JOURNAL OF Neuroscience Research

Genetic and functional analysis of a Pacific Hagfish opioid system

Alden Y. Huang, Anna M.W. Taylor, Atefeh Ghogha, Mochtar Pribadi, Qing Wang, Tanya S.J. Kim, Catherine M. Cahill, Giovanni Coppola, Christopher J. Evans

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Accepted: 8-June-2020

Editor: Trang, Tuan
Reviewer 1: Stevens, Craig
Reviewer 2: Leduc-Pessah, Heather

1st Editorial Decision 04/22/2020

22-Apr-2020

Dear Dr Evans:

Thank you for submitting your manuscript to the Journal of Neuroscience Research. We've now received the reviewer feedback and have appended those reviews below. I'm glad to say that the reviewers are overall very enthusiastic and supportive and found your study an excellent contribution to the field of opioid research and enhances our understanding of vertebrate opioid systems.

They did make some suggestions for clarification, and I expect that these points should be relatively straightforward to address. If there are any questions or points that are problematic, please feel free to contact me. I am glad to discuss.

We ask that you return your manuscript within 30 days. Please explain in your cover letter how you have changed the present version. If you require longer than 30 days to make the revisions, please contact Dr Cristina Ghiani (cghiani@mednet.ucla.edu). You can submit your revised manuscript directly by clicking on the following link: *** PLEASE NOTE: This is a two-step process. After clicking on the link, you will be directed to a webpage to confirm. ***

https://mc.manuscriptcentral.com/jnr?URL_MASK=5305a86fff5241c6b070354ecda6afcf

Thank you again for your submission to the Journal of Neuroscience Research; we look forward to reading your revised manuscript.

Best Wishes,

Dr Tuan Trang
Associate Editor, Journal of Neuroscience Research

Dr Cristina Ghiani
Co-Editor-in-Chief, Journal of Neuroscience Research

Editors Comments:

we would really appreciate if you could include the following:

GRAPHICAL ABSTRACT
Please upload a graphical abstract, which we are asking of all authors submitting original research articles. This is intended to provide readers with a visual representation of the conclusions and an additional way to access the contents and appreciate the main message of the work. What we require is a .tif image file and a .doc text file containing an abbreviated abstract. For the image, labels, although useful, must be kept to a minimum and the image should be 400 x 300, 300 x 400, or 400 x 400 pixels square and at a resolution of 72 dpi. This can be one of the

figures from your article, or something slightly different, as long as it represents your study. Instructions for this can be found in our author guidelines online at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1097-4547/homepage/ForAuthors.html

CONFLICT OF INTEREST AND AUTHOR CONTRIBUTIONS
Please add to your paper (after the Discussion and Acknowledgments, immediately before the References) a conflict of interest statement and a statement of authors' contributions. The statement must follow the CRediT Taxonomy. You can find examples of such statements in the author guidelines on-line at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1097-4547/homepage/ForAuthors.html

Reviewer: 1

Comments to the Author
This paper describes the significant finding of an opioid receptor and endogenous opioid peptides in the Pacific hagfish, an extant representative of an early-evolved vertebrate superclass called cyclostomata. Cyclostomes, including the hagfish and lamprey, are thought to share a common ancestor to all living vertebrate species. The authors use extensive genetic techniques to clone an opioid receptor-like sequence from the brain of the hagfish, verify this sequence with a contig assembly of the hagfish genome, and express the putative opioid receptor-like protein (esMOP) in mammalian cell culture. The confirmed the functionality of the hagfish opioid receptor-like protein by phosphor-MAPK assays using met-enkephalin, morphine or etorphine (with or without naloxone). esMOP internalization was also assessed using a FLAG-tagged esMOP protein in HEK cells. The authors also discovered coding sequences for two enkephalin precursor proteins in the hagfish (esPENKL1, esPEMKL1) with peptides predicted from esPENKL1 further tested on the above mentioned assays.

The discussion is broad and includes a special review of putative opioid receptors in invertebrate species. The authors go a long way to show that it is unlikely any true opioid receptors are expressed or present in the genome of invertebrate species and that the receptor characterized in the hagfish most likely represents the earliest example of an opioid receptor in the animal kingdom.

Overall, this paper represents an excellent contribution to the field of opioid research and enhances our understanding of vertebrate opioid systems. However there are a few comments and corrections the authors should consider:

1) Page 11, lines 6.5 and throughout pp. First of all, this paragraph starts correctly with the terms “Opioid receptor type.” MOP, DOP, KOP. and NOP are types of opioid receptors. The authors use this terminology correctly here but most of the paper use “subtypes” to refer to type of opioid receptors. Avram Goldstein made this clear in numerous instances and the term subtype is reserved for designations like mu1, mu2 or alternatively transcripts. The analogy is the adrenergic receptors where alpha and beta are types of adrenergic receptors and beta1, beta2, etc. are subtypes of receptors. Pls find and replace “subtype” with “type” in this context throughout the paper.

2). Page 12, line 19, “abhorrent pharmacology of bioassays and the inherent problems with immune detection (Baker, 2015).” The Baker paper discusses only antibodies and yes there are inherent antibody problems. However, “abhorrent pharmacology” does not seem warranted. In the case of DOR, it was NOT abhorrent pharmacology that led to the characterization of DOR using mouse vas deferens (the d for delta came from deferens) and numerous other examples could be mentioned where bioassays were certainly not abhorrent and indeed represented the very best pharmacology at the time. This part of the statement should be deleted or if not at least more fully justified scientifically and suggest not using the term “abhorrent.”

3) Page 11, lines 6.5 and throughout pp. Secondly, “substrate specificity” is a term of art of enzymology not pharmacology. Better use would be “receptor selectivity” and likewise replace specificity with selectivity throughout this pp. Also selectivity was not lost in receptors of lower vertebrates is not correct (it is thought they never had it!) so would be better written as “…opioid receptor type-selectivity is gained during evolution of vertebrates.”

4). Page 7, line 29. Authors surely don’t mean that the esMOP “whose deduced amino acid sequence most closely resembles murine MOP….” It would appear from the alignments (and from an evolutionary standpoint) that esMOP should most closely resemble Danio rerio MOP (zebrafish). Perhaps the authors meant that considering just the murine MOP, DOP, KOP, or NOP proteins, esMOP most resembled MOP. In any event, the original statement here is unclear and needs to be clarified and the percent homology given for drMOP vs. esMOP.

Other corrections:

Page 5, line 10, insert closed parenthesis after “../translate/”

Page 5, line 26, wrong in-text citation, replace (Stevens, 2004) with the original MEMOIR paper (Jean-Paul Ebejer, Jamie R. Hill, Sebastian Kelm, Jiye Shi, and Charlotte M. Deane, Memoir: template-based structure prediction for membrane proteins, Nucleic Acids Res. 2013 Jul; 41: W379–W383.)

Page 5, line 10, insert closed parenthesis and space after "../homologene/"

Page 11, line 8, insert "in" before mammals to read "studies performed predominantly in mammals."

Page 16, line 43.5, delete ". Ref " to read "..by Li (Li et al. 1996a) is boxed in green."

Page 16, line 52 species name of hagfish should be lower case to read "(Eptrateus stoutii)."

Reviewer: 2

Comments to the Author
JNR-2020-Mar-8686
Genetic and Functional Analysis of a Pacific Hagfish opioid system

Huang et al. describe the identification of a functional opioid receptor (esMOP) and endogenous opioid peptides in the Pacific Hagfish. Their findings provide the proposed earliest evidence for a functional opioid receptor system in vertebrates. To assess the evolutionary significance of this system, the authors compare the structure to the partial opioid receptors identified in lamprey and find the genes cluster together. While in contrast, there are no sequences with high genetic homology in invertebrates. These findings suggest that functional opioid receptors are likely a vertebrate-specific trait and provides a significant advance for the tracing of opioid receptors throughout evolution.

The main findings reported in the paper are the genetic sequences for the novel opioid receptor and ligands and computational models that assess their homology, structure and phylogenetic relationship to other species. Through these modalities, the authors do a thorough job of providing evidence that esMOP shares high sequence homology and critical structural features with the opioid receptor family. The authors go a step further by cloning and assessing the functional potential of esMOP as well as the opioid peptides described which is a great addition to the study. The scientific methods, analysis and discussion of the findings is sound and provides a significant advancement in the understanding of the evolution of opioid receptor systems. However, the manuscript would be improved by addressing the following comments prior to publication. A revised manuscript that addresses the points below would be appropriate to consider for publication.

Comments:

1. Figure 2 – "Comparison of MOP sequences from representative vertebrate species". This figure is a helpful addition to the manuscript and shows key areas of conservation across species and clear denotation of the TMs. Based on the results reported in the text (Page 7, lines 48-58) please double check the positioning of the predicted TMs relative to the sites described. These might be slightly off from each other, however please confirm since the text reports DRY is outside vs. within TMIII and xRRxxR is in an intracellular loop but appears to be in TMVI in the image.

2. Could Figure 3 be rotated to portrait orientation? It seems if it was slightly smaller and rotated it would still be adequate to observe the detail and would read better within the final manuscript.

3. The authors use the term esMOP to refer to the newly identified receptor but also hagfish MOP within the text. Are these denoting the gene/peptide vs. the receptor? It seems that they are used interchangeably within the text. If synonymous perhaps it could be simplified to one consistent term. In particular between Figures 4 & 5 descriptions in the text and in figure legends it is inconsistent.

4. The figure titles and legends for Figures 4 & 5 are very similar and do not clearly define the difference. It would be helpful to make it more clear in the title and in the legend that Figure 5 is a repeat phylogenetic tree with the additional incomplete lamprey opioid receptor sequences included as it is outlined in the text and to ensure the two figures have different titles.

5. The authors report in the text that Figure 4 is an un-rooted phylogenetic tree but in the figure legend that is has been rooted to the rhodopsin sequence, please clarify.

6. Could Figure 6 & 7 be rotated to portrait orientation and combined into a single figure? It seems like that orientation would work similar to Figure 8. The peptide sequence could also be added to Figure 8 for esPENKL2 for consistency and to highlight the signal peptide and cysteine motif as was done for esPENKL1.

7. It would be worth adding a reference for HEK cells not expressing endogenous opioid receptors since this is critical for the conclusions drawn from the subsequent experiments.

8. As there are already many Figures in this manuscript, Figures 9&10 (possibly even 11) could be combined. They share a common theme and could easily be formatted together in a single figure with consistent sizing of panels and images. Figure titles for figure 9 & 10 are already the same – otherwise please provide more specific titles.

9. The use of functional assays for the proposed opioid receptor and peptides is an excellent addition to the paper. However, this section of the results requires significant work.
Results on Page 10 Lines 6-30 needs to be revised.
It seems as though the Figure the text is referring to is mislabelled as 10 but should be 9 and Figure 10 is not described in the text at all.
Please clarify the use of etorphine in one assay vs. Met-enkephalin in the other.
Please provide a short justification of the use of MAPK experiment for MOP activity (Page 10, Lines 12-14) to provide context of the signaling activity for readers who may not be familiar with this pathway.
Please revise the conclusion reported that there is a significant increase in phosphorylated MAPK in response to hagfish peptides (Page 10 line 18) as this data was not quantified or statistically analyzed.
For Figure 10 – Control HEK cells should also have a Beta-actin control shown for loading control. Exposure/ image quality between Figure A and B appears quite different.
Please also provide some context for the use of the internalization assay and how it related to other opioid receptor function.

10. In addition to the above, the statistical analysis in Figure 9 A is not described within the paper. The specific statistical analysis done should be described in the Figure legend and in the methods.

11. A few typos in the writing (some examples – Page 7 line 34, Page 11 line 19) please review carefully before re-submission.

Authors' Response 05/21/2020

Reviewer: 1

Comments to the Author

Overall, this paper represents an excellent contribution to the field of opioid research and enhances our understanding of vertebrate opioid systems.

We thank the reviewer for the positive comments concerning the papers contribution to opioid research and for comments to improve the paper.

1) Page 11, lines 6.5 and throughout pp. First of all, this paragraph starts correctly with the terms "Opioid receptor type." MOP, DOP, KOP. and NOP are types of opioid receptors. The authors use this terminology correctly here but most of the paper use "subtypes" to refer to type of opioid receptors. Avram Goldstein made this clear in numerous instances and the term subtype is reserved for designations like mu1, mu2 or alternatively transcripts. The analogy is the adrenergic receptors where alpha and beta are types of adrenergic receptors and beta1, beta2, etc. are subtypes of receptors. Pls find and replace "subtype" with "type" in this context throughout the paper.

We apologize for the oversight of this nomenclature. The reviewer is correct, and the manuscript has been edited accordingly.

2). Page 12, line 19, "abhorrent pharmacology of bioassays and the inherent problems with immune detection (Baker, 2015)." The Baker paper discusses only antibodies and yes there are inherent antibody problems. However, "abhorrent pharmacology" does not seem warranted. In the case of DOR, it was NOT abhorrent pharmacology that led to the characterization of DOR using mouse vas deferens (the d for delta came from

deferens) and numerous other examples could be mentioned where bioassays were certainly not abhorrent and indeed represented the very best pharmacology at the time. This part of the statement should be deleted or if not at least more fully justified scientifically and suggest not using the term “abhorrent.”

We agree with the reviewer that this term could be misinterpreted. We have clarified that we are referring to only invertebrate pharmacology. This sentence now reads:

However, given unusual pharmacology in the invertebrate bioassays (some opioid-like effects require very high ligand concentrations and some are non-naloxone reversible or naloxone has agonist effects) and the inherent problems with immune detection (Baker, 2015), genome sequence authentication is necessary to rigorously confirm signal identity.

3) Page 11, lines 6.5 and throughout pp. Secondly, “substrate specificity” is a term of art of enzymology not pharmacology. Better use would be “receptor selectivity” and likewise replace specificity with selectivity throughout this pp. Also selectivity was not lost in receptors of lower vertebrates is not correct (it is thought they never had it!) so would be better written as “…opioid receptor type-selectivity is gained during evolution of vertebrates.”

Thank you for this suggestion. This has been corrected to receptor selectivity.

4). Page 7, line 29. Authors surely don’t mean that the esMOP “whose deduced amino acid sequence most closely resembles murine MOP….” It would appear from the alignments (and from an evolutionary standpoint) that esMOP should most closely resemble Danio rerio MOP (zebrafish). Perhaps the authors meant that considering just the murine MOP, DOP, KOP, or NOP proteins, esMOP most resembled MOP. In any event, the original statement here is unclear and needs to be clarified and the percent homology given for drMOP vs. esMOP.

This was a misunderstanding in the way the original text was written. The sentence now reads:
… whose deduced amino acid translation most closely resembles (in the mouse genome), the MOP with a homology of 63.92% (compared to 57.95% and 53.98% for murine DOP and KOP, respectively).

Other corrections:

Page 5, line 10, insert closed parenthesis after “../translate/”

Corrected

Page 5, line 26, wrong in-text citation, replace (Stevens, 2004) with the original MEMOIR paper (Jean-Paul Ebejer, Jamie R. Hill, Sebastian Kelm, Jiye Shi, and Charlotte M. Deane, Memoir: template-based structure prediction for membrane proteins, Nucleic Acids Res. 2013 Jul; 41: W379–W383.)

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Page 16, line 43.5, delete “. Ref “ to read “..by Li (Li et al. 1996a) is boxed in green.”

Corrected

Page 16, line 52 species name of hagfish should be lower case to read “(Eptrateus stoutii).”

Corrected

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Reviewer: 2

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The main findings reported in the paper are the genetic sequences for the novel opioid receptor and ligands and computational models that assess their homology, structure and phylogenetic relationship to other species. Through these modalities, the authors do a thorough job of providing evidence that esMOP shares high sequence homology and critical structural features with the opioid receptor family. The authors go a step further by cloning and assessing the functional potential of esMOP as well as the opioid peptides described which is a great addition to the study. The scientific methods, analysis and discussion of the findings is sound and provides a significant advancement in the understanding of the evolution of opioid receptor systems. However, the manuscript would be improved by addressing the following comments prior to publication. A revised manuscript that addresses the points below would be appropriate to consider for publication.

We thank the reviewer for their positive remarks.

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We thank the reviewer for noticing this. Unfortunately, it is unclear exactly where transmembrane domains (TMD's) begin or end, and it is likely the membrane inserted residues change both with posttranslational modifications such as the palmitoylation (eg close to the DRY sequence) and also state of receptor activation. We debated this issue but decided that we shouldn't guess and that we had to go with the prediction program which predicts these sequences in transmembrane domains. We have however, made the point in the figure legend that these TMDs are only predicted by the program and that inclusion or not in the TMD's is speculative. We added to the legend:

Red bars denote transmembrane domains predicted by TsPred. However, in should be recognized that residues transitioning transmembrane domains to extracellular or intracellular domains are speculative and likely to be regulated both by posttranslational modifications and/or state of activation of the receptor.

2. Could Figure 3 be rotated to portrait orientation? It seems if it was slightly smaller and rotated it would still be adequate to observe the detail and would read better within the final manuscript.

Thank you for this suggestion. We have reoriented this figure in the revised version.

3. The authors use the term esMOP to refer to the newly identified receptor but also hagfish MOP within the text. Are these denoting the gene/peptide vs. the receptor? It seems that they are used interchangeably within the text. If synonymous perhaps it could be simplified to one consistent term. In particular between Figures 4 & 5 descriptions in the text and in figure legends it is inconsistent.

The terms are synonymous and we have corrected everything to read the esMOP with the exception of the introduction and the titles of the figure legends.

4. The figure titles and legends for Figures 4 & 5 are very similar and do not clearly define the difference. It would be helpful to make it more clear in the title and in the legend that Figure 5 is a repeat phylogenetic tree with the additional incomplete lamprey opioid receptor sequences included as it is outlined in the text and to ensure the two figures have different titles.

We have changed the titles to be more descriptive, namely:

Figure 4. Phylogenetic relationship between the hagfish MOP and other Opioid Receptor Types.

Figure 5. Phylogenetic close relationship between the hagfish MOP and lamprey opioid receptor-like sequences.

5. The authors report in the text that Figure 4 is an un-rooted phylogenetic tree but in the figure legend that is has been rooted to the rhodopsin sequence, please clarify.

This was an error in the text and has been rectified. Thank you for catching this.

6. Could Figure 6 & 7 be rotated to portrait orientation and combined into a single figure? It seems like that orientation would work similar to Figure 8. The peptide sequence could also be added to Figure 8 for esPENKL2 for consistency and to highlight the signal peptide and cysteine motif as was done for esPENKL1.

We originally submitted these figures as portrait and the journal asked us to change to landscape for resolution.

7. It would be worth adding a reference for HEK cells not expressing endogenous opioid receptors since this is critical for the conclusions drawn from the subsequent experiments.

We have added the following sentence in the methods: Note, HEK293T cells do not express opioid receptors (Keith et al., 1996)

8. As there are already many Figures in this manuscript, Figures 9&10 (possibly even 11) could be combined. They share a common theme and could easily be formatted together in a single figure with consistent sizing of panels and images. Figure titles for figure 9 & 10 are already the same – otherwise please provide more specific titles.

We will ask the journal whether this is feasible and not lose resolution.

9. The use of functional assays for the proposed opioid receptor and peptides is an excellent addition to the paper. However, this section of the results requires significant work.

Results on Page 10 Lines 6-30 needs to be revised.
It seems as though the Figure the text is referring to is mislabeled as 10 but should be 9 and Figure 10 is not described in the text at all.

Thank you for identifying that we mislabeled Figure 10 where it should have been Figure 9. We do cite the data in Figure 10 earlier in the same paragraph referring to the results where cells were treated with morphine or etorphine with and without naloxone.

Please clarify the use of etorphine in one assay vs. Met-enkephalin in the other.

Etorphine was used because it is a potent agonist at all opioid receptors – and can also activate ORL-1 receptors albeit at high concentrations. In subsequent assays when we observed Met Enkephalin was likely made in the Hagfish, we then assessed the activity of this peptide.

Please provide a short justification of the use of MAPK experiment for MOP activity (Page 10, Lines 12-14) to provide context of the signaling activity for readers who may not be familiar with this pathway.

This is a signaling pathway activated by opioid receptors that we have published on previously and it is now referenced. We cite the paper: Morphine induces desensitization of insulin receptor signaling. Li Y, Eitan S, Wu J, Evans CJ, Kieffer B, Sun X, Polakiewicz RD.Mol Cell Biol. 2003 Sep;23(17):6255-66. doi: 10.1128/mcb.23.17.6255-6266.2003.PMID: 12917346

Please revise the conclusion reported that there is a significant increase in phosphorylated MAPK in response to hagfish peptides (Page 10 line 18) as this data was not quantified or statistically analyzed.

We have removed all reference to statistics in the sentence and now report:
Of the predicted products derived from esPENKL1, phosphorylated MAPK was observed in cells treated with the putative hagfish opioid peptides Y13V, Y12L similar to that with Met-Enk, and to a lesser extent, Y7S. As expected, there was no increase in activity in cells treated with the predicted non-opioid peptide Y7N (Figure 9b).

For Figure 10 – Control HEK cells should also have a Beta-actin control shown for loading control. Exposure/ image quality between Figure A and B appears quite different.

Figures A and B are showing cells with and without expression of MOR. Panel B should not have any change from control which is why it may be faint. Also the blots were run at different times. Some blots were re-stained with anti-beta-actin (1:5000; Sigma-Aldrich) to validate equivalent loading and protein transfer, especially in cells that had MOR expressed. However, other blots where cells were not expressed with receptor did not have b-actin re-stained.

Please also provide some context for the use of the internalization assay and how it related to other opioid receptor function.

Internalization was used in addition to MAPK activation to show functionality of the receptor. We state:
Functionality of esMOP was further confirmed by agonist-induced trafficking of HA-tagged esMOP expressed in HEK293T cells (Figure 11).

10. In addition to the above, the statistical analysis in Figure 9 A is not described within the paper. The specific statistical analysis done should be described in the Figure legend and in the methods.

Data for the MAPK signal was analyzed by a one-way ANOVA with Dunnett's post hoc analysis to identify specific differences from the 0.01 uM dose. Data are presented as means +/- SD.

11. A few typos in the writing (some examples – Page 7 line 34, Page 11 line 19) please review carefully before re-submission.

Thank you. The paper has been gone through by three authors.
