EJN European Journal of Neuroscience FENS Federation of European Neuroscience Societies

Subpopulations of vomeronasal sensory neurons with coordinated coexpression of type 2 vomeronasal receptor genes are differentially dependent on Vmn2r1

Sachiko Akiyoshi, Tomohiro Ishii, Zhaodai Bai & Peter Mombaerts

Review timeline:

Submission date:	16 November 2017
Editorial Decision:	08 January 2018
Revision received:	29 January 2018
Accepted:	15 February 2018

Editor: C. Giovanni Galizia

1st Editorial Decision 08 January 2018

Dear Dr. Mombaerts,

Your manuscript has been reviewed by external reviewers as well as by the Section Editor, Dr. C. Giovanni Galizia, and ourselves.

The reviews collectively indicate that your experiments generated new and important information. However, there are several issues that need to be clarified/resolved before we can consider your manuscript further for publication in EJN.

As you can see, your paper was well received by three reviewers, all complimenting you for your study and expressing the hope that the new lines will help to elucidate receptor expression mechanisms in more detail in the future. Each of them raise a few minor points that need to be addressed in a revised version. The suggestions are valid, and each point should be carefully considered. The comments about statistical documentation (number of animals used, parametric/non-parametric tests) are of particular importance. In addition, the point about the lack of the use of unbiased stereological methods to count cells needs to be carefully addressed and justified.

We also noted the following points that need to be addressed.
- The abstract could do with a proper concluding statement
- Please include the total number of animals used?
- Please include the dilutions of secondary antibodies
- Please include the dose of anaesthetic.

If you are able to respond fully to the points raised, we would be pleased to receive a revision of your paper within 30 days.

Thank you for submitting your work to EJN.

Kind regards,

Paul Bolam & John Foxe
co-Editors in Chief, EJN

Reviews:

Reviewer: 1 (Trese Leinders-Zufall, University of Saarland, Germany)

Comments to the Author
In their manuscript, Sachiko and colleagues make a first step to tackle the reason and function of co-expression of specific olfactory receptors (GPCRs) in the vomeronasal organ using various mouse strains.

They developed two new mouse strains having a deletion in Vmn2r1 (ΔC1 and ΔC2-GFP) that they crossbred with their various fluorescent-tagged Vmn2r mice, of which some are known receptors for chemosensory

ligands. The functional role of the co-expressed C1 receptors is unknown and can only be addressed by using these specific knockout animals.

The lack of the Vmn2r1 differentially causes a reduction in some Vmn2r expressing neurons, whereby Vmn2rs known to be co-expressed with a murine major-histocompatibility-complex H2-Mv receptor appear to be more affected. Furthermore, the data demonstrate that the lack of Vmn2r1 does not cause any new axonal wiring excluding a function in axon guidance. No compensatory expression of other family C Vmn2rs was found. This latter phenomenon is used to substantiate the lab's proposed model of V2R coordinated and dependent model.

The authors did a good job with a manuscript that is well written and even with the many numbers and receptor abbreviations could be followed relatively easy. I just stumbled over one typo on page 4 (last line of 2nd paragraph: 'there are no still reports…'.

In conclusion, the authors present here new mouse lines to now start investigating the physiological role of Vmn2r1, since the data in this manuscript indicate that the receptor can be excluded as a receptor necessary for axon guidance.

Reviewer: 2 (Enrique Lanuza, University of Valencia, Spain)

Comments to the Author
The manuscript by Akiyoshi et al reports interesting findings regarding the expression of different types of vomeronasal receptors in mice. I have no major concerns about the work, but I have three methodological observations:
- The number of animals use in each experiment is not reported. The authors state (page 11) that for all experiments at least 3 mice were used. But the reader can not know the actual number of animals in each experiment. I think that this information is relevant and should be provided.
- The cell numbers were compared using the unpaired t-test. However, this statistical test requires a normal distribution of the data and homogeneity of variance, and the authors do not mention whether the data meet these assumptions. If this is not the case, the authors should use a non-parametric test, such as the Mann-Whitney test. In any case, when the number of animals is so low, non-parametric tests are recommended.
- Finally, the method for counting labelled cells (counting labelled profiles every 5th section [or 10th, or 12th] and multiplying the obtained number by 5 [or 10, or 12], see page 8) is biased and it is likely to overestimate the result (Coggeshall and Lekan, 1996, J. Comp. Neurol 364: 6-15). However, the overestimation is probably similar in the experimental and control groups (unless the size or shape of the labelled cells differed between the two groups, which is unlikely), and thus unbiased methods would be important when estimating the total number of cells, but not so much for the comparison of experimental and control groups, as performed in this work. Thus, I only recommend explaining that the provided numbers are positive "cell profiles", and not "cells", and should not be taken as an estimation of the total number of positive cells.

Reviewer: 3 (Daisuke Kondoh, Obihiro University of Agriculture and Veterinary Medicine, Japan)

Comments to the Author
Comment to authors:
In this study, authors established two Vmn2r1-deficient mouse strains with a null mutation. I believe that these novel strains are useful in the future to reveal why a single V2R cell expresses two types of receptors, which is an exception to the "one cell-one receptor" manner. In addition, authors attempted to prove the role of Vmn2r1 in C1-type V2R cells and to adjust the relationships between C1-type and C2-type of V2Rs. This manuscript includes very important findings, but I recommend for authors to conform and revise several points described below.

Major comment 1.
Page 19, Line 17-21: Authors probably should describe the results correctly. In Fig. 6A, 24 of the 26 C1 type of V2Rs showed a significant decrease, and among them, 5 were DE genes in one strain. I think that authors excluded Vmn2r94 and Vmn2r104, but if even so, the reminder text about this exclusion should be added in Line 17.
In addition, the phrase "(median count < 100 in WT mice)" should be inserted after "no difference" in Line 21.

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Although it is possible that various hypotheses are established according to this fact, authors appear to present their self-serving hypothesis that Vmn2r1 mediates final mutation of cells to readers. Actually, the C1-negative C1 type V2Rs may lower at younger ages, but the difference of a C1-positive rate between C1 type V2Rs which stated in Line 7-11 does not seem to support this hypothesis. This paragraph should be deleted.

Major comment 3.
Page 26, Line 5-9: This paragraph is not suitable here. I recommend that the sentence “suggesting that the mutant allele yields unstable transcript. Alternately, transcription of the mutant allele may extinguish with time” is shifted in Page 13, Line 4, and that the paragraph in Page 26, Line 5-9 is deleted.

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Page 25 Line 5: According to Major comment 2 and 3, this subsection should be re-entitled “Varying reductions of gene expression of C1 type V2Rs in ΔC1 mice”, and authors should discuss this molecular mechanism and/or meaning in biological functions in more detail, if possible.

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Page 12, Line 24: Location of citation of Fig. 1F is incorrected. This panel should be cited in the next sentence.

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Minor comment 6
Fig 5A and C: Please indicate the whole AOB and glomerular layer of aAOB and anterior and posterior parts of pAOB with dashed lines.

Authors’ Response 29 January 2018

We thank the reviewers for a thorough and critical reading of our ms and for their constructive comments. They did not identify fatal flaws in our experimental design or reasoning, and did not recommend new experiments.

Reviewer 1 writes "*The authors did a good job with a manuscript that is well written and even with the many numbers and receptor abbreviations could be followed relatively easy*." Indeed, as the V2R gene family is very large, and as we used many mouse strains and reagents, we took great care at ensuring that the reader can follow our many analyses.

Below we copy-and-paste the text of editors and reviewers verbatim and in italics, and respond to each point.

We believe and hope that reviewers and editors will now be satisfied with our ms, and that the ms will be deemed worthy of publication in EJN.

Editors

We also noted the following points that need to be addressed.
- The abstract could do with a proper concluding statement

This point is well taken. We have deleted characters in the Abstract to make space for a concluding statement, which is part of the final sentence of the Conclusion after the Discussion: "Thus, there appears to be a fixed probability of gene choice for a given C2 type of V2R."

- Please include the total number of animals used?

The information about the number of animals used was mentioned in the figure legends in the original ms. We have done now at all instances in the Results section as well. We studied 4 mice per genotype when cell counts were made.

- Please include the dilutions of secondary antibodies

Done. It was 1:1000 for all secondary antibodies.

- Please include the dose of anaesthetic.

Done. It was 210 mg/kg body weight ketamine and 10 mg/kg body weight xylazine.

Reviewers

Reviewer: 1

In their manuscript, Sachiko and colleagues make a first step to tackle the reason and function of co-expression of specific olfactory receptors (GPCRs) in the vomeronasal organ using various mouse strains.

We respectfully note that our ms is not about olfactory receptors (ORs) but about vomeronasal receptors (VRs, in full Vmn2rs).

They developed two new mouse strains having a deletion in Vmn2r1 (ΔC1 and ΔC2-GFP)

We respectfully note that it is not ΔC2-GFP but ΔC1-GFP.

that they crossbred with their various fluorescent-tagged Vmn2r mice, of which some are known receptors for chemosensory ligands. The functional role of the co-expressed C1 receptors is unknown and can only be addressed by using these specific knockout animals.

The lack of the Vmn2r1 differentially causes a reduction in some Vmn2r expressing neurons, whereby Vmn2rs known to be co-expressed with a murine major-histocompatibility-complex H2-Mv receptor appear to be more affected.

We respectfully point out that the opposite is actually the case: subpopulations of VSNs that express Vmn2rs that are coexpressed with H2-Mv molecules are <u>less</u> affected than those that do not coexpress H2-Mv molecules.

Furthermore, the data demonstrate that the lack of Vmn2r1 does not cause any new axonal wiring excluding a function in axon guidance. No compensatory expression of other family C Vmn2rs was found. This latter phenomenon is used to substantiate the lab's proposed model of V2R coordinated and dependent model. The authors did a good job with a manuscript that is well written and even with the many numbers and receptor abbreviations could be followed relatively easy. I just stumbled over one typo on page 4 (last line of 2nd paragraph: 'there are no still reports…'.

Thanks - corrected.

In conclusion, the authors present here new mouse lines to now start investigating the physiological role of Vmn2r1, since the data in this manuscript indicate that the receptor can be excluded as a receptor necessary for axon guidance.

The public availability of these novel mouse strains will facilitate such experiments in other laboratories. Cryopreserved sperm samples of the four novel strains described in this ms have been deposited with The Jackson Laboratory years ago. As soon as the PubMed ID of our paper is available (which is typically a few days after the paper appears online), the two novel strains will be publicly available to the community, without any strings attached and without MTA.

Reviewer: 2

The manuscript by Akiyoshi et al reports interesting findings regarding the expression of different types of vomeronasal receptors in mice. I have no major concerns about the work, but I have three methodological observations:

- The number of animals use in each experiment is not reported. The authors state (page 11) that for all experiments at least 3 mice were used. But the reader can not know the actual number of animals in each experiment. I think that this information is relevant and should be provided.

The information about the number of animals used was mentioned in the figure legends in the original ms. We have done now at all instances in the Results section as well. We studied 4 mice per genotype when cell counts were made.

- The cell numbers were compared using the unpaired t-test. However, this statistical test requires a normal distribution of the data and homogeneity of variance, and the authors do not mention whether the data meet these assumptions. If this is not the case, the authors should use a non-parametric test, such as the Mann-Whitney test. In any case, when the number of animals is so low, non-parametric tests are recommended.

Most but not all biological parameters follow a normal distribution. It would be an enormous amount of work, and without precedent in the field of V1R or V2R genes, for us to have to show that the distribution of cell numbers for a given V2R gene across mice is normal and has homogeneity of variance. In fact, although V2R genes have been described in 1997, there are a very small number of papers that provide any cell numbers for a given V2R gene.

At the suggestion of Reviewer 2, we have now used the Mann-Whitney test, and have revised Figures 2 and 3 accordingly. What was significant for the unpaired t-text, is still significant for the Mann-Whitney test, and the same for non-significances; the p values are different.

- Finally, the method for counting labelled cells (counting labelled profiles every 5th section [or 10th, or 12th] and multiplying the obtained number by 5 [or 10, or 12], see page 8) is biased and it is likely to overestimate the result (Coggeshall and Lekan, 1996, J. Comp. Neurol 364: 6-15). However, the overestimation is probably similar in the experimental and control groups (unless the size or shape of the labelled cells differed between the two groups, which is unlikely), and thus unbiased methods would be important when estimating the total number of cells, but not so much for the comparison of experimental and control groups, as performed in this work. Thus, I only recommend explaining that the provided numbers are positive "cell profiles", and not "cells", and should not be taken as an estimation of the total number of positive cells.

Indeed, the absolute numbers do not matter so much - it is the difference between WT and mutant. According to Coggeshall and Lekan JCN 1996, the use of unbiased stereological methods is not necessary when there are no obvious changes in the size or shape of the counted objects between genotypes. In our cell counts, cell bodies of VSNs (but not fragments of dendrites or axons) were counted as labeled objects.

We measured the size of counted cell bodies, and no differences were seen between genotypes.

For the information of Reviewer 2, the average sizes of cell bodies counted (WT vs MUT) are:

V2r1b-GFP, 4 wk (119.9 µm2 vs 113.2 µm2, ns)
V2r1b-GFP, 10 wk (142.9 µm2 vs 92.48 µm2, ns)
V2rf2-GFP, 10 wk (143.1 µm2 vs 122.9 µm2, ns)
V2Rp5 IHC, 10 wk (139.8 µm2 vs 132.4 µm2, ns)
V2rf4-Venus, 10 wk (149.0 µm2 vs 140.9 µm2, ns)

V2rf1-Cherry (xΔC1), 10 wk (167.2 µm2 vs 141.5 µm2, ns)
V2rf1-Cherry (xΔC1-GFP), 10 wk (155.4 µm2 vs 140.3 µm2, ns)

We have now added the following sentence at the end of the section on IHC and cell counts in the VNO of the Materials and Methods: "The numbers given are numbers of VSN cell profiles, but for the sake of simplicity, they are referred to as numbers of VSNs."

We believe that repeating numerous times "numbers of VSN cell profiles" throughout the ms (in the text and in the figures) would be awkward and perhaps even unconventional. We hope that Reviewer 2 is on our side, and that s/he accepts that the pragmatic use of "numbers of VSNs" is sufficiently clear.

Reviewer: 3

Comments to the Author
Comment to authors:
In this study, authors established two Vmn2r1-deficient mouse strains with a null mutation. I believe that these novel strains are useful in the future to reveal why a single V2R cell expresses two types of receptors, which is an exception to the "one cell-one receptor" manner. In addition, authors attempted to prove the role of Vmn2r1 in C1-type V2R cells and to adjust the relationships between C1-type and C2-type of V2Rs. This manuscript includes very important findings, but I recommend for authors to conform and revise several points described below.

Major comment 1.
Page 19, Line 17-21: Authors probably should describe the results correctly. In Fig. 6A, 24 of the 26 C1 type of V2Rs showed a significant decrease, and among them, 5 were DE genes in one strain. I think that authors excluded Vmn2r94 and Vmn2r104, but if even so, the reminder text about this exclusion should be added in Line 17.

This point is well taken. We have now corrected this statement: 5 (instead of 3) genes were DE in one but not the other strain. We believe that it is better to summarize the analysis by referring to the C1 type of V2R genes that were DE in both strains: these were and are 19 of the 26 genes.

In addition, the phrase "(median count < 100 in WT mice)" should be inserted after "no difference" in Line 21.

We believe that Reviewer 3 is mistaken. We write on page 19: "Genes with a median count <100 in WT samples were considered as not expressed in whole VNO mucosa at the age of analysis, and deleted from data." In NanoString analyses, such a threshold is routinely taken. The "(median count < 100 in WT mice)" does not refer to the absence of a difference in expression of 25 of the 27 C2 type of V2R genes.

Major comment 2.
Page 25, Line 5: The first paragraph of the subsection entitled as "Varying percentages of colabeling with anti-C1 antibody" is not suitable for this study. The fact that "Not all VSNs expressing a given C1 type of V2R are colabeled with anti-C1 antibody" (Page 25, Line 6) is revealed even without the present findings.

We published some of these findings already in Ishii and Mombaerts, 2011, but present in this ms more such analyses, of WT and V2R-tagged mice, also by ISH. We thought it is useful to summarize the data (in increasing percentages), to highlight the point that not all VSNs expressing a given C1 type of V2R are colabeled with anti-C1 antibody. Often authors combine old and new data together in their Discussion. We do not devote much space to these statements.

Although it is possible that various hypotheses are established according to this fact, authors appear to present their self-serving hypothesis that Vmn2r1 mediates final mutation of cells to readers.

We do not understand what Reviewer 3 means with his comment. If s/he means "maturation" instead of "mutation", we kindly point out that we have not stated nor implied that Vmn2r1 mediates final maturation of VSNs. We wrote and write: "We speculate that with increasing age, a higher fraction of existing VSNs may start to coexpress Vmn2r1, perhaps as final maturation or induction by an activity-dependent mechanism." It is a speculation, not even a hypothesis. We are puzzled why this hypothesis would be "self-serving". Moreover, but we offer an alternative, but not mutually exclusive hypothesis right after it.

Actually, the C1-negative C1 type V2Rs may lower at younger ages, but the difference of a C1-positive rate between C1 type V2Rs which stated in Line 7-11 does not seem to support this hypothesis.

We have now specified the statement that at younger ages, the percentage of colabeling is lower, as follows: "At 3 wk, this percentage of colabeling is lower (Ishii and Mombaerts, 2011): 68.2% for V2rf2 (in V2rf2-GFP mice), and 81.3% for V2r1b (in V2r1b-GFP mice)."

This paragraph should be deleted.

Respectfully, we prefer to keep the paragraph. It is important for the reader to realize that not all VSNs expressing a particular C1 type of V2R coexpress Vmn2r1; that these percentages vary according to the V2R analyzed; and that the published data indicate that the percentage of coexpression is lower at 3 wk than at 10 wk. These new and old data are suitable for the Discussion and may inspire specific new experiments.

Major comment 3.
Page 26, Line 5-9: This paragraph is not suitable here. I recommend that the sentence "suggesting that the mutant allele yields unstable transcript. Alternately, transcription of the mutant allele may extinguish with time" is shifted in Page 13, Line 4, and that the paragraph in Page 26, Line 5-9 is deleted.

This point is well taken. Done.

Major comment 4.
Page 25 Line 5: According to Major comment 2 and 3, this subsection should be re-entitled "Varying reductions of gene expression of C1 type V2Rs in ΔC1 mice", and authors should discuss this molecular mechanism and/or meaning in biological functions in more detail, if possible.

We have now rephrased the title of this subsection as "Varying percentages of colabeling with anti-C1 antibody and varying reductions in the ΔC1 or ΔC1-GFP background". It is not possible for us to discuss meaningfully the molecular mechanism and/or biological meaning; it would be unsubstantiated speculation. Unfortunately there is no correlation between the percentage of colabeling in WT mice and the percentage of reduction in the ΔC1 or ΔC1-GFP background.

Major comment 5.
Page 26, Line 10: The first paragraph of the subsection entitled as "Possible function of Vmn2r1" seems to adequately reflect to author's own findings. However, this discussion is only the repetition of summary in one of Results part (Page 17, Line 21-24). And, the second paragraph is obviously exaggerated, because it depends on only one citation, not the present findings. This subsection should be deleted.

We respectfully disagree. We do not repeat a summary of the Results. We refer to the frequently-overlooked but important paper of Ebrahimi & Chess, 2000 that a minimal number of olfactory sensory neurons expressing a given odorant receptor is required to maintain glomeruli in the olfactory bulb; the same is likely to be the case for VSNs, but this point has, to our knowledge, not been made yet. The fact that we found normal glomeruli for V2rf2+ VSNs and in the appropriate region of the bulb, is relevant for the novel and perhaps unexpected conclusion that Vmn2r1 has no major biological function for axon guidance. This paragraph goes nicely with the next paragraph, starting with "Results from a heterologous HEK293 expression system..."

What function could Vmn2r1 then have? There is indeed only one citation (DeMaria et al., 2013) and we found it important and relevant to cite it. We qualify the importance from the heterologous HEK293 data by stating "If these responses can be extended in vivo or ex vivo to VSNs of WT mice and can be shown to be reduced or abolished in the ΔC1 or ΔC1-GFP background,"

Thus, this subsection is relevant and our statements are qualified. We prefer to keep it.

Minor comment 1
Page 12, Line 24: Location of citation of Fig. 1F is incorrected. This panel should be cited in the next sentence.

This point is well taken - done.

Minor comment 2
Page 14, Line 2: The explanatory text that V2r1b-GFP+ neurons at 0d were detected by IHC against GFP should be added. Readers will misapprehend that they can compare the numbers of V2r1b-GFP+ neurons directly between 0d and 4wk or 10wk, although the detection methods were different.

This point is well taken - done.

Minor comment 3
Page 20, Line 7: MT mice ---- WT mice

Corrected.

Minor comment 4
Page 25, Line 3-4: The sentence "By ISH ~ Vmn2r76" is not needed and should be deleted.

We do not understand what "not needed" means. These are the only ISH data on cell numbers for C2 type of V2R genes. We find it useful and meaningful to keep this sentence in the Discussion, as it indicates cell-autonomous effects of the Vmn2r1 knockout mutation. We provide three lines of evidence for cell-autonomous effects, based on three distinct methods, which we summarize in the Discussion in three successive sentences (By IHC analysis, ...; By NanoString analysis, ...; By ISH analysis, ...). The sentence that is "not needed" is the third and last line of evidence for our finding of cell-autonomous effects of the Vmn2r1 knockout mutations, and must be kept.

We have now added "C2 type of V2R genes" for the sake of clarity.

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Fig 5A and C: Please indicate the whole AOB and glomerular layer of aAOB and anterior and posterior parts of pAOB with dashed lines.

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