Peer Review File

Manuscript Title: Sensory specializations drive octopus and squid behavior

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

This is the second of two excellent papers detailing how a nicotinic acetylcholine receptor (nAChR) morphed into a receptor for different substances that are utilized in the hunting behavior of octopus or squid. Like the first paper, multiple methods are used to make a strong case that chemotactile receptors are used to identify prey. Interestingly, the receptors in squid are closer to ancestral molluscan nAChRs and that the orthologous receptors in octopus have evolved further to detect substrate molecules. It is fascinating to see how the binding site of this receptor evolved from a largely hydrophilic site with an aromatic cage, to one that is mainly a hydrophobic site.

The experiments are nicely done and the paper is clear and beautifully illustrated.

One point that the authors should address is: how do these receptors relate to the large molluscan expansion of nAChRs? One paper that I do not see in the references and that would seem to be very relevant is Jiao et al (2019) (https://bmcgenomics.biomedcentral.com/articles/10.1186/s12864-019-6278-9) that documents an expansion of nAChRs in gastropod mollusks, often in tandem arrays of intronless receptors, just as described here in octopods and squid. Are the sequences that they used in the phylogeny presented in figure 1? I see that they use sequences from Lottia gigantea in their phylogeny. The authors might go into a little more detail on the relationship of the CRTs to these molluscan receptors.

The nerve recordings seem to be summed activity so that it is difficult to know if single neurons respond to both substances or if individual neurons are selective to one or the other substance. In other words, if one substance (terpene) is an attractant and the other (denatonium) is aversive. Have the authors tried to sample individual afferents by waveshape to tease apart whether there are separate bitter and terpene sensitive neurons sending this information forward?

The rejection of denatonium-laced prey by squid is like the vertebrate distaste for bitter substances. It would be interesting to compare human bitter receptors (I think these are GPCRs) with these novel receptors to compare how they interact with bitter substances. And why do we or squid avoid bitter substances? Are they harmful in some way? Humans come to like various bitter things (Brussel sprouts come to mind) I wonder if in time squid would eat them too if all their food was bitter and they had no choice in the matter. Just some “food for thought” but nothing the authors need to do on their MS.

Harold Zakon

Referee #2 (Remarks to the Author):

The manuscript Kang et al., “Sensory specializations drive octopus and squid behaviour” outlines the function of Decapodiformes chemotactile receptors (CRs) and their role in environmental sensing. Overall, the paper describes very interesting functional findings on the diversity and role of CRs in Decapodiformes. Commenting on the evolutionary part of the story, we think that the findings can be better represented and reanalyzed.

Firstly, the tree representation in Fig 1b does not necessarily imply the "ancestral-ness" of squid CRs (i.e., CRBs). The tree topology generally shows split in octopus CRs and squid CRs, which, as the authors describe, may have driven adaptations to different environments and ecological challenges (which, we find, is a very exciting finding). In the "traditional" sense, the finding may simply be that there were independent expansions of CRs in squids and octopuses. One could generally call all CRBs simply squid CRs. Then, CRBT clade may also be interpreted as the most ancestral clade (since it has both squid and octopus genes). In this view, squids may be seen as more derived. Secondly, the tree itself would benefit from a better representation by at least showing support values or labeling supported branches. The authors may select only the sequences shown in Fig 1b and construct an independent tree to validate the branching pattern and further support CRB/CRT/CRX groupings.

The general interpretation of the results should also be reconsidered. For example, sentences like "duplication and specialization via structural modification of specific functional regions that is driven by lineage-specific behavior" should be reworded as it is not how evolution works ("associated with lineage-specific behavior"?). Overall, the findings, taken from the evolutionary perspective, seem "expected" (differential expansion of gene families), therefore, while very interesting, may seem incremental to what the authors have already published a few years back.

Minor points:
- Check for correct usage of words “cephalopods” and “coleoid cephalopods” (if not present in Nautilus). "Lineage-defining" may be "lineage-specific", etc.
- It should be “Decapodiformes” or “Octopodiformes” rather than then “decapod”, “octopod”.
- Species names in the transcriptomic section should be corrected to “Sepioloidea lineolata”, “Nautilus pompilius”.
- It would help the reader to have a list of all tissues collected or analyzed in one table. This is important as Nautilus predation can be seen "similar" to squids and octopuses and so its important to rule out the possibility of simply undersampled Nautilus tissues.
- Along these lines, in the Methods section it would be beneficial to list how many hits were obtained in the iterative searches for CR orthologs in the outgroups to the coleoid cephalopods. Again, to solidify the findings that no sequence was missed due to bad genome annotation in, especially, Nautilus.
- Line 62: “based on previous and subsequent functional experiments” reference lacking?
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Referee #3 (Remarks to the Author):

The set of two manuscripts, from Allard, Kang and colleagues describes how, at the molecular level, squid and octopus detect bitter and hydrophobic compounds through specialized sensory receptors. These sensory receptors have evolved from nicotinic acetylcholine receptors, and the papers characterize two of them in detail with functional and structural approaches. Because the two papers go together, I'm reviewing them together.

The Kang et al. paper first identifies a subgroup of decapod-specific receptors. One of them, CRB1, is found to be sensitive to bitter compounds, which also generate arm and tentacle movements and cause a behavioral response. The cryoEM structure of CRB1 reveals that the bitter compound denatonium sits in the orthosteric site, where neurotransmitters usually bind. The geometry of the site is altered to provide specificity for that type of compound. Finally, the authors provide a side-by-side comparison of the orthosteric site between a human nAChR, CRB1 and the octopus receptor CRT1, while relating structural differences to evolution of cephalopods.
The Allard et al. paper briefly introduces the CR family and then jumps directly in the description of the cryoEM structure of CRT1. The architecture is described, and then a bizarre parenthesis on determinants of Ca2+ permeation is proposed. The authors then come back to a more classical identification/description of the binding site, which happens to be the orthosteric site. The hydrophobic part of the detergent used for purification sits there, is found to be a potent agonist but does not trigger arm response. The structural analysis is expanded in regards with the evolution of the CR family.

I first want to congratulate the authors: the work is impressive, both in its diversity and in its depth. I was in awe reading about two new subtypes of sensory receptors characterized at the structural, functional, ex vivo and behavioral levels. I can tell how much work went into some single panel of an ED figure (e.g Allard Fig ED 3).

Major remark #1
However, the way the two stories are presented was, in my opinion, unsatisfactory. I understand the logic of trying to get two papers from the excellent set of data presented, but to my eyes it is not working in the present form. Bits of related information that may well go together are separated in the two papers (e.g. the last sections, starting line and 149 and line 156 in Kang et al. and Allard et al. respectively). Sometimes this dual presentation yields redundancies (e.g. Fig 4c-e with Fig. 3c). Sometimes information is missing (or maybe just too hard to find); in many instances I would have liked to have had a pedestrian side-by-side comparison of CRB1, CRT1 and a7. In more specific comments below, I try to point out places where scattering the data over two papers looked the

most problematic.
My point is not to suggest that a looong article would be more appropriate than two letters. I don't know. However, I want to underline that, in the current presentation, a reader has to jump constantly from one to the second paper and back. This back and forth is detrimental to an/my overall understanding of the content.

Major remark #2
As the papers present a new family of CRs, I was sometimes disappointed not to find more information on some of their 'basic' features. First and foremost, desensitization. It is present in most pLGIC involved in neurotransmission. Is it also a constitutive feature of those sensory receptors? Is there a sustained nerve response, an arm motion when compounds are applied for a long time? It was not straightforward to guess from the electrophysiology traces whether/how much desensitization was present, and the topic was barely discussed. To my eyes, including a section on desensitization would be 10 times more relevant than exploring Ca2+ permeability. Such a section could simply show the functional effect of prolonged applications of bitter compounds or terpenes and educate the reader on what is known or remains to be discovered.
There is also a conformational aspect to that remark. Both the CRT1 and the CRB1 pores seem to be in an open state (although I'm not sure it was stated explicitly for CRB1, maybe because the authors don't want to be too conclusive about it). In cryoEM, one observes the state stabilized by experimental conditions, and it's not always the state one would expect. But it should be discussed explicitly.
Second example, which is actually more a naïve question. Are the ECDs of the CRs exposed to salt water? If yes, then there are probably things to say about the electrostatics of that domain. If not, how do the compounds diffuse from the environment to the receptors? In particular for CRT1 and hydrophobic compounds, one wonders how terpenes would go from the prey to the receptor (partition to the membranes?). Here what is missing is maybe a few sentences to educate readers like myself, not familiar with sensory systems of marine organisms.

Specific remarks (roughly following reading through Kang et al. then through Allard et al.).

Line 45. "We then leverage…" I wasn't sure what information that sentence brought. What are the "conserved principles of evolutionary innovation" discovered?

CRs are found across coleoid cephalopods
It is not straightforward to understand the amount of novelty here, and the author's Cell paper from 2020 is not cited in this paragraph. Do I get right that the description of 3 CR groups (CRB, CRT, CRX, line 61) was not proposed before? (I understood that only when I reached line ~100).

CRB1 structural adaptation enable bitterant sensing
Very clear section, loved it!
As the authors mention there were 3 CRs in squid sensory cells and 13 in octopus, I wondered if there would be any evidence of the formation of heteromers (e.g difference of functional properties from native cells and heterologous expression). Even in the absence of evidence, one sentence could be helpful to warn the reader that heteromers are frequent in this family and that 13 CR subunits could lead to a daunting variety of receptors

Structure of CRB1
Line 131, "cation-permeable" is not explained. I guess it can be seen from the sequence of M2 that carries the determinant of cationic selectivity? I'm not sure whether this is shown anywhere but in Fig. 2c of the companion article.
By the way, there is no sequence of CRB1 in any SI figure. It should be included in the alignment provided (currently Fig. ED1 of the companion paper).
Also, looking at the alignment, I was surprised for CRT1 to see a lysine at position 20' of the pore. It is not usual for a cationic receptor, is it? I don't know the literature for nAChR but for the 5-HT3R, mutation of the equivalent E20' to a positive charge strongly impacts the speed of desensitization (PMID: 16096341)
What is the equivalent residue in CRB1 (It would help to have a pore view similar to Allard Fig. 2c…) ?

Comparing Fig ED5 of this paper and the equivalent Fig ED3 of the companion paper, one wonders if other detergents were also tested to try to identify conditions stabilizing a symmetric TMD in CRB1. If yes, it could be mentioned.
In any case, it would be appropriate to tell the reader a bit more about CRB1 TMD! I get from the SI that it is asymmetric. How much do the ECD and TMD deviate from symmetry, with comparisons to CRT1 and a7?
More generally, I wished there were more comparison panels in the spirit of Kang Fig. 4c and overlays as in Allard ED6 a-e. Overlays of different zones of CRB1 with CRT1 should be proposed. There are 11 panels showing close-ups on the binding site (my preferred ones were Kang. Fig 4c and ED8, which look great and are informative). Maybe some optimization of those would give enough space to show other overlays/comparisons.

Upon the first look at Fig. 3, tilt angle of the helices versus the membrane normal seemed large compared to nAChRs. Is that true or just a visual impression ? One more argument to have overlays + domain/subunit RMSDs.

Most of those structural remarks relate to the major issue #1.

Kang Fig 3d
This is where I started to wonder about desensitization. Are the traces showing desensitization (+ rebound currents due to pore block ? large at negative potentials!) at 50 and 100 uM of denatonium? This should be explicitly characterized, as it can have physiological relevance (see below remark on Allard ED7).

Allard. Conserved elements for signal transduction.
This section felt awkward. While it is fully interesting to look at determinants of Ca2+ permeability and at the omega loop conformation, it felt like a quite specialized topic for pLGIC aficionados made its way into a work that otherwise speaks to a way more general audience.
Why not, but aren't there tons of more general info to fit into the limited main text of a/two Nature paper(s)?

Line 126 "Diosgenin was curiously bound…"

The authors describe a ligand bound to the site where ligands bind in virtually every pLGIC. Can this be called a surprise? Do they mean that, because the CR1 ligands are hydrophobic, the receptor could have evolved binding sites in its TMD? Then ok, but this in turn requires the authors to elaborate on why M4 is not there, on densities surrounding the TMD, etc...
Do the authors think that there might be more than one binding site for the hydrophobic agonists? In that respect, the mutagenesis data presented in Fig 4d was interesting but not conclusive. With every mutant producing spontaneous activation, is it possible to rule out the existence of a second site? Was it not possible to find mutants that abolish the GDN or ntk response without producing this spontaneous activation (e.g. polar residues replacement of the aromatics)?

Allard Fig. 4b
What are the other zones with high selection, ie around residue 200 and 350. Could an analysis of those regions point to other important structural adaptations?
This panel was easy to understand and informative, maybe more than the Kang Fig 4a panel that, as far as I understood, just illustrate which subfamily appeared first and could have been in SI.

Allard Extended Data Figure 7.
Panel a shows the response to GDN in patch-clamp experiments, with a EC50 of 125 nM.
Panel b shows the neural response and arm behavior with apparently no response to 5 uM of GDN. One sentence in the legend is there to acknowledge this discrepancy, I suppose, which is also hinted at in the main text. Those two allusions felt insufficient. The serendipitously identified agonist GDN, the one seen in the structure, does not yield a neural or behavioral response, even if it triggers a response of CR1 not drastically different from agonists that do yield a behavioral response. Could the authors propose an hypothesis explaining this discrepancy?
Panel a made me wonder again about desensitization and pore block, that seem to be present for all three agonists tested, yet with differences. What is the physiological scenario? Very transient current because of the combined effect of desensitization and pore block? Is this why, in the arm experiments where the compound stays in the petri dish, there seems to be motion only for a few seconds (e.g. Fig ED3h)? But then, can the animal sense a prolonged contact with a prey through CRs or just the initial contact and then CRs are desensitized ?
Here, I do not expect the authors to provide complementary experiments, but rather to be more explicit/educational about the current ones.
What is this slow tail current with GDN at high concentrations? Is the interval between applications long enough here?

Allard Extended Data Figure 5
Please choose between cylindrical helix and cartoon representation. I was confused and thought one was AF and the other the experimental structure, as the difference in color was lost in the version I printed. It becomes clear only when zooming on the PDF.
The AF2 model seems to possess a helical M4 that packs on the rest of the TMD bundle. Yet it is absent in the cryoEM structure. Can one infer from the sequence that M4 could be not as tightly attached to the rest of the TMD as it is in other pLGIC? I see that the residues involved in the M2-M4 salt bridge are conserved, anything noticeable about hydrophobic/aromatic residues (made me think of PMID: 28348412)?
Why doesn't this figure include a panel on the prediction for CRB1? It could be interesting, especially

for the TMD. The asymmetric experimental conformation is unlikely to be present in the prediction, but would this TMD have a rather low pLDDT score that could be indicative of its dynamic nature? Whether one subunit structure was predicted or the whole pentamer should be mentioned (the latter being preferable).

Hugues Nury, Institut de Biologie Structurale, Grenoble, France

Author Rebuttals to Initial Comments:

SQUID

Referees' comments:

Referee #1 (Remarks to the Author):

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We thank Reviewer 1 for his enthusiasm for our broad comparative approach which integrates structural biology, biophysics, and animal behavior to probe the adaptative function of newly described chemotactile receptors. Indeed, a central message of our study is how CRs diverge from nAChRs to function in sensory roles and how receptor subtypes support distinct behaviors in squid and octopus. In this context, we are especially grateful to the reviewer for bringing an important citation (Jiao et al, 2019) to our attention. We have included this citation and added context regarding the position of CRs in relationship to the expansion of molluscan nAChRs reported in Jiao et al (2019). We find that CRs represent the most recent radiation nested within the expansion of molluscan nicotinic-like receptors, which includes characterized chemoreceptors consistent with their new role in sensation (Extended Data for CR position relative to *Lottia* nAChRs). In support of this conclusion, we distinguished CRs from nAChRs based on a number of criteria, including: (1) the most derived clade encompassing known chemoreceptors CRT1 and CRB1 (2) absence of canonical acetylcholine binding regions; (3) genomic architecture (4) expression in sucker sensory cells versus organs in which canonical nAChRs are typically found; (5) extensive heterologous an native functional analyses; and (6) structure and ligand selectivity of the founding members of two CR lineages. Thus, our results robustly support CRs as novel radiation of sensory receptors. Molluscan expansions have unknown functions, but may represent an enhanced diversity of nicotinic receptors for neurotransmission (they contain residues important for binding acetylcholine) or may also be involved in other functions, yet expression and physiological analyses are required to assess their functional roles.

The nerve recordings seem to be summed activity so that it is difficult to know if single neurons respond to both substances or if individual neurons are selective to one or the other substance. In other words, if one substance (terpene) is an attractant and the other (denatonium) is aversive. Have the authors tried to sample individual afferents by waveshape to tease apart whether there are separate bitter and terpene sensitive neurons sending this information forward?

The rejection of denatonium-laced prey by squid is like the vertebrate distaste for bitter substances. It would be interesting to compare human bitter receptors (I think these are GPCRs) with these novel receptors to compare how they interact with bitter substances. And why do we or squid avoid bitter substances? Are they harmful in some way? Humans come to like various bitter things (Brussel sprouts

come to mind) I wonder if in time squid would eat them too if all their food was bitter and they had no choice in the matter. Just some “food for thought” but nothing the authors need to do on their MS.

Harold Zakon

We strongly agree with Reviewer 1: an important future direction of this work is to determine which natural, ecologically relevant stimuli and/or combinations of stimuli represent appetitive or aversive stimuli, and how distinct sensory signals are differentially encoded in the complex cephalopod peripheral nervous system.

To begin to address this question, we established a nerve recording preparation in order to probe which physical and chemical stimuli are sensed and transduced in cephalopod arms and tentacles. While we are not yet able to distinguish individual nerve fibers (we have tried), remarkably, we found that lineage-specific CR agonists, but not classical appetitive stimuli such as amino acids or sugars, resulted in robust neural responses, suggesting that CR-mediated chemotactile sensation is a prevalent sensory modality in cephalopods.

Regarding CR pharmacology, so far, we have identified agonists that are classically thought of as aversive stimuli and we have not yet identified a specific cloned receptor or heteromeric combination that mediates apparent appetitive prey-seeking behaviors. Therefore, it is tempting to speculate that the CRs characterized to date mediate sensation of aversive stimuli with the intriguing possibility that octopus arms explore seafloor cracks and crevices using tactile cues / mechanoreceptors (van Giesen, 2020) and chemotactile sensation triggers avoidance of harmful substances or objects. Alternatively, cephalopods may rely on appetitive cues that differ from those identified for other organisms, and therefore await identification. Or, we have yet to identify CRs for appetitive cues. Moving forward, our long-term goal is to distinguish among these and other possibilities by using extensive analyses spanning natural product chemistry to identify small molecule agonists, receptor pharmacology and neurophysiology, and probing whether such molecules elicit distinct arm and animal behavioral responses. We, too, are excited for these important future directions in cephalopod neuroscience.

As an even further-reaching future direction, we agree that comparing cephalopod chemotactile sensation to other chemosensory systems is an exciting prospect made possible by structure-function analyses in this study. Indeed, such comparisons have significant potential to uncover reveal conserved principals of sensory system adaptation from ancient systems within invertebrates to more derived senses like mammalian taste. As we continue to understand natural ligands, identify and characterize additional CRs, and probe their behavioral valence, we will be positioned to compare binding sites and other functional features across cephalopod sensory receptors and beyond!

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We thank the reviewer for suggesting ways to improve the presentation and scope of our evolutionary findings and the enthusiasm regarding how these analyses complement the major focus of our study in functionally probing the diversity and role of CRs across coleoid cephalopods with distinct behaviors. We have now moved Figures 1a-b to Figures 4a-b and improved their presentation. Additionally, we now have a new figure, Extended Data Figure 9, summarizing evolutionary analyses addressing topology inference as well as time estimates for species and sequences. The reviewer’s helpful comments and suggestions greatly improved the clarity, analyses, and discussion of receptor evolution.

Firstly, the tree representation in Fig 1b does not necessarily imply the "ancestral-ness" of squid CRs (i.e., CRBs). The tree topology generally shows split in octopus CRs and squid CRs, which, as the authors describe, may have driven adaptations to different environments and ecological challenges (which, we find, is a very exciting finding). In the "traditional" sense, the finding may simply be that there were independent expansions of CRs in squids and octopuses. One could generally call all CRBs simply squid CRs. Then, CRBT clade may also be interpreted as the most ancestral clade (since it has both squid and octopus genes). In this view, squids may be seen as more derived. Secondly, the tree itself would benefit from a better representation by at least showing support values or labeling supported branches. The authors may select only the sequences shown in Fig 1b and construct an independent tree to validate the branching pattern and further support CRB/CRT/CRX groupings.

We regret that our previous presentation used too similar of colors to indicate different cephalopods and distinct CR groups, as it might have prevented a comprehensive understanding of the phylogenetic relationships between CRs. We have now changed the color scheme in the now Fig. 4b so that the CRs lineage does not read as simply splitting between octopus and squid CRs. Although the CRB subfamily is specific to squid and the CRX family specific to octopuses, the CRT clade comprises representative sequences from octopuses and squids. Therefore, CRB is not strictly interchangeable with all squid CRs in the same sense as CRX is not synonymous with all octopus CRs. CRB is the earliest divergent, oldest lineage of the CRs and it is sister to the CRB-CRX clade. Hence, there is not a “CRBT” clade, because a group with these two lineages would be rendered paraphyletic. Additionally, although CRT clade is the only family encompassing squid and octopuses CRs, it is the sister clade to CRX, and as such, a more nested lineage which cannot be interpreted as the most ancestral clade. Consequently, considering these groupings that sometimes span Decapodiformes and Octopodiformes, we felt it was appropriate to define CR subtypes based on functional properties rather than species.

We thank the reviewer for suggesting ways to improve the representation of the trees. We now include support values for all branches of the tree in Extended figure 8a, showing that CRs clade has a maximum bootstrap value of 100, and that all internal nodes of the CRs have high bootstrap values (over 90). Thus, validating the branching pattern and further supporting the CRB/CRT/CRX groups. Moreover, we now include a densitree (Extended Figure 8d) depicting the distribution of bootstrap topologies only for the CR sequences, which lends support to CRB as the earliest divergent lineage sister to the CRT-CRX clade.

The general interpretation of the results should also be reconsidered. For example, sentences like "duplication and specialization via structural modification of specific functional regions that is driven by lineage-specific behavior" should be reworded as it is not how evolution works ("associated with lineage-specific behavior"?). Overall, the findings, taken from the evolutionary perspective, seem "expected" (differential expansion of gene families), therefore, while very interesting, may seem incremental to what the authors have already published a few years back.

We agree with the reviewer regarding our interpretation of evolutionary results and have altered the text to convey that evolutionary adaptations are “associated” with functional properties. Our previous work (van Giesen et al. 2020) was restricted to Octopodiformes species, was concerned only with topologies, and established that octopus CRs are divergent sensory receptors from acetylcholine receptors. In this work, we have significantly expanded our phylogenetic sampling from octopods to representatives from all major clades of cephalopods and outgroup mollusks. This extensive, deep-time sampling has allowed us to characterize the tempo and mode of CRs evolution, as well as to establish that functional and structural differences in diverse sensory receptors correspond to distinct lineages, thus substantiating the description of new gene families. In this context, our analyses provide the first comprehensive study of CR evolution. Nevertheless, we agree that this is not the most significant advance provided by our manuscript. These results are important in contextualizing the evolution of CRs across the cephalopod radiation, complementary to the major focus of our work which spans atomic, biophysical, and physiological levels to understand how structural adaptations support behavior.

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We have addressed all “minor points” by either altering or removing the text, as advised by the reviewer. We also added the suggested table describing tissues used for transcriptomic analyses. Regarding the outgroup analyses, we further described our methods for iterative searches, which used jackHMMER with an e-value of 10e-5. Additionally, we performed sequence analyses of the closest relatives from nautilus and other molluscs with attention to the orthosteric binding site to note that some of the key residues which facilitate acetylcholine binding were present in nautilus receptors and absent in CRs. We do not rule out that some of the *Nautilus* sequences in the “non-alpha” sister group of the CRs may represent sensory receptors, but a more exhaustive expression and functional characterization is needed to test these sequences. In addition, CRs are primarily enriched in sucker cups, an evolutionary innovation of coleoid cephalopods, which might explain why no *Nautilus* sequences are nested within the CR group.

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The Allard et al. paper briefly introduces the CR family and then jumps directly in the description of the cryoEM structure of CRT1. The architecture is described, and then a bizarre parenthesis on determinants

of Ca2+ permeation is proposed. The authors then come back to a more classical identification/description of the binding site, which happens to be the orthosteric site. The hydrophobic part of the detergent used for purification sits there, is found to be a potent agonist but does not trigger arm response. The structural analysis is expanded in regards with the evolution of the CR family.

I first want to congratulate the authors: the work is impressive, both in its diversity and in its depth. I was in awe reading about two new subtypes of sensory receptors characterized at the structural, functional, ex vivo and behavioral levels. I can tell how much work went into some single panel of an ED figure (e.g Allard Fig ED 3).

Major remark #1
However, the way the two stories are presented was, in my opinion, unsatisfactory. I understand the logic of trying to get two papers from the excellent set of data presented, but to my eyes it is not working in the present form. Bits of related information that may well go together are separated in the two papers (e.g. the last sections, starting line and 149 and line 156 in Kang et al. and Allard et al. respectively). Sometimes this dual presentation yields redundancies (e.g. Fig 4c-e with Fig. 3c). Sometimes information is missing (or maybe just too hard to find); in many instances I would have liked to have had a pedestrian side-by-side comparison of CRB1, CRT1 and a7. In more specific comments below, I try to point out places where scattering the data over two papers looked the most problematic.
My point is not to suggest that a looong article would be more appropriate than two letters. I don't know. However, I want to underline that, in the current presentation, a reader has to jump constantly from one to the second paper and back. This back and forth is detrimental to an/my overall understanding of the content.

We thank Reviewer 3 for his excitement regarding the diversity, depth, and sheer volume of work in our studies! We share the reviewer's enthusiasm for the foundational nature of these studies in describing new sensory receptors from structure, molecular and cellular function, to comparative animal behavior.

Indeed, organizing the amount of data in these two studies into comprehensive stories was challenging and we appreciate the reviewer's constructive feedback toward improving presentation. We have incorporated many of the suggestions, which has resulted in a much stronger analysis. In our initial submission, we intended for the octopus structure manuscript to be read before the comparative study (the manuscript numbering however was in the opposite order, which was our oversight). The first study presents the seminal structure-function comparison of octopus CRT1 with α7 and describes the structural basis by which a family of novel sensory receptors evolved in cephalopods. The second manuscript focuses on the discovery and characterization of a new CR family in squid to comparatively ask how sensory receptor tuning drives new behaviors. Together, these comprehensive and complementary studies provide a basis for understanding how subtle structural analysis give rise to new receptors functions to facilitate novel organismal behaviors suited to specific ecological contexts. Nevertheless, we completely agree that these two studies can and should be organized so that each stands independently, without forcing the reader to reference the other.

To that end, we have reorganized the presentation of these paired but independent manuscripts. In the octopus structural study, we now present desensitization results and make clearer comparisons between the experimental and predicted atomic models. Our most significant reorganization takes place in the comparative squid manuscript, which we feel greatly improves the clarity and independent message of this novel and broadly accessible study. We now begin with comparative analysis of octopus and squid predatory behavior, discovery and characterization of squid receptors, squid CR structure and function, then leverage this information to probe evolution across cephalopods by comparing structural features, phylogeny, and function toward understanding how protein adaptations support novel lineage-specific behaviors. Beyond the extensive sequence evolution, physiology, and behavior, in the structural component of the comparative study, we have included more side-by-side comparisons to highlight regions of conservation and functional divergence related to receptor function and discuss organismal relevance. For example, we now compare the electrostatic properties of the different receptors to relate the

environment to the pharmacology and behavior. Further, we now include AlphaFold2-based predictions for all models, with a new comparative analysis that gets at why the octopus CRT1 receptor has at best a very loose interaction M4 helix, versus squid CRB1 and α7. Collectively, this reorganization makes a clear distinction between the focus on the emergence of novel traits in octopus versus asking how structural adaptations in receptor proteins adapt to support the lifestyle of ecologically and behaviorally distinct taxa.

Major remark #2
As the papers present a new family of CRs, I was sometimes disappointed not to find more information on some of their 'basic' features. First and foremost, desensitization. It is present in most pLGIC involved in neurotransmission. Is it also a constitutive feature of those sensory receptors? Is there a sustained nerve response, an arm motion when compounds are applied for a long time? It was not straightforward to guess from the electrophysiology traces whether/how much desensitization was present, and the topic was barely discussed. To my eyes, including a section on desensitization would be 10 times more relevant than exploring Ca2+ permeability. Such a section could simply show the functional effect of prolonged applications of bitter compounds or terpenes and educate the reader on what is known or remains to be discovered.

Our study shows that despite remarkable conservation of general architecture between nicotinic and CRs, subtle divergence in specific regions drives vastly different functional roles in neurotransmission vs. sensation. In this respect, we think analyzing structure-function relationships for similarities between CRs and nAChRs is important for testing and contextualizing this observation. Among the important conserved tractable structural features is the outer vestibule, which controls calcium permeation, an important molecule in signal transduction with implications for cellular excitability and neural signaling. It is also the first region that cations encounter as they travel toward the transmembrane pore, so it felt natural to describe the vestibule first. Following analysis of conserved features, we then turn our attention to notable differences, with particular attention to ligand binding between neurotransmission and environmental sensation.

In this context, we thank the reviewer for the excellent suggestion of further analyzing CR desensitization, experiments that we have now included in both manuscripts (ED Fig. 7 in Allard et al and ED Fig. 3 in Kang et al). As the reviewer mentions and we previously described in our initial study of CRs, desensitization is minimal. While we do not understand the structural basis for this difference compared with α7, we observe that high agonist concentration elicits inhibition followed by large wash-off or “tail” currents. This effect is stronger at negative holding voltages and is present in binding site mutants, consistent with another site of action. Both results suggest the mechanism can likely be attributed to pore block, yet the physiological implications are unclear. We reason that minimal desensitization could contribute to prolonged nerve and arm responses that last seconds or longer, although there are numerous signaling events between receptor activity and arm movement. We hypothesize that lack of strong receptor desensitization in CRs could reflect an adaptive feature of channel function toward mediating sustained chemotactile sensation, but this is highly speculative as we do not yet understand cellular or neural transduction in this system.

There is also a conformational aspect to that remark. Both the CRT1 and the CRB1 pores seem to be in an open state (although I'm not sure it was stated explicitly for CRB1, maybe because the authors don't want to be too conclusive about it). In cryoEM, one observes the state stabilized by experimental conditions, and it's not always the state one would expect. But it should be discussed explicitly.
Second example, which is actually more a naïve question. Are the ECDs of the CRs exposed to salt water? If yes, then there are probably things to say about the electrostatics of that domain. If not, how do the compounds diffuse from the environment to the receptors? In particular for CRT1 and hydrophobic compounds, one wonders how terpenes would go from the prey to the receptor (partition to the membranes?). Here what is missing is maybe a few sentences to educate readers like myself, not familiar with sensory systems of marine organisms.

The reviewer correctly interpreted our caution in assigning a functional state to the structure of CRB1. It felt speculative and was not central to our evolutionary analysis. However, with the new results, we can

say more. As mentioned above, the channel does not substantially desensitize in the sustained presence of agonist. The pore in the structure has a minimal diameter of 7 Å that would permit passage of hydrated cations and is in line with estimates of open pore diameter for other cationic pLGICs (e.g., PMID: 6247422). The functional result is consistent with the pore size indicating an activated state / conducting conformation. The asymmetric nature of the pore conformation was the basis of our caution. While there is a precedent in a recent 5-HT_3 paper for an asymmetric homopentamer (PMID 33594077), there is a possibility that the conformation relates to EM sample preparation and is not physiologically meaningful (membrane mimetic, air-water interface, freezing process; the pore is very widely open). To address the sample preparation part of the question, we now include in ED Fig. 5a-d a comparison of TMD structures in different membrane mimetics, including detergents vs. nanodiscs. None resulted in a well-ordered, symmetric TMD, which suggests GDN is as good as other common choices for stabilizing the receptor TMD. We now include an explicit discussion of conformational state. To support this discussion, we include several new figure panels (two bottom rows in ED Fig. 7, in Kang et al) where we compare pore profiles, and symmetry and deviations thereof in CRT1 vs. CRB1 in the TMD and ECD. We additionally present electrostatic analysis of the permeation pathways in this figure.

Yes, the ECDs are exposed directly to salt water. We appreciate this helpful question as it motivated more electrostatic analyses. We were interested to see that, based on simple analysis, the vestibules and pores are less electronegative compared to α7, when these estimates are made in 140 vs. 500 mM NaCl. In future work, we are curious to see if these differences relate to lower single channel dynamics in the cephalopod receptors. How do compounds from the prey surface reach the receptor binding site? We favor the idea that these molecules are not freely diffusing, like a neurotransmitter or hormone. Instead, solubility and/or localization keeps molecules close to surfaces allowing cephalopods to use chemotactile sensation for detection. Indeed, we previously showed that octopuses sense terpenes in a contact-dependent manner (van Giesen 2020).

Specific remarks (roughly following reading through Kang et al. then through Allard et al.).

Line 45. "We then leverage…" I wasn't sure what information that sentence brought. What are the "conserved principles of evolutionary innovation" discovered?

We agree that this sentence was somewhat redundant; we have removed it.

CRs are found across coleoid cephalopods
It is not straightforward to understand the amount of novelty here, and the author's Cell paper from 2020 is not cited in this paragraph. Do I get right that the description of 3 CR groups (CRB, CRT, CRX, line 61) was not proposed before? (I understood that only when I reached line ~100).

We clarified that the description of the three groups is new, as is the characterization of CRs in squid and cuttlefish.

CRB1 structural adaptation enable bitterant sensing
Very clear section, loved it!
As the authors mention there were 3 CRs in squid sensory cells and 13 in octopus, I wondered if there would be any evidence of the formation of heteromers (e.g difference of functional properties from native cells and heterologous expression). Even in the absence of evidence, one sentence could be helpful to warn the reader that heteromers are frequent in this family and that 13 CR subunits could lead to a daunting variety of receptors

Indeed, we described that octopus CRs are capable of forming heteromers, which could facilitate tremendous diversity in ligand binding and signal transduction (van Giesen, 2020). We propose that such sensory receptor organization could be well-suited for encoding diverse signals among the octopus's distributed nervous system. Yet, we do not yet understand the functional or behavioral implications in

octopus and have not yet observed heteromerization in squid (although we might not have identified and cloned the subunits that form correct combinations). We added text to describe potential receptor diversity and the physiological implications.

Structure of CRB1
Line 131, "cation-permeable" is not explained. I guess it can be seen from the sequence of M2 that carries the determinant of cationic selectivity? I'm not sure whether this is shown anywhere but in Fig. 2c of the companion article.

Thank you for pointing out this vaguely supported statement. The conclusion of cation permeability is based primarily on ion substitution experiments, but also as pointed out on sequence in the M2 helices. With glutamates at the -1' position as in most nicotinic receptor subunits and 5-HT_{3A}, the channel would be expected to select for cations. We now include calls to the IV curves to support the conclusion of cation permeability.

By the way, there is no sequence of CRB1 in any SI figure. It should be included in the alignment provided (currently Fig. ED1 of the companion paper).

Absolutely, we agree, thank you. This comment made us think more about how to improve the existing alignment, as well. We now include a sequence alignment as Extended Data Figure 8 in Allard et al. that has a CRT1 vs. α7 alignment adjacent to a subunit structure, to highlight rapidly evolving residues. We further include a new alignment with CRB1 in Kang et al. as a Supplementary Data Figure.

Also, looking at the alignment, I was surprised for CRT1 to see a lysine at position 20' of the pore. It is not usual for a cationic receptor, is it? I don't know the literature for nAChR but for the 5-HT3R, mutation of the equivalent E20' to a positive charge strongly impacts the speed of desensitization (PMID: 16096341). What is the equivalent residue in CRB1 (It would help to have a pore view similar to Allard Fig. 2c…) ?

Both CRB1 and CRT1 have a lysine at 20' in M2. As reviewer 3 pointed out, the 20' position is often negatively charged in cationic channels such as 5-HT_3 (usually aspartate, one asparagine) and α subunits of nicotinic receptors (usually glutamate). A lysine 20' is found in β2 and β4 nicotinic receptor subunits of the cationic permeable heteromeric α4β2 and α3β4 receptors, which is consistent with CRB1 and CRT1. Increasing the number of lysines in an α4β2 nicotinic receptor was shown to decrease relative calcium permeability (PMID: 17132685). Accordingly, this is a position that can be used to tune gating (desensitization) and conductance. An interesting question we are pursuing relates to heteromeric assembly, where these subunits with positive charges at 20' might be expected to act like nicotinic beta subunits (with regard to permeation and gating contributions from that site).

Comparing Fig ED5 of this paper and the equivalent Fig ED3 of the companion paper, one wonders if other detergents were also tested to try to identify conditions stabilizing a symmetric TMD in CRB1. If yes, it could be mentioned.

Yes, great point, we have now added this comparison for CRB1 in Kang et al ED Fig. 5a-d (results described above).

In any case, it would be appropriate to tell the reader a bit more about CRB1 TMD! I get from the SI that it is asymmetric. How much do the ECD and TMD deviate from symmetry, with comparisons to CRT1 and a7?

Great question, addressed in the same ED Figure and discussed above.

More generally, I wished there were more comparison panels in the spirit of Kang Fig. 4c and overlays as in Allard ED6 a-e. Overlays of different zones of CRB1 with CRT1 should be proposed.

There are 11 panels showing close-ups on the binding site (my preferred ones were Kang. Fig 4c and ED8, which look great and are informative). Maybe some optimization of those would give enough space to show other overlays/comparisons.

We appreciate reviewer 3's comments on optimizing panels, which makes the figures more straightforward and informative. We condensed panels on the binding site as suggested and focused more on the different zones of comparative studies, including pore conformation and TMD interactions, as described above in replies to earlier queries. An example of condensed binding site comparisons is in Kang et al (Squid) Fig. 4, where we incorporate electrostatics into the same details present in the original figure. We now include a comprehensive set of subunit superpositions in Kang et al (Squid) ED Fig 8.

Upon the first look at Fig. 3, tilt angle of the helices versus the membrane normal seemed large compared to nAChRs. Is that true or just a visual impression ? One more argument to have overlays + domain/subunit RMSDs.

We agree that this is an important point to address; the overlays of subunits and the corresponding rmsd values are now included (Kang et al (Squid) ED Fig 8). The M4 helix angles (as a reference point) of CRB1 and α7 putative activated state are similar; less than 1 degree different compared to a reference plane.

Most of those structural remarks relate to the major issue #1.

Kang Fig 3d
This is where I started to wonder about desensitization. Are the traces showing desensitization (+ rebound currents due to pore block ? large at negative potentials!) at 50 and 100 uM of denatonium? This should be explicitly characterized, as it can have physiological relevance (see below remark on Allard ED7).

Similar to octopus CRT1, squid CRB1 minimally desensitizes at low concentrations but exhibits some (likely) pore block at high concentrations. We now characterize this phenotype at low and high concentrations (ED Fig. 3). Consistent with pore block, high concentrations of denatonium show the most pronounced block at negative potentials, which is followed by a large wash-off current. Minimal desensitization seen in CRB1 is consistent with prolonged nerve and behavioral responses, as described above.

Allard. Conserved elements for signal transduction.

This section felt awkward. While it is fully interesting to look at determinants of Ca2+ permeability and at the omega loop conformation, it felt like a quite specialized topic for pLGIC aficionados made its way into a work that otherwise speaks to a way more general audience.
Why not, but aren't there tons of more general info to fit into the limited main text of a/two Nature paper(s)?

As described in response to major remark #2, we emphasized the strikingly similar structural features underlying calcium permeation to demonstrate that many facets of the conserved channel architecture result in similar function. We think this important general point helps demonstrate how subtle differences in specific regions drive functional differences that correspond with new organismal functions – neurotransmission to sensation.

Line 126 "Diosgenin was curiously bound…"
The authors describe a ligand bound to the site where ligands bind in virtually every pLGIC. Can this be called a surprise? Do they mean that, because the CR1 ligands are hydrophobic, the receptor could have evolved binding sites in its TMD? Then ok, but this in turn requires the authors to elaborate on why M4 is not there, on densities surrounding the TMD, etc...
Do the authors think that there might be more than one binding site for the hydrophobic agonists? In that respect, the mutagenesis data presented in Fig 4d was interesting but not conclusive. With every mutant producing spontaneous activation, is it possible to rule out the existence of a second site? Was it not

possible to find mutants that abolish the GDN or ntk response without producing this spontaneous activation (e.g. polar residues replacement of the aromatics)?

We understand the point. It should not be a surprise to find that an agonist binds where every other agonist (except for protons) binds to this superfamily. Before we obtained the structure with this ligand bound, we were, frankly, expecting agonists to bind this receptor in the TMD. They are all quite hydrophobic. So, for us, it was quite a surprise, but we did an ineffective job of explaining this to readers. We have revised the text to more clearly explain why we found it surprising. It is remarkable that what is usually a polar crevice in the neurotransmitter receptors (and to a degree in CRB1) has been flattened and made hydrophobic and electrostatically neutral, such that steroid-like molecules can bind there and activate the channel. We did not see densities suggestive of any ligands binding in the TMD, for example at anesthetic- or neurosteroid-like sites, however we cannot rule out the possibility of a second site. We agree that the functional data is not completely conclusive, with the result that all mutants, while they decrease sensitivity to agonist, also cause a gain of function.

Allard Fig. 4b
What are the other zones with high selection, ie around residue 200 and 350. Could an analysis of those regions point to other important structural adaptations?

Good question. Using a cutoff of LRT of ≥3, 24 residues meet this criterion of positive diversifying selection, meaning these positions are mutating faster than predicted by random chance. Approximately half of these positions are on the six classical loops that contribute to the orthosteric agonist site. 6 are in post-M3 disordered regions (5 between potential MX and potential M4, and one in potential M4 helix span). 6 are scattered through the ECD. We looked carefully at each of them and found they do not cluster together or participate in a known network that connects to evolved biophysical properties. The clustering observed in loops that participate directly in agonist binding are comparatively easy to understand. A note that we slightly modified this bar graph figure panel (Allard (Octopus) Fig. 4b and Fig. ED 8) to remove the signal peptide from the numbering scheme; the numbering now matches that in the atomic model.

This panel was easy to understand and informative, maybe more than the Kang Fig 4a panel that, as far as I understood, just illustrate which subfamily appeared first and could have been in SI.

We agree that the Kang et al (Squid) Fig. 4a could be improved in its presentation. As the reviewer suggests and we described above, we have reorganized the manuscript to more clearly show when CRs originated in coleoid cephalopods and that the CRB1 family appeared first within a phylogenic tree. The original fourfold degenerate site distance analysis (4DTv) is an important additional analysis to ask about the evolutionary timeline and we have moved it to ED.

Allard Extended Data Figure 7.
Panel a shows the response to GDN in patch-clamp experiments, with a EC50 of 125 nM. Panel b shows the neural response and arm behavior with apparently no response to 5 uM of GDN. One sentence in the legend is there to acknowledge this discrepancy, I suppose, which is also hinted at in the main text. Those two allusions felt insufficient. The serendipitously identified agonist GDN, the one seen in the structure, does not yield a neural or behavioral response, even if it triggers a response of CR1 not drastically different from agonists that do yield a behavioral response. Could the authors propose an hypothesis explaining this discrepancy?

As mentioned above, we serendipitously found GDN – the detergent used for biochemical purification – bound to CRT1 in the cryo-EM structure, thereby facilitating identification of the ligand binding pocket and additional structurally similar (non-detergent) hydrophobic ligands. As the reviewer notes, GDN was not as effective on arm nerves and behavior as it was on expressed receptors. We agree that it is important to further comment on these observations and have added discussion in the ED to reflect some of these important technical considerations that could underlie this result:

1) Perhaps most likely, GDN is a detergent and we used it at a lower concentration than other ligands sampled (5 μM is near its CMC). All ligands (either previously characterized or newly identified in this study following the GDN observation) were used at 50 μM or higher for behavioral assays. Furthermore, these concentrations are likely overestimations because all ligands have relatively poor solubility and were added via pipetting toward arms stabilized in a custom-made recording chamber. Perhaps needless to say, this is a challenging preparation developed for and used for the first time during this study. Remarkably, the ligand sensitivity of CRs is well reflected in arm nerve activity and autonomous behavior, further substantiating a role for these receptors in chemotactile sensation.
2) Isolated receptor sensitivity may not directly correlate with behavioral responses because we do not yet know how different receptor activity is encoded at the level of distinct sensory cell electrical tuning, synaptic release, circuit integration, and muscle recruitment. It is very possible (and perhaps likely considering the newly carried out sequence evolution analysis) that certain ligands bind as-yet uncharacterized CRs. In this case, simultaneous activation of multiple CRs could elicit synergistically amplified or silenced responses depending on circuit level integration. Describing the nervous system organization downstream of CRs is an important future step toward understanding how the arms process sensory signals to carry out autonomous behavior.

Panel a made me wonder again about desensitization and pore block, that seem to be present for all three agonists tested, yet with differences. What is the physiological scenario? Very transient current because of the combined effect of desensitization and pore block? Is this why, in the arm experiments where the compound stays in the petri dish, there seems to be motion only for a few seconds (e.g. Fig ED3h)? But then, can the animal sense a prolonged contact with a prey through CRs or just the initial contact and then CRs are desensitized ?

We discuss this point above (major remark #2). Indeed, all ligands elicit relatively minimal desensitization in CRs and nerve and arm responses persist for many seconds or sometimes longer. While the kinetics of ligand detection and predation remain to be determined, particularly in the context of other influencing sensory cues (vision, touch), we have observed that octopuses reduce touch duration in the presence of ligands to modulate chemotactile exploration (van Giesen 2020). Thus, initial contact along the lengths of the arms could be most important for facilitating predation within the cracks and cervices of the sea floor. Nevertheless, we agree with the reviewer that this is an interesting point and expect that future analyses of cellular transduction at the level of voltage-gated channel amplification, synaptic transmission, and circuit level integration will provide further context for how receptor level filtering modulates arm behavior. We added text to clarify arm desensitization.

Here, I do not expect the authors to provide complementary experiments, but rather to be more explicit/educational about the current ones.
What is this slow tail current with GDN at high concentrations? Is the interval between applications long enough here?

As the reviewer notes, GDN currents have slow off kinetics, even with long intervals between application. This could be biological, however GDN is a detergent, and, consistent with detergents being notoriously problematic for patch-clamp recordings, it is difficult to wash off and cells do not tolerate it well at high concentrations near the CMC (when tail currents become most prominent). Overall, GDN served as an unanticipated and useful tool for discovery but is not the most tractable model ligand (nor physiological) for biophysical studies and their relationship to behavior.

Allard Extended Data Figure 5

Please choose between cylindrical helix and cartoon representation. I was confused and thought one was AF and the other the experimental structure, as the difference in color was lost in the version I printed. It becomes clear only when zooming on the PDF.

Sorry about that, we certainly agree. We have changed those two panels to be clearer and bring in one subunit colored by prediction confidence, as the reviewer suggests (pLDDT score) below.

The AF2 model seems to possess a helical M4 that packs on the rest of the TMD bundle. Yet it is absent in the cryoEM structure. Can one infer from the sequence that M4 could be not as tightly attached to the rest of the TMD as it is in other pLGIC? I see that the residues involved in the M2-M4 salt bridge are conserved, anything noticeable about hydrophobic/aromatic residues (made me think of PMID: 28348412)?
Why doesn't this figure include a panel on the prediction for CRB1? It could be interesting, especially for the TMD. The asymmetric experimental conformation is unlikely to be present in the prediction, but would this TMD have a rather low pLDDT score that could be indicative of its dynamic nature? Whether one subunit structure was predicted or the whole pentamer should be mentioned (the latter being preferable).

These are great questions that pushed us to look more closely at the predicted and experimental models for all three structures (CRT1, CRB1, α7). We look in detail at the CRT1 octopus receptor in Allard et al ED Fig. 5. We further perform a new extensive analysis of interactions among M1/M3/M4 helices and find that, while α7 and CRB1 have many hydrophobic contacts stabilizing M4 in contact with the rest of the TMD bundle, nearly all of these are missing in CRT1, consistent with M4 being structurally unobserved. These results are shown in Kang (Squid) ED Fig. 9.

Hugues Nury, Institut de Biologie Structurale, Grenoble, France

Reviewer Reports on the First Revision:

Referee #1 (Remarks to the Author):

This is an exciting paper based on large amounts of data utilizing different techniques from behavior, physiology, biophysics, molecular biology and cryo-EM structural studies. The authors responded thoughtfully to the comments of all three reviewers which has made this and the companion paper more readable and better integrated.

I have no further comments.

Referee #2 (Remarks to the Author):

Thank you for carefully addressing our previous comments.

Perhaps a single remaining "major" comment is on the phylogenetic tree in EDF10, where S. lineolata is placed as sister to D. pealei and E. berryi. This is probably wrong placement, since S. lineolata has been identified as sister to E. berryi in several phylogenies including mitochondrial and transcriptome-based studies (forming Sepiolida order, whereas Doryteuthis is in Myopsida, several papers, e.g., Sanchez et al., 2021 and Anderson and Ligdren 2021 etc). The position in this manuscript could be erroneous due to the evolutionary model used as well as mtDNA. While you can also use IQ-tree for this, similar as you did to assess the relationships among CR, it maybe more advisable and less confusing to the readers to exclude that tree and cite divergence estimates provided by any other paper (unless you want to redo the tree with many species and on coding genes).

My other comments are only minor:
Line 70: in the phrasing it is not clear why CRB1 is a “founding member” of the CRB or CR.
Line 159: DecapodiformeS

In some place S. lineolata appear as S. lineolate
Extended Figure 10d: green should be CRX
Figure 4b should be “Decapodiformes”
Make sure that all EDFs are cited correctly in the text (there is some confusion regarding EDF10 vs 8)

Referee #3 (Remarks to the Author):

The points raised in the first round of review were satisfactorily addressed in the revised versions of the manuscripts by Allard et al. and Kang et al.
The evolutions of the two manuscripts were also explained in detail in exhaustive rebuttals.
The manuscripts appeared to me to be both more accessible and more complete, thanks to the changes and additions made in the review process.

As requested, I paid attention to the statistics and error bars, which were described appropriately.

Hugues Nury

Author Rebuttals to First Revision:

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Referee #2 (Remarks to the Author):

Sensory specializations drive octopus and squid behavior

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Make sure that all EDFs are cited correctly in the text (there is some confusion regarding EDF10 vs 8)

We removed the phylogenic tree from Extended Data Fig. 10 and cited the suggested studies. We also addressed the minor text changes throughout.

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