Peer Review File

Manuscript Title: Structural basis of sensory receptor evolution in octopus

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referee expertise:

Referee #1: ion channels, evolution

Referee #2: evolution, cephalopods

Referee #3: structural biology, pentameric LGICs

Referees' comments:

Referee #1 (Remarks to the Author):

This paper is an excellent example of a “soup to nuts” study from details of receptor structure and ligand binding to neurophysiology and behavior in the service of elucidating how a receptor molecule—a nicotinic acetylcholine receptor—jettisoned a highly conserved sensitivity to the neurotransmitter acetylcholine and evolved novel binding to naturally-occurring “greasy” ligands in an octopus chemoreceptor. This is also likely to be the beginning of a long and rich story as there are 27 tandemly arranged duplicate genes of this receptor type on one chromosome so there is likely to be interesting divergence of sensitivities across receptors.

The result are novel and beautifully done. The paper is very readable, the separate sections are well integrated, and the analyses and results are clearly described and illustrated.

I would like to see further discussion of the stimuli. The main stimulus was fish juice. From a brief look at a few papers on Wikipedia (I’m not expert here), I see that stomach contents show that Octopus vulgaris mainly eat crustaceans and mollusks and only rarely fish. The octopuses in the lab are fed crabs. Are nootkatone and zearalenone merely used as pharmacological agents to represent terpenes? Are they components of anything in the octopus’ diet? What is the effect of placing a sucker on the carapace of the crabs that the octopuses are fed in the lab? Does this stimulate the receptor?

The movement of the arm is scored. Is it possible to tell if these are appetitive or aversive movements? Can the arm make this type of decision? Or is this possible without communication with the central nervous system?

While the alpha7 nicotinic acetylcholine receptor functions as a homomer, many vertebrate nAChRs are heteromeric. Is there evidence, besides the similar responses in the expressed receptor and

native receptor, that CRT1 functions as a homomer in the octopus? Is it known if more than one receptor type is expressed in one sucker?

In fig. 1 the middle panel shows multiple CRT1 expressing cells in a sucker. Does each sucker express CRT1 in some cells or is CRT1 specialized so that it is expressed in only a few suckers?

Referee #2 (Remarks to the Author):

The paper by Allard et al., "Structural basis of sensory receptor evolution in octopus" describes the cryo-EM structure of the previously reported chemotactile receptors in octopus. Its main finding is that the family is structurally diverse and distinct from the closest reported nicotinic acetylcholine receptors.

In our review, we comment on the evolutionary analyses and conclusions. Previous paper by the authors a few years back was the key step to set the stage for CR analysis. In that paper, the authors described CR phylogenetically and functionally. As more genomic data becomes available, it is very interesting to see more details about CR evolution emerge. The finding that CRs show more neutral evolution, compared to nAChR, is striking and also within the expected range if a gene is tandemly duplicating via reverse transcription (in the same genomic vicinity). As phylogenetic distance is still relatively large (octopus and squid lineages split over 200 million years ago), it would make sense to include also other cephalopod (and non-cephalopod) species in the tree shown in Fig 1b. In this respect, a very minor point is to conduct selection analysis on the subgroups of the CRs to better estimate the selective pressure. Alternatively, plotting omega (dN/dS) values on the tree in Fig 1b (or separately in the supplement) could be able to resolve any periods of positive selection. Underlying data (trees with support values, alignments, etc) for this analysis should also be included in the Supplement.

Generally, the evolutionary analyses in this manuscript are based on the previous study and in our view the value of this manuscript would be much higher in this respect if combined together with the companion paper on the evolution of CRs in squids and octopuses (however, see also comments there).

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, Franc

Author Rebuttals to Initial Comments:

Referees' comments:

Referee #1 (Remarks to the Author):

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We thank Reviewer 1 for his appreciation regarding our approach that spans the evolution of protein structure to unique animal behavior. Indeed, we agree this is a foundational study for future experiments analyzing the diversity of CRs, their ligands, and the evolution of sensory systems in the context of ecology. We share the reviewer’s interest regarding the potential diversity of CR ligands and their significance to animal behavior. Two important future directions of this work are: (1) the identification of natural CR ligands in the octopus’s native environment; and (2) to determine which natural stimuli (and combinations of stimuli) represent appetitive or aversive stimuli. Indeed, aspects of these important questions remain poorly understood even in well-studied chemosensory systems.

We initially probed the “taste by touch” sensory system to discover receptors and cells in octopus using chemically complex extracts of diverse prey organisms, including fish, crabs, and shrimp. All of these stimuli resulted in physiological responses at the level of receptors, cells, tissues, and animals. These extracts represent concentrated, complex mixtures, which likely contain both appetitive and aversive signals for cephalopods, and therefore casted a wide net for discovery and represent a useful “positive control” for testing receptors. In our more specific chemical screening efforts, to date, we have identified agonists that are classically thought of as aversive. For example, the terpene nootkatone is now an approved insect repellent, and polygodial is a defense chemical secreted by marine invertebrates. We therefore speculate that the CRs characterized to date mediate sensation of aversive stimuli during chemotactile exploration, but given the complexity of CRs and their rapidly evolving binding pocket, we anticipate that specific as-yet unidentified receptors or receptor combinations could mediate appetitive sensation. This remains to be determined following the discovery of additional receptors and behaviorally relevant ligands.

Although artificial agonists are commonly used to study chemosensory systems in many animals, we believe the octopus represents an interesting model to explore natural pharmacology. Considering the incredible diversity of terpenes, we hypothesize that similar ecologically relevant terpenes and other CR agonists await discovery. Thus, in ongoing studies in our labs, we are pursuing the identification and characterization of natural product ligands isolated from bacteria and fungi cultured from the octopus’s environment (including the crab carapace!). We anticipate such studies will inform our understanding of

how CRs evolved in the context of the octopus' unique ecological niche. We updated our conclusions section to discuss the topic of potential ligand diversity.

The movement of the arm is scored. Is it possible to tell if these are appetitive or aversive movements? Can the arm make this type of decision? Or is this possible without communication with the central nervous system?

We developed and used this behavioral assay to ask whether distinct chemicals elicit general arm autonomous behavior. Remarkably, molecules active on CRs were sufficient to elicit arm behavior while non-active molecules were ineffective. While responses were specific to CR agonists, so far, these molecules elicited qualitatively similar movements. It could be that all CR ligands elicit similar arm movement, as-yet undiscovered agonist-receptor pairs elicit distinct movements, or the central nervous system controls more sophisticated appetitive or aversive motion. Our long-term direction is to identify a greater diversity of agonists, receptors, and to use more sophisticated markerless tracking analyses to test whether specific stimuli elicit distinct behaviors in isolated arms and animals. We agree that such studies will reveal important insight regarding the unusual organization and function of the distributed octopus nervous system.

While the alpha7 nicotinic acetylcholine receptor functions as a homomer, many vertebrate nAChRs are heteromeric. Is there evidence, besides the similar responses in the expressed receptor and native receptor, that CRT1 functions as a homomer in the octopus? Is it known if more than one receptor type is expressed in one sucker?

In our previous study that first characterized octopus CRs (van Giesen et al 2020), we used RNA *in situ* hybridization to determine that individual sensory cells express either single or multiple CR subunits, suggesting they could form homo- or heteromeric channel complexes. Furthermore, we found that a small number of CR subunit pairs could physically interact in heterologous expression systems. Considering the number of subunits, the potential diversity of CR complexes is significant. Thus, as a future direction, we are exploring spatial transcriptomic and biochemical approaches to determine which CRs interact within native octopus sensory cells, and which interactions influence receptor function and chemotactile sensation. We hypothesize that heteromerization could influence channel formation and/or properties, reminiscent of α/β subunits of neuronal nicotinic receptors, or ORCO for insect odorant receptors. Such interactions could alter the binding pocket, thereby differentially encoding a wide range of stimuli. We clarified this exciting possibility in the text.

In fig. 1 the middle panel shows multiple CRT1 expressing cells in a sucker. Does each sucker express CRT1 in some cells or is CRT1 specialized so that it is expressed in only a few suckers?

We sometimes visualized CRT1-negative cells using neural markers (α-HRP) which appear to weakly label putative sensory cells in octopus tissue, suggesting CRT1 is not expressed in all cells. However, these markers are not well established in octopus and could only label a subset of cells or could miss low expression in some types. At this point, we can conclude that CRT1 appears to be expressed in many, but perhaps not all sensory cells. Future spatial transcriptomic and biochemical approaches mentioned above for heteromer analyses may provide additional insight.

Referee #2 (Remarks to the Author):

The paper by Allard et al., "Structural basis of sensory receptor evolution in octopus" describes the cryo-EM structure of the previously reported chemotactile receptors in octopus. Its main finding is that the family is structurally diverse and distinct from the closest reported nicotinic acetylcholine receptors.

In our review, we comment on the evolutionary analyses and conclusions. Previous paper by the authors a few years back was the key step to set the stage for CR analysis. In that paper, the authors described CR phylogenetically and functionally. As more genomic data becomes available, it is very interesting to

see more details about CR evolution emerge. The finding that CRs show more neutral evolution, compared to nAChR, is striking and also within the expected range if a gene is tandemly duplicating via reverse transcription (in the same genomic vicinity).

We thank Reviewer 2 for insightful questions and comments regarding the evolution of CRs. Indeed, an interesting finding from this study is that CRs show higher rates of neutral evolution. Furthermore, the dn/ds ratio shown in Fig. 1c indicates selective pressure acting on protein-coding genes. As Reviewer 2 suggests, genes tandemly duplicated by retrotranscription exhibit higher rates of evolution. Because we showed that CRs are functional, higher rates of evolution likely result from diversifying selection driving the specialization of ligand binding sites among receptor subtypes. We have further emphasized this point in the revised text.

As phylogenetic distance is still relatively large (octopus and squid lineages split over 200 million years ago), it would make sense to include also other cephalopod (and non-cephalopod) species in the tree shown in Fig 1b.

We strongly agree with the reviewer that analyses of other cephalopods and non-cephalopods is incredibly interesting in terms of cross-species comparisons and determining origins for CRs. In this respect, we carry out this distinct and extensive analyses in Kang et al. We further describe our rationale below.

In this respect, a very minor point is to conduct selection analysis on the subgroups of the CRs to better estimate the selective pressure. Alternatively, plotting omega (dN/dS) values on the tree in Fig 1b (or separately in the supplement) could be able to resolve any periods of positive selection. Underlying data (trees with support values, alignments, etc) for this analysis should also be included in the Supplement.

We thank the reviewer for suggesting further analyses to gain insights in CRs diversification and resolve periods of positive selection for the main octopus CR groups. We now include tests between distinct models partitioning omega values for CRs, the major CR clades, and distinct omega values for each branch (new Table S1). In addition, we plot omega values across the phylogeny in the new Fig. S1. Finally, we include supporting values for branches in the tree in Figure S2. Furthermore, all sequence data will be submitted to GenBank and available upon acceptance.

Generally, the evolutionary analyses in this manuscript are based on the previous study and in our view the value of this manuscript would be much higher in this respect if combined together with the companion paper on the evolution of CRs in squids and octopuses (however, see also comments there).

These evolutionary analyses provide a foundation from which we could ask how related Cys-loop receptors subtly diverge in their structure to mediate new function. Beyond initial characterization, our previous study (van Giesen et al, 2020) showed that CRs are present in several species of octopus and diverged from acetylcholine receptors. The major focus of this study is to use CRs as a model system to understand how structural adaptations drive new sensory function. In this context, in addition to extensive structural and physiological characterization, we now also probe the evolutionary basis for how CRs emerged from ancestral nicotinic receptors. We show that these genes are intronless, tightly clustered in a single array in two clusters in Chrm. 15, and derived from retrotranscription, suggesting a mechanism underlying their evolution and novel structural and functional features, the focus of this manuscript. We have now added a new Extended Data Figure 1 mapping the number of introns across acetylcholine receptor-like genes, emphasizing that all CRs are intronless genes and depicting their genomic architecture. Additionally, for this paper, we included branch and site models of evolution characterizing selection for CRs compared to other nAChRs (Fig. 1c) and across octopus CRs sequences (Fig. 4b). As previously outlined, these analyses complement and are tailored to enhance the scope of the central topic of this paper, the first structural basis of chemotactile sensation in octopus and sensory receptor adaptation. We believe this is accomplished by providing an evolutionary framework for the diversification of key amino acids in the

transition from neurotransmitter receptors to a sensory receptor that contains a promiscuous, rapidly evolving binding pocket for environmental chemical cues.

Figure 1b and c focus only on the diversification of CRs from acetylcholine receptor-like genes in *O. bimaculoides*, the species in which they were only recently discovered, functionally characterized, and we now present the first in class structure. The *O. bimaculoides* genome also encodes the highest number of CRs, thereby providing statistical power to explore patterns of heterogeneous selection across amino acid sites, rather than only across branches in the phylogeny. Indeed, identification of residues under stronger selection across CRs of *O. bimaculoides* informed our characterization of key sites involved in functional receptor properties and ligand binding. These data complement the first CR structure and provide an independent line of evidence to suggest potential tuning sites within the structure of these divergent receptors.

We agree with the reviewer that probing the function and diversification of CRs across cephalopods is incredibly interesting. In this respect, we extensively explored this topic in our accompanying manuscript by analyzing the expression of CRs within a model squid, extending those transcriptomic profiling experiments to other cephalopods, characterizing native cellular and neurophysiological function, cloning and characterizing the first squid CR, identifying its ligands, predatory behavior, and determining the cryo-EM structure for structure-function comparisons with the first octopus CR structure. In the context of the comparative squid study, we also analyzed CR evolution across the cephalopod radiation, rather than investigating patterns of selection in a single, well-studied model organism, as we did for the octopus-focused manuscript. The comparative analysis is distinct as it is focused on phylogenetic sampling by including species from the major lineages of cephalopods: octopuses, cuttlefish and squids, and nautiloids, as well as using a bivalve mollusk as an outgroup. These distinct phylogenetic analyses provide a deep-time perspective on the evolution of CRs across the cephalopod radiation, complementary to the focus of the comparative manuscript, which spans structure to lineage-specific behavior. Therefore, we believe these analyses are most clearly and cohesively presented separately, because they provide evolutionary foundations to understand the origin and adaptation of CRs at different phylogenetic levels.

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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:

Referees' comments:

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):

We thank the authors for their work on the revised manuscript version. Just a few minor points:

Line 178-179: This sentence might better be moved after the next paragraph since the time calibrated tree appears in the companion manuscript.
Line 731: this should be “mapped acetylcholine receptor-LIKE coding sequence”?

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

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We made these suggested text changes.

Sensory specializations drive octopus and squid behavior

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)

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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