Author's response to reviews

Title: The asthma candidate gene NPSR1 mediates isoform specific downstream signalling

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Version: 3 Date: 11 March 2011

Author's response to reviews: see over
Dear Prof Kere,

Your manuscript has now been peer reviewed and the comments are accessible in PDF format from the link below. Do let us know if you have any problems opening the file.

Referee 1:  
http://www.biomedcentral.com/imedia/1111879559493331_comment.pdf
Referee 2:  
http://www.biomedcentral.com/imedia/7335997845029561_comment.pdf

Editorial request:
data deposition: Where appropriate, authors should adhere to the standards proposed by the Microarray Gene Expression Data Society and must deposit microarray data in one of the public repositories, such as ArrayExpress, Gene Expression Omnibus (GEO) or the Center for Information Biology Gene Expression Database (CIBEX).

We would be grateful if you could address the comments in a revised manuscript and provide a cover letter giving a point-by-point response to the concerns. We are aware that the referees have recommended additional experiments and analysis. If you require further time to complete these, we can extend your resubmission deadline up to 3 months.

Please also highlight (with 'tracked changes'/coloured/underlines/highlighted text) all changes made when revising the manuscript to make it easier for the Editors to give you a prompt decision on your manuscript.

Please also ensure that your revised manuscript conforms to the journal style (http://www.biomedcentral.com/info/ifora/medicine_journals). It is important that your files are correctly formatted.

We look forward to receiving your revised manuscript by 28 January 2011. If you imagine that it will take longer to prepare please give us some estimate of when we can expect it.

You should upload your cover letter and revised manuscript through http://www.biomedcentral.com/manuscript/login/man.asp?txt_nav=man&txt_man_id=1443247055467995. You will find more detailed instructions at the base of this email.

Please don’t hesitate to contact me if you have any problems or questions regarding your manuscript.

With best wishes,

Robin.

Robin Cassady-Cain, PhD
on behalf of:

Tim Shipley, PhD
Executive Editor
BMC-series Journals
BioMed Central
Floor 6, 236 Gray’s Inn Road
London, WC1X 8HL
Dear Editor,

Thank you very much for the opportunity to revise the manuscript according to the Reviewers’ insightful comments. They were helpful in improving the manuscript in several ways. We have now added additional FACS experiments showing equal intensity of the receptors on the cell membrane before and after ligand stimulation, and cAMP and Ca\(^{2+}\) experiments verifying our data that NPSR1-A has stronger signalling properties than NPSR1-B. Statistics has also been added when appropriate. As per editor’s request, we have now submitted the expression array data to ArrayExpress, accession: E-MEXP-2782.

We believe that the manuscript is now substantially improved from the previous version, and hope that it is now acceptable for BMC Pulmonary Medicine.

Sincere regards,

Juha Kere

Corresponding author

Reviewer’s report
Title: The asthma candidate gene NPSR1 mediates isoform specific downstream signalling
Version: 2 Date: 7 December 2010
Reviewer: Girolamo Calo
Reviewer’s report:
In this paper Pietras et al investigated the signaling properties of two variants of the neuropeptide S (NPS) receptor (NPSR) named NPSR1-A and B. Most of the experiments were performed in HEK-293 cells however similar results were obtained in A549 and SH-SY5Y cells. No major qualitative differences were found between NPS stimulated NPSR1-A and B responses. In general NPSR1-A elicited maximal effects larger then those caused by NPSR-B. The paper is easy to read, the methods are clear and the results nicely described. The discussion is adequate. In the following lines the referee comments are listed in order of appearance rather than importance.

In the background section the following paper should be quoted and the relative information made available to the reader. Allen IC, Pace AJ, Jania LA, Ledford JG, Latour AM, Snouwaert JN, Bernier V, Stocco R, Therien AG and Koller BH (2006) Expression and function of NPSR1/GPRA in the lung before and after induction of asthma-like disease. Am J Physiol Lung Cell Mol Physiol 291:L1005-1017. This paper is now cited and relative information, together with supporting information, described in the manuscript

In the same section it should be mentioned that the 107 Ile/Asn SNP of the human NPSR has been investigated in detail and several groups reported higher potency of NPS at the NPSR107Ile than at the NPSR107Asn isoform (see for a review of the available results Guerrini R, Salvadori S, Rizzi A,
Regoli D and Calo G (2010) Neurobiology, pharmacology, and medicinal chemistry of neuropeptide S and its receptor. Med Res Rev 30: 751-77. The reader would probably appreciated to know which aa is present in position 107 of the investigated NPSR1-A and B proteins. *It is now reported in our manuscript that Ile107 is the more potent isoform and that the same isoform also is present in our NPSR1-A and -B construct used to generate our experimental data.*

PP11 – receptor binding saturation experiments would allow to precisely calculate NPS Kd and Bmax values on membranes from NPSR1-A and B expressing cells. These experiments are in our opinion worthy of being performed. *We agree that it should be interesting to define both Kd and Bmax. However, since the aim of these experiments was to study the functional outcome between these two receptors, we believe that the relational of the data from the different signal transduction pathways answer our questions regarding this.*

PP12 – My guess is that the reader would expect to see concentration response curves to NPS in NPSR1-A and B measured with a calcium assay and a cAMP assay at least in HEK-293 cells. These data would allow to compare the NPS sensitivity of the cells used in this investigation with those used in several different laboratories. In addition these same experiments would allow the authors to compare their results on gene expression with those of classical signaling studies. These data will increase the overall meaning of this investigation. *Concentration-response curve with a cAMP assay have now been added. Due to technical problems the Ca\(^{2+}\) assay could only be performed at one concentration, nevertheless we believe that these data has a relevant contribution to manuscript.*

PP13 “the similar EC50 values between the isoforms verify an equal amount of receptor protein on the plasma membrane”. This statement is in our opinion meaningless. In fact, the result of increasing the number of a given receptor would be a proportional increase of maximal effect elicited by the agonist (with no changes of its potency). This holds true till the maximal response that a given preparation is reached; then a further increase of receptor number will produce a leftward shift of the concentration response curve to the agonist. The authors may verify this by reading the relative chapter in Kenakin T (2004) A Pharmacology Primer, Elsevier Academic Press, San Diego. *This sentence is now removed from the manuscript.*

We hope the authors will find our comments fair and useful for improving their article.

**Level of interest:** An article of importance in its field

**Quality of written English:** Acceptable

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**

I declare that I have no competing interests.

**Reviewer’s report**

**Title:** The asthma candidate gene NPSR1 mediates isoform specific downstream signalling

**Version:** 2 **Date:** 4 January 2011

**Reviewer:** Alex Therien

**Reviewer’s report:**

This manuscript provides novel data on the isoform-specific nature of downstream signaling in NPSR1. The major conclusion is that NPS stimulation leads to a similar if less robust response in cells expressing NPSR1-B compared to NPSR1-A. Although the data are of some interest to researchers in...
this field, there are a number of points that should be addressed prior to acceptance of the manuscript.

Major compulsory revisions:

1- The most glaring gap in this manuscript is the lack of statistical analysis of the data. The primary conclusion of the manuscript is that NPSR1-A signals more strongly than NPSR1-B but with the exception of a single gene, CGA, the differences between the two isoforms in Table 1 are in the order of 2-fold or less. In light of such small differences, it is important to demonstrate that the expression levels of at least some of the genes are significantly higher in NPSR1-A. Also, none of the data in Figs 3, 4, 5 and supplemental figure 1 appear to have been statistically validated. This is particularly important for CD69, which the authors claim is the only gene that is more highly expressed in NPSR1-B-expressed cells. However, the modest increase (1.5 fold at the 4h time point) does not warrant such a conclusion in the absence of statistical significance. Any discussion pertaining to CD69 (page 18, end of first paragraph) is not informative without this analysis. Another important point is the author's claim that the stronger signaling in NPSR1-A is retained in other cell types, SH-SY5Y and A549. Are the differences in these cells significant? Again, the small differences make a statistical analysis that much more important, in particular since expression levels of the two NPSR1 isoforms were not compared in these two cell types.

Statistical verification are now added to Figure 3, 4, 6 (former Fig 5) and supplementary Figure 1, were a * indicates a significance level of p ≤ 0.05.

2- The authors show that phosphorylation of the C-terminal tail of the isoforms is not the mechanistic basis for the differences in gene expression patterns. However, this does not offer an explanation, but rather rules out one of many possibilities. The system described here offers an opportunity to flesh this out further by looking at expression levels at the cell surface (by FACS) over time and following stimulation with NPS. If the authors can show that stimulation with NPS does not differentially affect cell-surface NPSR1 expression levels between the two isoforms, this would considerably strengthen the claim that differences are not due to altered turnover and/or recycling of the receptors. Measuring expression levels before and after NPS stimulation (and perhaps at a few timepoints in between) is an important control in any case since it would more convincingly demonstrate that the differences in downstream gene expression patterns are indeed not due to differential expression of the two isoforms in HEK-293 cells (as suggested by the FACS analysis in Fig 2).

An additional FACS experiment has now been added to the manuscript (Figure 2 c) showing the cell-surface expression of NPSR1-A and -B, before NPS stimulation, after 3h and after 6h. The results suggest no differences between the two receptor forms before or after NPS stimulation.

Minor Essential Revisions:

1- Related to point 2 above, how many hours after transfection was the FACS analysis in Fig 2 done? I assume it is 24h, coinciding with the time-point at which cells are stimulated with NPS in subsequent experiments, but I did not see this stated anywhere.

We do apologies for that mistake and the FACS analysis time point post transfection is now stated in the methods section in the manuscript.

2- The sentence on page 16 "Thus, it is highly motivated to understand its functions in more detail" should be re-written.

The sentence has been re-written to “It is therefore important to understand its functions in more detail”.

3- The sentence on page 18 "...over expression of the NPSR1-B isoform in asthmatic airways might thus drive this pathway toward allergic inflammation" is highly speculative in particular in light of the lack of statistical analysis showing that CD69 is indeed more highly stimulated in NPSR1-B-expressing cells.
We agree that the statement is highly speculative but since the statistical analysis show significant difference between NPSR1-A and -B in CD69 expression after 4h of NPS stimulation we allow this speculation to withstand.

Discretionary revisions:
NONE

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Acceptable

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**
I declare that I have no competing interests