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August 23, 2016

Dear Editors,
Thank you for reviewing our manuscript entitled "Transient receptor potential vanilloid 1 (TRPV1)-immunoreactive signals in murine enteric glial cells" (Manuscript No. 28433) that we submitted for publication.

The manuscript has been thoroughly revised to address the points raised by the reviewers. Please find attached the revised version of the manuscript in which the changes are highlighted, and our point-by-point responses to the reviewers' comments. We hope that the revised manuscript is now acceptable for publication.

We would like to thank the referees for their helpful comments and suggestions.

Yours sincerely,

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Point-by-point responses to the reviewers' comments.

We are very grateful for the comments and criticisms raised by the reviewers. We have revised our manuscript as follows in response to the reviewers' comments. The reviewers' comments are shown in *italic*. The quotations from the revised manuscript are shown in red color.

1. Replies to comments from reviewer 03529851

COMMENTS TO AUTHORS

The manuscript by Yamamoto M et al. is well written, and shows immunoreactivty for TRPV1 labelling in EGC. I have the following questions that would be important to clarify. The TRPV1 KO mouse is a functional knock out, and the protein may still express, we have seen some of immunoreactivity on the KO animals, as well as others. Of course the antibodies make the most important point here. The authors nicely provide the information on the IgG sources, and the peptides targeted for immuno-detection. It would be important to indicate whether the peptide for the immuno-labeling is the same as the deletion region of the KO TRPV1? To bring more value to the paper, it would be nice if authors could show some functional responses of TRPV1 from the large or small intestine, or the cultured cells, which the authors used in their study. Even Ca2+ responses elicited by capsaicin application to the WT but not KO mice would suffice.

The reviewer addressed two points and our answers are as follows:

It would be important to indicate whether the peptide for the immuno-labeling is the same as the deletion region of the KO TRPV1?

Answer: As the reviewer has pointed out, this strain is developed by disruption of an exon coding a part of the fifth and all of the sixth transmembrane domains of TRPV1 (Caterina MJ et al, Science 2000; 288:306-313). The antibody used in the present study is a monoclonal antibody targeted to the c-terminal of the protein, which is preserved in the mutated TRPV1 in KO mice. Accordingly, although the catalogue of the supplier (Jackson Labs) stated that "no gene product

(mRNA or protein) is detected in dorsal root ganglia", we detected mRNA and protein of mutated TRPV1 by RT-PCR and immunohistochemistry in the intestinal samples. However, immunohistochemical signal in KO mice is faint, even in case detected, compared to that in WT mice, at least in the same staining and signal development protocols on the same slide on which the specimens from KO and WT mice were mounted together. Similar observations have been reported by Yamada et al, J Histochem Cytochem 2009; 57:277-287) using anti-TRPV1 antibodies raised against the N- and C-terminal of TRPV1 protein. To clarify this point, we have included the following additional statements in the legend of Table 1 in the revised manuscript.

TRPV1 KO mice used in the present study preserved this sequence but the signal of immunohistochemistry in KO mice is faint, even in case detected, compared to that in WT mice, in the same staining and signal development protocols on the same slide on which the specimens from KO and WT mice were mounted together. Similar observations have been reported by Yamada et al, J Histochem Cytochem 2009; 57:277-287.

To bring more value to the paper, it would be nice if authors could show some functional responses of TRPV1 from the large or small intestine, or the cultured cells, which the authors used in their study. Even Ca2+ responses elicited by capsaicin application to the WT but not KO mice would suffice.

Answer. We understand the reviewer's suggestion, and to be honest, we had been trying to show Ca^{2+} responses in EGCs. In our laboratory, we routinely use Ca^{2+} influx assay on recombinant cells as reported in Tsuchiya K et al, Neurogastroenterol Motil 2016 Jun 10. doi: 10.1111/nmo.12877. However, we found that standard protocols for Ca^{2+} influx assay may not be applicable for the estimation of Ca^{2+} influx in EGC. The reason for this is unclear, but some discussion in the manuscript might be worthwile. The isolated EGCs in the present study are cultured *in vitro* for a long period (11 days) because freshly isolated cells are small and round, and expressed little GFAP; consequently, identification as EGC is difficult, as is analysis by standard cell biology techniques. The *in vitro* cultured EGCs may not

obtain the functions and phenotypes of authentic EGCs developed *in vivo*. Accordingly, as shown in Figure 7, TRPV1 immunosignals *in vitro* in cultured EGCs are abundant in cytoplasm in a vesicular form, rather than in the plasma membrane. As discussed in the manuscripts, intracellular TRPV1 has some possible biological functions. However, it is plausible that standard Ca^{2+} influx assays optimized for evaluation of Ca^{2+} influx through ion channels located in the plasma membrane may not be suitable for investigation of intracellular TRPV1 function. We think that the investigation of the function of TRPV1 in EGCs requires elaborate experiments after extensive preliminary examination of experimental protocols, which would be beyond the scope of the present study. In the meanwhile, we recognize the present research does not establish the functional presence/significance of TRPV1 in EGCs. Therefore, we titled this paper "Transient receptor potential vanilloid 1 (TRPV1)-immunoreactive signals in murine enteric glial cells".

2. Replies to comments from reviewer 03215423

COMMENTS TO AUTHORS

The authors are to be commended for the work in the manuscript entitled "Transient receptor potential vanilloid 1 (TRPV1)-immunoreactive signals in murine enteric glial cells". This is an interesting paper highlighting the expression profiles of TRPV1 murine enteric glial cells. Despite these interesting findings there is significant work that needs to be performed before I can warrant it acceptable for publication. Major critiques: 1) Do the authors have any functional TRPV1 data correlating the expression studies...calcium imaging or patchclamp data to capsaicin to further verify TRPV1 expression in EGCs. 2) Please provide negative controls for all staining experiments. 3) Do the authors have any functional data detailing the purpose of TRPV1 in the LI and SI EGCs? What physiological GI processes are they contributing to? 4)The authors demonstrate a difference in GFAP-IR at different PDs... do the authors see changes in TRPV1 expression the same time points? 5) Functional data (calcium imaging or patch clamp data) in freshly isolated cells from the LI or SI would greatly

enhance the conclusions of the current manuscript and validate their functional expression patterns. Minor critiques: 1) The manuscript should be proof read for grammatical errors. 2) Consistency in figure labeling - merge or Merge, etc. 3) Is Figure 2 necessary... could the enlarged view be incorporated in to Figure 1.

Point-by-point responses to the reviewer's comments are as follows:

Major critique 1) *Do the authors have any functional TRPV1 data correlating the expression studies...calcium imaging or patchclamp data to capsaicin to further verify TRPV1 expression in EGCs.*

Answer. We understand the reviewer's criticism. For Ca^{2+} influx assay, we had been trying to show Ca^{2+} responses in EGCs. In our laboratory, we routinely use Ca^{2+} influx assay on recombinant cells as reported by Tsuchiya K et al, Neurogastroenterol Motil 2016 Jun 10. doi: 10.1111/nmo.12877. However, we found that standard protocols for Ca^{2+} influx assay may not be applicable for the estimation of Ca^{2+} influx in EGC. The reason for this is unclear, but some discussion in the manuscript might be worthwile. The isolated EGCs in the present study are cultured *in vitro* for a long period (11 days) because freshly isolated cells are small and round, and expressed little GFAP; consequently, identification as EGC is difficult, as is analysis by standard cell biology techniques. The *in vitro* cultured EGCs may not obtain the functions and phenotypes of authentic EGCs developed *in vivo*. Accordingly, as shown in Figure 7, TRPV1 immunosignals *in vitro* in cultured EGCs are abundant in cytoplasm in a vesicular form, rather than in the plasma membrane. As discussed in the manuscripts, intracellular TRPV1 has some possible biological functions. However, it is plausible that standard Ca^{2+} influx assays optimized for evaluation of Ca^{2+} influx through ion channels located in the plasma membrane may not be suitable for investigation of intracellular TRPV1 function. As for the pachclamp assay, we did not perform this procedure because it is suitable mainly for evaluation of ion flux through channels located in the plasma membrane (Kubota K et al, Am J Physiol Gastrointest Liver Physiol 308: G579–G590,

2015). The investigation of TRPV1 function in EGCs would require elaborate experiments after extensive preliminary examination of experimental protocols, which would be beyond the scope of the present study.

***Major critique* 2)** *Please provide negative controls for all staining experiments.*

Answer. According to the reviewer's suggestion, we have added the Supplementary Figure S6 which shows the images of negative controls of Figures 1, 3, 4 and 7 and Supplementary Figures S3 and S5. Negative controls of Figure 2, Supplementary Figures S1 and S2 were omitted because Figure 2 is the enlarged view of Figure 1 and Supplementary Figure S1 is from the screening experiment having no negative controls. However the specificity of the selected antibody was shown by the negative controls for Figure 1. For Supplementary Figure S2, images of mock cells expressing no TRP channels are good negative controls of immunostaining which showed no immunosignal in spite that they were stained with both of primary and secondary antibodies. In response to this revision, we have added the following descriptions to the legend of Table 1:

"The negative controls of immunostaining performed in this study were shown in Supplementary Figure S6."

The legend of Supplementary Figure S6:

Supplementary Figure S6

Negative controls of immunostaining performed in this study. For Figure 3, DAPI image was added to show the presence of the cells.

Furthermore, in the present study all immunostaining experiments were performed as double immunostaining using a cocktail of primary antibodies for 2 different proteins selected from TRPV1, GFAP, PGP9.5 and αSMA and the cocktail of fluorescence-labeled or biotinylated secondary antibodies for anti-rabbit and anti-mouse IgGs. Because the original manuscript is unclear in this point, we have included additional explanatory text in several places in Materials and Methods. For example:

"The preparations were then placed in a mixture of primary

antibodies…",
"incubated with a mixture of the relevant secondary antibodies…"

Major critique 3) *Do the authors have any functional data detailing the purpose of TRPV1 in the LI and SI EGCs? What physiological GI processes are they contributing to?*

Answer. This is quite an important problem but we have no data concerning the functions and physiological significance of TRPV1 in EGCs. TRPV1 on astrocytes in CNS have purported to influence glial activation, cell migration, cell death, etc. Because the change in GFAP signals in TRPV1 KO mice was observed only temporally, TRPV1 might be involved in the postnatal formation of myenteric plexus via regulating EGC migration and death. However, this remains speculative at present.

Major critique 4) *The authors demonstrate a difference in GFAP-IR at different PDs... do the authors see changes in TRPV1 expression the same time points?*

Answer. We performed double immunostaining with GFAP and TRPV1 in the experiments represented in Figure 4. However there were no statistically significant difference in TRPV1-IR between time points. Further a weak relationship between GFAP-IR and TRPV1-IR might exist but we have not obtained statistically significant results showing this.

Major critique 5) *Functional data (calcium imaging or patch clamp data) in freshly isolated cells from the LI or SI would greatly enhance the conclusions of the current manuscript and validate their functional expression patterns.*

Answer. We understand the reviewer's suggestion. But at present, we have not succeeded in demonstrating functions of TRPV1 EGC as described in our answer to the reviewer's Major critique 1).

Minor critique 1) *The manuscript should be proof read for grammatical errors.*

Answer. According to the reviewer's criticism, the revised manuscript has been carefully reviewed by an experienced medical editor whose first language is English and who specializes in the editing of papers written by physicians and scientists whose native language is not English.

Minor critique 2) *Consistency in figure labeling - merge or Merge, etc.*

Answer. We have revised the manuscript according to the reviewer's comment.

Minor critique 3) *Is Figure 2 necessary... could the enlarged view be incorporated in to Figure 1.*

Answer. Figure 2 is shown for detailed examination of the co-staining pattern. If, in the final publication, Figure 1 is provided in a high resolution image which can be enlarged enough to be sufficiently detailed, Figure 2 can be omitted. We would like to leave this decision to the editor.