doryteuthis (decapods). The study primarily focuses on Octopus bimaculoides, a species that is well-

established for its genomic data and the availability of molecular genetic protocols. The introduction is well-written, though there are specific details that warrant attention (as outlined below). The results are presented with clarity and precision, supported by a thorough description of the materials and methods, ensuring the reproducibility of the findings. The authors have investigated a sufficient number of specimens to strengthen their conclusions.

While some of the findings claimed by the authors are not novel—such as the description of the ganglia of the arm nerve cords (ANC), which have been documented previously, including in ontogenetic studies—this does not detract from the overall contribution of the study. However, these prior discoveries should be acknowledged.

The discussion is balanced, with only a few points requiring further attention. Specifically, there is a need for a more detailed definition of segmentation, including how segments are defined in different groups such as arthropods, annelids, and vertebrates. Reviews by Ariel Chipman on segmentation emphasize that the segmentation processes in these groups vary significantly, suggesting that the process itself may not be homologous across these taxa. The authors touch upon this topic in their discussion, but it is crucial to ensure that readers of the manuscript fully understand (the importance) of these distinctions.

Details:

I. 37-47: The authors present the current understanding but do not mention that the ANC has long been known to be composed of individual ganglia, a discovery made by Graziadei (1971) and Young (1971).

L. 75: "Nerves exiting the ANC nerves can be divided into the oral nerves, which innervate the sucker, and 75 central and aboral nerves, which together innervate the brachial musculature (Fig 2e, f, Extended Data Fig. 76 4a)."

This was already described before by Graziadei (1971) and review in Nixon and Young 2003 (l. 280):

"The axial nerve cord forms a prominent part of each arm and extends to the extreme tip. It consists of a series of ganglia. Many neurons of each ganglion send axons to innervate the corresponding sucker. The axial nerve is linked by nerve bundles to peripheral nerve centres, consisting of our intramuscular nerve cords lying in the intrinsic musculature of the arm, and to sucker ganglia, one lying below the base of each sucker. The arm muscles are innervated by motoneurons of the axial nerve centres (Graziadei 1971)."

Also, Young, in his classical work, showed in Fig. 3.10. "section of the arm with the brachial ganglia, showing characteristic alternate arrangement, each ganglion corresponding to one sucker. Bundles of nerve fibres connect each ganglion to its neighbour, constituting short interconnecting systems." (Young, 1971).

The authors' findings are nonetheless very interesting, but they need to acknowledge that previous researchers had already described these structures, albeit in less detail."

Recent ontogenetic studies also reported these ganglia:

Nödl et al. (2016) for example show in Fig. 6B the "extent of" what they call "single ganglia".

See also Fig. 8 (late differentiation)

Other publications that should be discussed since they directly touch the topic:

The authors should also discuss the results by

- Zullo et al. (2019) who studied motor control pathways in the nervous system of *O. vulgaris* (J. Comp. Phys A 205: 271-279).

- Wollesen et al. (2009) who already described F-actin labeling the vasculature of the brain in the pygmy squid *Xiphoteuthis* notoides.

Wollesen, T., Loesel, R., Wanninger, A. 2009. Pygmy squids and giant brains: Mapping the complex cephalopod CNS by phalloidin staining of vibratome sections and whole-mount preparations. *Journal of Neuroscience Methods* 179: 63–67.

I. 73: These results indicate that unlike in the vertebrate...

I. 178:

"Segments are present in the arm and tentacle club in *D. pealeii*, yet they are indistinct in the stalk 178 which is devoid of suckers. Interestingly, there are fewer segments per sucker in the arm of *D. pealeii* 179 compared with that of *O. bimaculoides*. Differences in ecological niche and behavioral repertoire, including 180 prey hunting strategies, could drive this variation10,35."

Just out of curiosity: Maybe the authors could comment on the situation in nautiloids which are closely related to coleoids but which do not possess suckers. Would they also expect segmentation of the ANC here?

I. 183: "In particular, segmentation in molluscs has been proposed for basally branching polyplacophorans (chitons) and monoplacophorans."

Please refrain from citing this reference, as the monoplacophoran sequences were later revealed to be contaminated, a fact that the lead author themselves acknowledged. This means the hypothesis was fundamentally flawed from the start. Instead, if you need to cite a reference supporting the unification of Monoplacophorans and Polyplacophorans, use Wilson, Rouse, and Giribet (2010), which is based on more reliable sequence data (Reference 43).

It's also crucial to note that Monoplacophorans and Polyplacophorans are not basally branching taxa. In the phylogenies presented by Wilson et al. (2010) and others (e.g., Smith et al., Kocot et al.), a sister group relationship is observed, which, by definition, does not allow for a basal branching position (in a sister group relationship, none of both clades can be basically branching).

You might mention that monoplacophorans are basally branching within conchiferans according to the phylogeny of Kocot et al. (2020, 'New data from monoplacophorans...'), but in this phylogeny, polyplacophorans would not be considered basally branching.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)

The manuscript „Neuronal segmentation in cephalopod arms” by Cassady S. Olsen, Natalie Grace Schulz and Clifton W. Ragsdale adds detailed information on the structure of the nervous system in cephalopod arms. The authors studied the nerve fibre distribution in arms of mainly Octopus, but also a decabrachiate species for comparison, by means of injections, immunolabelling and in situ hybridization; all the methods used are documented in high detail. In their study, they found that the nerve cord in each arm is superficially a medullary cord as previously described, but is furthermore arranged in several thin segments, which are best visualized by the localization of cell somata, which are separated by collagenous fibre material as well as vasculature. Olsen et al. furthermore managed to trace individual nerves and could thereby show that the branching pattern is repeated in several segments along the cephalopod arm. Additionally, the comparative part of the study also shed light on the difference between the arms and tentacles in decabrachiate cephalopods, which taken together with the other findings suggests that segmentation is also used in molluscs to facilitate complex control of reiterating muscles and in this case suckers.

While the methods used and the results obtained deliver a great picture of the nervous system as a whole and the segments of the nerve cord in higher detail, one of the weaknesses is the manual tracing of individual nerves, such as illustrated in Figure 2g: If it was done similar to what is shown in this figure, I am worried that not all processes have been labelled and therefor also some possible connections between nerves, such as possible between the green and magenta-labelled nerve have not been accounted for. Them and other projections not being accounted for in the later analyses might not be too much of a problem for the analyses conducted in this study, but could possibly give a wrong impression about connectivity between individual segments. It would also be very interesting to check the fascinating findings on an ultrastructural level or after employing some specific pre- and postsynaptic markers to see whether the innervation patterns really hold true (i.e. nerve endings identified by acTUBA really connect to other nerves by containing synapses, ...). Based on their findings, I also think their statement of segmentation to deal with the control of motor patterns is justified, and I am curious whether more detailed studies of vasculature and musculature together with the nervous system will strengthen this model. Overall this is my only major comment to an otherwise really exciting and well-documented study (some small details are commented on directly in the attached PDF), which I highly recommend for publication.

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I find the revised manuscript to be satisfactory and a significant contribution. The histology is so beautiful that it is almost distracting. I look forward to seeing this work in print.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

The authors have successfully addressed all of my concerns. Congratulations on this interesting and innovative publication!

(Remarks on code availability)

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This contribution documents convincingly the segmented nature of the octopus peripheral nervous system in the arm cords. Moreover, it reveals a remarkable high resolution of the segmentation far beyond that suggested by the serial order of the suckers. The report is well written and largely clear, save for minor points detailed below.

We thank the reviewer for the positive comments on our study! We have addressed the specific points below.

Certainly, a significant contribution to both literature and the language is the word “suckerotopy” introduced in line 21 of the Summary and later in line 108 of the text. However, its meaning is not quite clear in the Summary. It would be clearer if a more precise descriptor better than “spatial topography” were added to the Summary, or even replaced the new word.

We have updated the language used to introduce “suckerotopy” in the abstract.

Page 3, lines 53-53: the description of the ANC "orienting in turn to each sucker" does not make clear sense.

We have updated the introduction to clarify our description.

Methods:

Page 12, line 256: possible missing time duration for PBST rinse in "PBST 1x,"

There is no missing time duration here as it is a quick rinse, but we have changed the language to avoid confusion.

The description of washes in the histological procedures (e.g., 3x15 min) is laboratory jargon that could be clarified the first use by saying 3x15 min (3 times for 15 minutes each).

We have added in this clarification after the first use.

Pages 11-12, lines 236, 244, 257: for consistency either "minutes" or "min" should be used everywhere, for example, "3x10 minutes", "3x5 minutes", 3x15 minutes"

We have made the methods consistent with “minutes”.

Page 12, line 249: "rinsed twice in [not 'is'] 100% methanol"

Thank you for noting this typo! It has been fixed.

Page 18, line 398: "was set to 1"

We have made our description of the normalization more explicit.

The transcripts cloned for in situ hybridization might be clearly identified. Otherwise, unless one is completely up on the literature, the abbreviations are meaningless. For instance, SYT1 as synaptotagmin.

We have spelt out the gene acronyms the first time they are used as well as in Table 1. In addition, although not mentioned by the reviewers, we noticed that our protocol for DiI labeling was inadvertently omitted from the initial submission. We have added a paragraph to the methods with this protocol.

Figures:

Some of the stain text labels blend with the background image, for instance in Fig 3(e). In these cases, it may help to flip the order of the identified stains, such as for Fig 3(e) switching the positions of the F-actin and SMI-31 label texts so they are more readable.

We have gone through all figure panels and switched the text around to make it more readable when needed.

Also, it would be helpful to clarify in certain figure legends, such as for Figures 2 and 3, what the arrowheads are specifically identifying.

We have made sure to specify in every figure legend what the arrows and arrowheads indicate.

Page 27, Figure 2: it may help to have an inset showing the longitudinal slice location in (e) and (f), similar to 3(d) or 3(g).

We have added in an inset to demonstrate slice location.

Pages 29 and 31, Figure 3 and Extended Data Figure 1: it may help to modify the white inset figure in (e),(f),(g), possibly by delineating certain sections of the ANC more, such as the CBT and CBLs (much like in Extended Data Figure 6 (d)), to make it clearer which part of the ANC the horizontal slices are from.

To better orient readers to the white inset, we have added a panel to Extended Data Figure 1 (now Supplementary Figure 1) with the regions of the ANC labeled. We have also thickened the lines to make the territories in the inset more readable when small.

Page 34, Extended Data Figure 3: In (h), the labeling may be clearer here if dots are used for denoting Motor neuron territories, instead of diagonal lines.

As suggested, we have changed the diagram to dots denoting motor neuron territories.

Line 86 – “banching”?

Thank you for noting this typo! It has been fixed to “branching”.

Reviewer #2 (Remarks to the Author):

Comments to Authors

Olson et al. examine the neuroanatomy of the adult arms in two representatives of the coleoid cephalopods, *Octopus* (octopods) and *Doryteuthis* (decapods). The study primarily focuses on *Octopus bimaculoides*, a species that is well-established for its genomic data and the availability of molecular genetic protocols. The introduction is well-written, though there are specific details that warrant attention (as outlined below). The results are presented with clarity and precision, supported by a thorough description of the materials and methods, ensuring the reproducibility of the findings. The authors have investigated a sufficient number of specimens to strengthen their conclusions.

While some of the findings claimed by the authors are not novel—such as the description of the ganglia of the arm nerve cords (ANC), which have been documented previously, including in ontogenetic studies—this does not detract from the overall contribution of the study. However, these prior discoveries should be acknowledged.

Thank you for raising this concern. We have reworked the introduction to better situate our current study in the context of the existing literature. In addition, as detailed below, we have addressed the reviewer's specific concern about the "description of the ganglia".

The discussion is balanced, with only a few points requiring further attention. Specifically, there is a need for a more detailed definition of segmentation, including how segments are defined in different groups such as arthropods, annelids, and vertebrates. Reviews by Ariel Chipman on segmentation emphasize that the segmentation processes in these groups vary significantly, suggesting that the process itself may not be homologous across these taxa. The authors touch upon this topic in their discussion, but it is crucial to ensure that readers of the manuscript fully understand (the importance) of these distinctions.

We have updated the discussion to include a reference to a 2019 review by Ariel Chipman and added a sentence emphasizing differences in segmentation generation.

Details:

l. 37-47: The authors present the current understanding but do not mention that the ANC has long been known to be composed of individual ganglia, a discovery made by Graziadei (1971) and Young (1971).

The nature of the anatomy of the axial nerve cord is that there are enlargements corresponding to each of the hundreds of suckers that line the arms.

There are two points here. First, we did not intend to give the impression that we were the first to describe these sucker-associated enlargements; we certainly are not. We needed, however, to make this anatomy clear before moving on to describe our novel finding about the segmental organization of the ANC. Accordingly, we have added appropriate references to indicate that the ANC enlargements have been well-known for decades.

The second point is that although many authors refer to these enlargements as ganglia, they are not ganglia as classically described, namely, “swellings” of neurons linked together by neuron-free connectives and commissures. “Ganglia” are distinguished from “medullary cords” which consist of neuronal cell bodies together with neuropil. This distinction was made explicit in the consensus nomenclature paper published by Richter et al. 2010 (see also Rossi and Graziadei, 1954). We follow this modern nomenclature convention and refer to ANC enlargements rather than to separate ganglia. To avoid confusion on this point, we have changed the text and added references.

L. 75: “Nerves exiting the ANC nerves can be divided into the oral nerves, which innervate the sucker, and 75 central and aboral nerves, which together innervate the brachial musculature (Fig 2e, f, Extended Data Fig. 76 4a).”

This was already described before by Graziadei (1971) and review in Nixon and Young 2003 (l. 280):

“The axial nerve cord forms a prominent part of each arm and extends to the extreme tip. It consists of a series of ganglia. Many neurons of each ganglion send axons to innervate the corresponding sucker. The axial nerve is linked by nerve bundles to peripheral nerve centres, consisting of our intramuscular nerve cords lying in the intrinsic musculature of the arm, and to sucker ganglia, one lying below the base of each sucker. The arm muscles are innervated by motoneurons of the axial nerve centres (Graziadei 1971).”

Also, Young, in his classical work, showed in Fig. 3.10. “section of the arm with the brachial ganglia, showing characteristic alternate arrangement, each ganglion corresponding to one sucker. Bundles of nerve fibres connect each ganglion to its neighbour, constituting short interconnecting systems.” (Young, 1971).

The authors' findings are nonetheless very interesting, but they need to acknowledge that previous researchers had already described these structures, albeit in less detail.”

This “L.75” sentence serves to orient the reader to the language we use to describe the subsets of nerves that exit the ANC, and it was not our intention to give the impression that we were the first to report nerve fibers leaving the axial nerve cord. Accordingly, we have added the needed references.

Recent ontogenetic studies also reported these ganglia:

Nödl et al. (2016) for example show in Fig. 6B the “extent of” what they call “single ganglia”.

See also Fig. 8 (late differentiation)

We have added in this citation when referring to ganglia.

Other publications that should be discussed since they directly touch the topic:

The authors should also discuss the results by

- Zullo et al. (2019) who studied motor control pathways in the nervous system of *O. vulgaris* (J. Comp. Phys A 205: 271-279.

- Wollesen et al. (2009) who already described F-actin labeling the vasculature of the brain in the pygmy squid *Xiphoteuthis notoides*.

Wollesen, T., Loesel, R., Wanninger, A. 2009. Pygmy squids and giant brains: Mapping the

complex cephalopod CNS by phalloidin staining of vibratome sections and whole-mount preparations. *Journal of Neuroscience Methods* 179: 63–67.

Thank you for suggesting these citations. We have been added them in at the appropriate locations.

l. 73: These results indicate that unlike in the vertebrate...

We believe this refers to a comment made on the PDF (comment #1, listed below) that came attached to the reviewers' comments. To address the concerns raised, we have substantially updated Extended data figure 3 (now Supplementary Fig. 3) to make the spatial overlap between sensory and motor markers more apparent, as explained below.

l. 178:

“Segments are present in the arm and tentacle club in *D. pealeii*, yet they are indistinct in the stalk 178 which is devoid of suckers. Interestingly, there are fewer segments per sucker in the arm of *D. pealeii* 179 compared with that of *O. bimaculoides*. Differences in ecological niche and behavioral repertoire, including 180 prey hunting strategies, could drive this variation^{10,35}.” Just out of curiosity: Maybe the authors could comment on the situation in nautiloids which are closely related to coleoids but which do not possess suckers. Would they also expect segmentation of the ANC here?

Nautiloids would provide a great point of comparison. While their tentacles don't have suckers, they have cirri and axial nerve cords (Sasaki et al., 2010). Because of their endangered status, however, members of this genus are not available for cellular and molecular studies. A single micrograph in Kier (1987) suggests that the nautilus ANC has enlargements for each cirri, but without any possibility of further study, it seems too much to speculate that the Nautiloid ANC could also have segments.

l. 183: “In particular, segmentation in molluscs has been proposed for basally branching polyplacophorans (chitons) and monoplacophorans.”

Please refrain from citing this reference, as the monoplacophoran sequences were later revealed to be contaminated, a fact that the lead author themselves acknowledged. This means the hypothesis was fundamentally flawed from the start. Instead, if you need to cite a reference supporting the unification of Monoplacophorans and Polyplacophorans, use Wilson, Rouse, and Giribet (2010), which is based on more reliable sequence data (Reference 43).

It's also crucial to note that Monoplacophorans and Polyplacophorans are not basally branching taxa. In the phylogenies presented by Wilson et al. (2010) and others (e.g., Smith et al., Kocot et al.), a sister group relationship is observed, which, by definition, does not allow for a basal branching position (in a sister group relationship, none of both clades can be basically branching).

You might mention that monoplacophorans are basally branching within conchiferans according to the phylogeny of Kocot et al. (2020, 'New data from monoplacophorans...'), but in this phylogeny, polyplacophorans would not be considered basally branching.

We have updated this section to remove the language “basally branching” and streamlined the

references on the controversy regarding segmentation in molluscs.

Reviewer #3 (Remarks to the Author):

The manuscript „Neuronal segmentation in cephalopod arms” by Cassady S. Olsen, Natalie Grace Schulz and Clifton W. Ragsdale adds detailed information on the structure of the nervous system in cephalopod arms. The authors studied the nerve fibre distribution in arms of mainly Octopus, but also a decabrachiate species for comparison, by means of injections, immunolabelling and in situ hybridization; all the methods used are documented in high detail. In their study, they found that the nerve cord in each arm is superficially a medullary cord as previously described, but is furthermore arranged in several thin segments, which are best visualized by the localization of cell somata, which are separated by collagenous fibre material as well as vasculature. Olsen et al. furthermore managed to trace individual nerves and could thereby show that the branching pattern is repeated in several segments along the cephalopod arm. Additionally, the comparative part of the study also shed light on the difference between the arms and tentacles in decabrachiate cephalopods, which taken together with the other findings suggests that segmentation is also used in molluscs to facilitate complex control of reiterating muscles and in this case suckers.

1. While the methods used and the results obtained deliver a great picture of the nervous system as a whole and the segments of the nerve cord in higher detail, one of the weaknesses is the manual tracing of individual nerves, such as illustrated in Figure 2g: If it was done similar to what is shown in this figure, I am worried that not all processes have been labelled and therefore also some possible connections between nerves, such as possible between the green and magenta-labelled nerve have not been accounted for. These and other projections not being accounted for in the later analyses might not be too much of a problem for the analyses conducted in this study, but could possibly give a wrong impression about connectivity between individual segments. It would also be very interesting to check the fascinating findings on an ultrastructural level or after employing some specific pre- and postsynaptic markers to see whether the innervation patterns really hold true (i.e. nerve endings identified by acTUBA really connect to other nerves by containing synapses, ...).

This is a great point. First, it's important to note that the image in Figure 2g is a maximum projection. Since it is a compression of a 3D space into a 2D image, the apparent connections are misleading. Nonetheless, many of these nerves touch the intramuscular nerve cords and other undescribed nervous elements embedded in the muscle. They most certainly receive connections this way, and we agree that it would be very interesting to investigate these connections at the ultrastructural level. Investigating the synaptic connectivity of the nerve fibers is outside the scope of the current study, but we have updated the discussion to prevent any confusion about the connectivity between segments. We have also updated the methods to include a better description of how the manual tracing of the nerves was done—i.e., once the nerve touched a peripheral mechanism, such as the intramuscular nerve cord, the tracing stopped.

Based on their findings, I also think their statement of segmentation to deal with the control of motor patterns is justified, and I am curious whether more detailed studies of vasculature and musculature together with the nervous system will strengthen this model.

The vasculature data presented here is the part of our extensive analyses that clarified the relationship of the vasculature to the segmentation. Specifically, we show that blood vessels are a component of the septa between the segments. We think that this is an anatomical feature of the septa, but one that is unrelated to the neuronal functions of the segmentation.

We have looked at the brachial musculature organization of adult and developing tissue and have been unable to detect any evidence for segmentation. This finding remains puzzling to us, but we, and we hope others, will investigate this point further in future studies.

Overall this is my only major comment to an otherwise really exciting and well-documented study (some small details are commented on directly in the attached PDF), which I highly recommend for publication.

We thank this reviewer and the others for their positive reception of our manuscript and for insightful and detailed comments that have strengthened it!

Comments from attached PDF:

1. Line 73: These findings are illustrated in Extended Data Fig. 3, however, it would be great to also show images of the entire ANC/arm crosssection, since the figure only seems to show details of individual labellings, which don't allow the assessment of the overall staining pattern or overlap of 2 or more markers on the same section

Because many of the cells are small, two-color methods do not work as well. To address reviewers' comments, we have substantially revised this figure to clarify the relationship between labeling and topography in the ANC. We have updated this figure to include the entire ANC for the two strongest expressors: a motor neuron marker (*NKX6*) and a sensory neuron marker (*PIEZO*). We have included arrowheads for the remaining genes to show where on the ANC the images were taken.

2. Figure 1, Line 545: Please explain what the arrow heads stand for - I guess it is the segmentation pattern?

We have added in a description of what the arrowheads stand for.

3. Figure 2, panel C: Would it be possible to show these figures larger? Otherwise the labelling needs to be adjusted to be of similar size to the one in the remaining panels

As suggested, we have made these panels larger.

4. Figure 2, Line 567: Maybe it is just me, but not the entire nerve seems to be colored in magenta or green - is there is reason for that? There seems to be a fibre leading from the green to the magenta nerve - without labelling for synapses though it is hard to say whether there is any connection

As noted and dealt with above [our response to reviewer point 1], this is a maximum projection image, which could give a false sense of apparent connections.

5. Figure 4, line 606: Would it be possible to give these ones another colour/shape? It would be easier to identify what is labelled that way, especially when it is also explained in the previous figures what the white arrowheads indicate

We have changed the shape of the arrowheads in panels

6. EDF 3, line 639: This is a bit tricky I think - not the entire ANC is shown in these images, and also for stating that these territories are overlapping it would be better to show this overlap by labelling multiple of these markers at the same time

We have addressed this point in response to the first comment on the PDF.

7. EDF 6, line 676: typo in Cerebrobrachial tract

Thanks for pointing out this typo! It's been corrected

8. EDF 7, panel aii: This figure is a bit hard to correlate with the previous one (ai) - is it maybe possible to add the sucker surface as dashed lines, so it is easier to see the overlay?

Since adding the sucker surface as dashed lines cluttered the image too much, we have instead added numbers to correlate the sucker image to the ANC image.

Reviewer #1 (Remarks to the Author):

I find the revised manuscript to be satisfactory and a significant contribution. The histology is so beautiful that it is almost distracting. I look forward to seeing this work in print.

Reviewer #2 (Remarks to the Author):

The authors have successfully addressed all of my concerns. Congratulations on this interesting and innovative publication!

Thank you for the generous comments and appreciation of our work!