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Receptor guanylyl cyclase-G is a novel thermosensory protein activated by cool temperatures

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Alexander Kohlmaier

1st Editorial Decision 01 September 2014

Thank you for submitting your manuscript "Receptor guanylyl cyclase-G is a novel thermosensory protein activated by cool temperature" to The EMBO Journal editorial office.

We have now received the reports of three expert reviewers, which you will find copied below. All the referees consider your findings of interest and importance. I am therefore pleased to inform you that we will consider your manuscript further, and I would at this stage like to invite you to revise the manuscript according to the referees' suggestions: I am highlighting here some key points that we consider most important for the revision: Apart from a number of constructive suggestions for textual changes by all referees, in our view investigating in some more molecular detail how cool temperature could possibly affect GC-G coformation is of particular importance: As most clearly voiced by referee #2, experimentally testing how temperature quantitatively affects dimerization grade (or GTP binding), or testing, for example, whether dimerization and the cool-response of activity can be molecularly disentangled, at least in vitro, would certainly add to the current mechanistic understanding. I would, therefore, be pleased if you invested the time and effort to experimentally address this reviewer's concern.

Besides this, we think that other specific suggestions raised by referees #2 and #3 and #1 are very constructive and addressable. In the interest of advancing the understanding of the function and regulation of GC-G though, in our eyes, the experiments suggested in referee #1's specific points 3 and 4, while interesting, might, potentially lead to a slight digression from the main focus - We do,

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therefore, not insist that you perform these specific experiments for consideration of the paper at the EMBO journal (Still the GC-A/G chimeric protein might be useful also as a control e.g. in the dimerization test suggested above).

Should you have questions regarding the decision or should you have comments about extent or feasibility of a specific referee point, please do contact me.

Congratulations to your work and thank you for the opportunity to consider this work for publication! I am looking forward to your revision.

REFEREE COMMENTS

Referee #1:

Chao et al. examined the function and molecular mechanisms of guanylyl cyclase-G (GC-G) by combining biochemistry, imaging, mouse genetics, and behavioral assays. Their experiments indicate that GC-G is directly activated by coolness, mediates the responses of GG cells to low temperature, and contributes to the ultrasonic vocalization of mouse pups in cold environment. This reviewer finds that the experiments were well carried out. Activation of a membrane guanylyl cyclase by low temperature is novel. The behavioral phenotypes are also interesting.

I have the following questions:
(1) The authors suggest that GC-G produces cGMPs and activates CNGA3-type channels. It will be ideal if the authors can apply a specific channel blocker L-cis-diltiazem to confirm the importance of CNGA3 channels.
(2) Previous studies from the same group indicate that GC-G is activated by bicarbonate (Chao et al., 2010). Since the GC-G mutants are available now, can the authors test the effect of bicarbonate on GC-G-/- neurons? At least the authors should discuss the relevance of the new results to their own previous observations.
(3) The authors examined the behavioral phenotypes of GC-G mutant pups. It will be valuable if the authors can report whether similar phenotypes can be observed from adult mutants. In light of the studies from other groups showing the role of GG neurons in freezing behavior, I am curious if cold environment facilitates freezing behavior and GC-G mutants have any phenotypes.
(4) The authors delved into the mechanisms of GC-G signaling by making a GC-A/G chimera (Fig 2). GC-A/G is also activated by coolness, although its cGMP products at both basal and coolness-evoked states are lower than those of GC-G. The extracellular domain of GC-A is maintained in a closed-state conformation and requires its peptide ligand ANP or BNP to activate. A similar approach in a previous study (Sun et al., PNAS 2009) reveals that both ANP and bicarbonate are needed to activate GC-A/D, which contains a GC-D cyclase domain for bicarbonate sensing. Does the addition of ANP have any impact on GC-A/G? If not, how to explain the unexpected properties of GC-A/G?

Referee #2:

The manuscript entitled
"Receptor guanylyl cyclase-G is a novel thermosensory protein activated by cool temperatures"
by Chao et al. describes carefully performed, very elegant in vitro/in vivo studies which demonstrate for the first time the activation of a mammalian membrane guanylyl cyclase receptor by cool temperatures and the role in the behavior of rodent pups and maternal-care behavior.

Guanylyl cyclase G (GC-G) is member of a familiy of 7 transmembrane cyclic GMP forming receptors (A-G) which all have distinct functions. They all exist as homodimers of single-span transmembrane proteins, containing an extracellular "ligand-binding domain" (ECD) and three intracellular domains, including the C-terminal guanylyl cyclase domain (GCD). The mechanism of

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activation of cGMP production by pGC receptors is ultimately unknown. While GC-A, B and C (possibly GC-D) are indeed "receptors" for extracellular hormones (ANP, BNP, CNP, guanylins), the others remain orphan receptors or possibly have no extracellular ligands (despite their ECD). The retinal GC-E and F are activated by intracellular calcium-binding proteins (GCAPs). GC-G, the last one discovered, has a very interesting tissue expression pattern: lung, skeletal muscle, kidney, brain and grüneberg ganglion. Up-to-know there is no known extracellular agonist for GC-G and its regulation is (to my knowledge) unknown.

The grüneberg ganglion (GG) is a special neuronal tissue in the rodent nose and contains sensors for odorants and cool temperature and thereby is involved in modulating behaviour. The present study describes for the first time that GC-G is expressed in specific subset of neurons within the GG. By the combination of biochemical studies in heterologous expression systems and with recombinant GC-G protein, the authors demonstrate (for the first time) that the cGMP-synthesizing activity of GC-G is directly activated by low temperatures (15oC). By directed mutagenesis they show that the temperature sensitive region of GC-G is located either in the hinge region (= dimerization domain) or in the guanylyl cyclase domain. It is fascinating that the substitution of these domains within GC-A (the receptor for cardiac natriuretic peptides) makes this hormone-dependent receptor, temperature responsive. Finally, elegant physiological studies in neonatal wildtype and GC-G deficient mice demonstrate that the GC-G-cGMP pathway activates specific cyclic nucleotide gated channels and calcium influx. Activation of the GC-G/cGMP/calcium pathway in the GG of neonatal mice exposed to lower temperatures (in the absence of their "warming mothers") stimulates the emission of ultrasound calls by the neonates, to recruit maternal-care behavior.
Although GC-G is not expressed in higher mammals like humans, this is a novel, original and exciting (cool!) study. This is the first report of a temperature sensitive pGC and, to my knowledge, of temperature-dependent synthesis of cGMP in mammals. With fine biochemistry and physiology, the authors demonstrate the role of GC-G in behaviour. The experiments are well conducted and clearly described. The manuscript is written in clear and comprehensive way. The study will be clearly very interesting and stimulating for other scientists working in the cGMP field or in neuroscience.

Some specific comments and questions:
- Which sequences within the hinge region and/or catalytic domain of GC-G are distinct from respective regions in other pGCs and could account for this selective temperature-responsiveness of GC-G
- The experiments with the deletion constructs lead to the valid hypothesis that cool temperatures may affect GC-G conformation to enhance dimerization (discussion, page 13). Could the authors follow this hypothesis at least in vitro, in overexpressing HEK293 cells?
- The in vivo experiments in Fig. 6 are a bit difficult to understand. Why did GC-G deletion affect the proportion of responsive pups but not overall the responsiveness of all pups? Does this mean that there is a supplementary ("rescue") pathway which has different activity between pups? Please explain/discuss in better way
- Figure 1C shows that the "baseline" activity of the ligand-stimulated pGCs (A,B,C; in absence of ligands) is much lower, as the activity of the "orphans" (D,E,F,G). Is there any explanation for this difference, i.e. for the "constitutive" activity of the later?
- In previous studies the authors reported the function of GC-G in kidney and sperm. Are the here presented novel results relevant for the regulation of GC-G in other organs? What is known about other mechanisms regulating this pGC.

Referee #3:

This is a fascinating manuscript. The data appears very solid from what I can tell. My only major critcism - easily fixable - is that the authors do a very poor job in putting their exciting findings into the broader context of receptor-type guanylyl cyclases (rGCs). To my knowledge, rGCs in vertebrates have so far "only" been shown to be receptors for small, endogenous ligands (peptides) within the body. However, a number of studies over the years in the nematode C.elegans have provided a number of tantalizing strong hints to receptor-type GC proteins being direct sensory

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receptors of various distinct external sensory modalities. For example, GCY-14 is thought to be a alkaline sensor (Murayama et al., 2013 PMID 23664973), GCY-9 is thought to be a CO2 sensor (Hallem et al., 2011; PMID 21173231) and various other C.elegans rGCs are through to be salt sensors in gustatory neurons (Oritz et al. 2009; PMID 19523832; Smith et al., 2013 PMID 23695300). Moreover, there are three rGC proteins in C.elegans which are involved in thermosensation (Inada et al. 2006; PMID 16415369 ; Wang et al., 2013 PMID 24081984), but there has never been any biochemical data to support their role as direct thermosensors.
The authors should describe all these studies either in the Introduction and/or Discussion, bring up the notion that rGCs are indeed probable sensory receptors and use them to point out that their data is the first to suggest that in vertebrates these proteins can also act as sensory receptors. It is also completely fine to point out that while genetic studies in C.elegans has strongly suggested a sensory role, it was never rigorously proven with the kind of approaches that the authors use in their study here.

1st Revision - authors' response 13 October 2014

To Referee #1:

We would like to thank the reviewer for thorough reading of this manuscript and for the thoughtful comments and constructive suggestions, which helped us to improve the quality of this manuscript.

(1) The authors suggest that GC-G produces cGMPs and activates CNGA3-type channels. It will be ideal if the authors can apply a specific channel blocker L-cis-diltiazem to confirm the importance of CNGA3 channels.

response: We appreciate the reviewer's suggestion. The importance of the cGMP-activated ion channel CNGA3 for the detection of cool temperatures in Grueneberg ganglion (GG) neurons has been confirmed in a recent report by using the CNG channel inhibitor L-cis-diltiazem (Brechbuhl *et al*, *Front Behav Neurosci* 7: 193, 2013). In this study, the authors showed that L-cis-diltiazem markedly inhibited the calcium transients induced by cool temperatures in GG neurons. This finding is cited in the discussion section of our revised manuscript (page 14; first paragraph).

(2) Previous studies from the same group indicate that GC-G is activated by bicarbonate (Chao et al., 2010). Since the GC-G mutants are available now, can the authors test the effect of bicarbonate on GC-G-/- neurons? At least the authors should discuss the relevance of the new results to their own previous observations.

response: As suggested, we have examined the effect of bicarbonate on GC-G-/- neurons in the GG. Consistent with our previous report (Chao et al., 2010), the bicarbonate-induced calcium influx observed in GG neurons endowed with GC-G is abolished in GG neurons lacking functional GC-G (see below). This finding has been included and discussed in the Discussion section (page 13, first paragraph).

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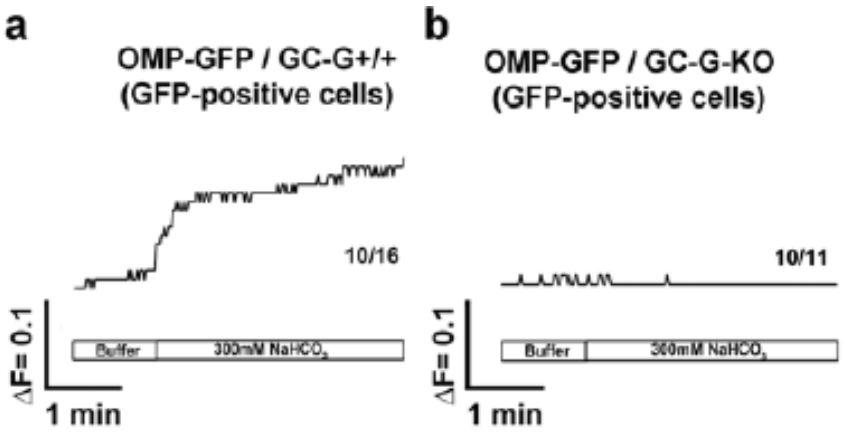

Figure R1. Bicarbonate-induced calcium influx in GC-G-positive and GC-G-KO neurons from the GG.

Representative ratiometric Ca2+ transients induced by bicarbonate in GFP-positive GG neurons from GC-G+/+ (a) and GC-G-KO (b) pups. The numbers in the bottom right corners are the number of cells with Ca2+ influx similar to what is shown in the respective graph (left) and total number of measured cells (right).

(3) The authors examined the behavioral phenotypes of GC-G mutant pups. It will be valuable if the authors can report whether similar phenotypes can be observed from adult mutants. In light of the studies from other groups showing the role of GG neurons in freezing behavior, I am curious if cold environment facilitates freezing behavior and GC-G mutants have any phenotypes.

response: In contrast to mouse pups, adult mice do not produce USV during aversive situations. They emit USV exclusively during (non-aggressive) social interactions, in particular during mating (reviewed by Portfors V (2007) Journal of the American Association for Laboratory Animal Science 46: 28-34).
To our knowledge, there is so far no evidence that coolness facilitates freezing behavior in mice. One might even wonder if it would make sense for mice to freeze at cool temperatures since this would probably enhance hypothermia of the body. Yet, we agree that it is of interest to examine whether there might be temperature-related effects on freezing behavior in general. We will examine this in our future studies; including experiments with GC-G-KO animals.

(4) The authors delved into the mechanisms of GC-G signaling by making a GC-A/G chimera (Fig 2). GC-A/G is also activated by coolness, although its cGMP products at both basal and coolness-evoked states are lower than those of GC-G. The extracellular domain of GC-A is maintained in a closed-state conformation and requires its peptide ligand ANP or BNP to activate. A similar approach in a previous study (Sun et al., PNAS 2009) reveals that both ANP and bicarbonate are needed to activate GC-A/D, which contains a GC-D cyclase domain for bicarbonate sensing. Does the addition of ANP have any impact on GC-A/G? If not, how to explain the unexpected properties of GC-A/G?

response: We have examined the effect of ANP on the chimeric GC-A/G protein. While ANP significantly activates GC-A enzymatic activity, this ligand peptide has no effect on stimulating the activity of the chimeric GC-A/G protein at both cool and warm temperatures (see below). Although it is unclear why ANP fails to stimulate GC-A/G, we speculate that artificial fusion of GC-A and GC-G in this version of the chimeric receptor may severely alter its structural conformation, inhibiting stimulation by ANP. However, further studies are needed to verify this hypothesis.

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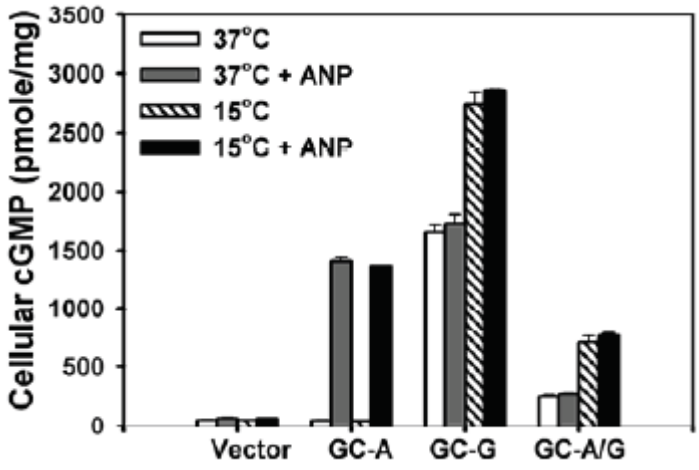

Figure R2. ANP could not further stimulate activation of the chimeric GC-A/G receptor at 15oC. HEK-293T cells expressing the indicated proteins were exposed to an ambient temperature of 37 or 15°C for 20 min in the absence or presence of ANP (1 _M); then the intracellular cGMP concentration
was measured.

To Referee #2:
We are very grateful to the referee for thorough review. We have revised this manuscript in the light of the very useful suggestions and comments.

(1) Which sequences within the hinge region and/or catalytic domain of GC-G are distinct from respective regions in other pGCs and could account for this selective temperature-responsiveness of GC-G

response: We have performed protein sequence alignment of GC-G with the other known murine GCs focusing on the hinge region and cyclase catalytic domain (see below). In fact, there are a few amino acid residues in the hinge region of GC-G which are distinct from the corresponding residues in other murine GCs. Yet, it is unclear whether these different amino acids account for activation of GC-G by coolness. In this context, it would be even difficult to analyze this by mutagenesis analyses since misfolding might be caused by any point mutation. However, further structural studies using NMR together with mutagenesis might shed lights on the importance of these residues in thermal responsiveness of GC-G.

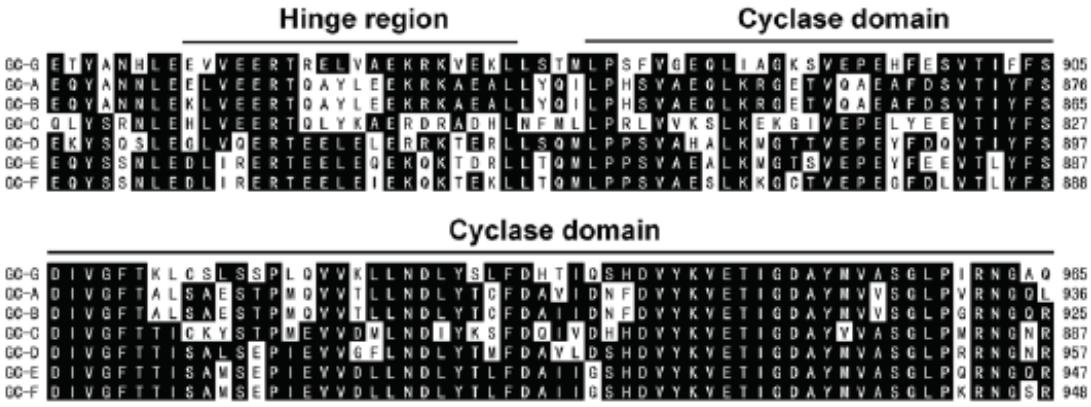

Figure R3. Protein sequence alignment of the hinge region and N-terminal cyclase domain among mammalian receptor GC proteins.

(2) The experiments with the deletion constructs lead to the valid hypothesis that cool temperatures may affect GC-G conformation to enhance dimerization (discussion, page 13). Could the authors follow this hypothesis at least in vitro, in overexpressing HEK293 cells?

response: As suggested, we performed co-immunoprecipitation experiments to evaluate whether the dimerization/oligomerization level of GC-G is enhanced at cool temperature. As shown in Fig. 4 and as described on page 7 (last paragraph) and page 8 (first paragraph) of the revised manuscript, dimerization/oligomerization of GC-G is indeed markedly increased at a cool temperature, whereas dimerization of GC-A remains unchanged at different temperatures. These results indicate that cool temperatures affect GC-G conformation, promoting dimerization/oligomerization. Thus, the increased enzymatic activity of GC-G at cool temperatures might be indeed based on an enhanced dimerization/oligomerization of GC-G evoked by coolness.

(3) The in vivo experiments in Fig. 6 are a bit difficult to understand. Why did GC-G deletion affect the proportion of responsive pups but not overall the responsiveness of all pups? Does this mean that there is a supplementary ("rescue") pathway which has different activity between pups? Please explain/discuss in better way.

response: Knockout of GC-G reduced the proportion of responding pups at P4 and P6, but not at P2 (Fig. 7a in the revised manuscript). However, even at P2, the number of USV calls per pup was clearly decreased (Fig. 7b in the revised manuscript). Thus, the overall responsiveness of pups is reduced at all ages tested. Moreover, in this context, it has to be pointed out that even in wild type pups, only a portion of the pups respond to cooling by generating USV (Fig. 7a in the revised manuscript). Yet, we agree that there may be an additional pathway which can somewhat compensate the loss of GC-G. For example, we have recently found that the thermosensitive ion channel TREK-1 seems to be involved (at least to a minor degree) in coolness-evoked responses in GG neurons (Stebe *et al*, 2014). In addition, it is possible that coolness-stimulated vocalization is regulated by other thermosensory cells. We have discussed these aspects in the Discussion section of the revised manuscript [page 17 (last paragraph) and page 18].

(4) Figure 1C shows that the "baseline" activity of the ligand-stimulated pGCs (A,B,C; in absence of ligands) is much lower, as the activity of the "orphans" (D,E,F,G). Is there any explanation for this difference, i.e. for the "constitutive" activity of the later?

response: The GC subtypes GC-D to GC-G are endogenously expressed in various sensory neurons (olfactory, retinal photoreceptor, and GG neurons). In our experiments, we heterologously expressed them in HEK cells. It is conceivable that the HEK-293T cell system lacks cellular regulatory factor(s) which fine-tune or even somewhat suppress basal activity of “sensory” GC subtypes. However, further experiments are needed to validate this hypothesis.

(5) In previous studies the authors reported the function of GC-G in kidney and sperm. Are the here presented novel results relevant for the regulation of GC-G in other organs? What is known about other mechanisms regulating this pGC.

response: In fact, expression of GC-G has been reported in mouse kidney and sperm. However, the precise function of GC-G in these organs remains unclear. Moreover, whether coolness-stimulated activity of GC-G is relevant for the regulation of this enzyme in these organs remains so far elusive. For the kidney, it is likely that coolness may not play an essential role because this organ is kept at a warn temperature in adult animals. In addition, we previously showed that GC-G activity is stimulated by bicarbonate (Chao *et al*, 2010), which has been validated by bicarbonate-induced

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calcium transients in the GG slices (please see above our response to issue #2 raised by Referee #1). Nevertheless, the biological significances of bicarbonate-regulated GC-G activity in these organs require further investigation.

To Referee #3:

(1) This is a fascinating manuscript. The data appears very solid from what I can tell. My only major critcism - easily fixable - is that the authors do a very poor job in putting their exciting findings into the broader context of receptor-type guanylyl cyclases (rGCs). To my knowledge, rGCs in vertebrates have so far "only" been shown to be receptors for small, endogenous ligands (peptides) within the body. However, a number of studies over the years in the nematode C.elegans have provided a number of tantalizing strong hints to receptor-type GC proteins being direct sensory receptors of various distinct external sensory modalities. For example, GCY-14 is thought to be a alkaline sensor (Murayama et al., 2013 PMID 23664973), GCY-9 is thought to be a CO2 sensor (Hallem et al., 2011; PMID 21173231) and various other C.elegans rGCs are through to be salt sensors in gustatory neurons (Oritz et al. 2009; PMID 19523832; Smith et al., 2013 PMID 23695300). Moreover, there are three rGC proteins in C.elegans which are involved in thermosensation (Inada et al. 2006; PMID 16415369 ; Wang et al., 2013 PMID 24081984), but there has never been any biochemical data to support their role as direct thermosensors. The authors should describe all these studies either in the Introduction and/or Discussion, bring up the notion that rGCs are indeed probable sensory receptors and use them to point out that their data is the first to suggest that in vertebrates these proteins can also act as sensory receptors. It is also completely fine to point out that while genetic studies in C.elegans has strongly suggested a sensory role, it was never rigorously proven with the kind of approaches that the authors use in their study here.

response: We thank the referee for the constructive and very kind comments. In the Introduction (page 3, last paragraph) and the Discussion (page 13) section of the revise manuscript, we have included the studies in *C. elegans* mentioned by the reviewer which suggest that receptor-type GCs can function as sensory receptors for various distinct external sensory modalities. In addition, in the revised version, we have pointed out that our data demonstrate that a receptor-like GC can act as a thermal sensor (page 14, second paragraph).

2nd Editorial Decision 28 October 2014

Thank you for submitting your revised manuscript "Receptor guanylyl cyclase-G is a novel thermosensory protein activated by cool temperatures" to The EMBO Journal. The manuscript has now been seen again by the three referees. You will find their comments pasted below. I am pleased to inform you that we will accept your manuscript for publication. Your article will be processed for publication in The EMBO Journal by EMBO Press and Wiley.

Congratulations to your work!

Referee #1:

This is an interesting study. The authors have addressed all my concerns.

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Referee #2:

The revised manuscript entitled "Receptor guanylyl cyclase-G is a novel thermosensory protein activated by cool temperatures" by Chao et al. describes carefully performed, very elegant in vitro/in vivo studies which demonstrate for the first time the activation of a mammalian membrane guanylyl cyclase receptor by cool temperatures and the role in the behavior of rodent pups and maternal-care behavior.
Guanylyl cyclase G (GC-G) is member of a familiy of 7 transmembrane cyclic GMP forming receptors (A-G) which all have distinct functions. They all exist as homodimers of single-span transmembrane proteins, containing an extracellular "ligand-binding domain" (ECD) and three intracellular domains, including the C-terminal guanylyl cyclase domain (GCD). The mechanism of activation of cGMP production by pGC receptors is ultimately unknown. While GC-A, B and C (possibly GC-D) are indeed "receptors" for extracellular hormones (ANP, BNP, CNP, guanylins), the others remain orphan receptors or possibly have no extracellular ligands (despite their ECD). The retinal GC-E and F are activated by intracellular calcium-binding proteins (GCAPs). GC-G, the last one discovered, has a very interesting tissue expression pattern: lung, skeletal muscle, kidney, brain and grüneberg ganglion. Up-to-know there is no known extracellular agonist for GC-G and its regulation is (to my knowledge) unknown.
The grüneberg ganglion (GG) is a special neuronal tissue in the rodent nose and contains sensors for odorants and cool temperature and thereby is involved in modulating behaviour. The present study describes for the first time that GC-G is expressed in specific subset of neurons within the GG. By the combination of biochemical studies in heterologous expression systems and with recombinant GC-G protein, the authors demonstrate (for the first time) that the cGMP-synthesizing activity of GC-G is directly activated by low temperatures (15oC), possibly because low temperatures facilitate the formation of homodimers (which is essential for guanylyl cyclase activity). By directed mutagenesis they show that the temperature sensitive region of GC-G is located either in the hinge region (= dimerization domain) or in the guanylyl cyclase domain. It is fascinating that the substitution of these domains within GC-A (the receptor for cardiac natriuretic peptides) makes this hormone-dependent receptor, temperature responsive. Finally, elegant physiological studies in neonatal wildtype and GC-G deficient mice demonstrate that the GC-G-cGMP pathway activates specific cyclic nucleotide gated channels and calcium influx. Activation of the GC-G/cGMP/calcium pathway in the GG of neonatal mice exposed to lower temperatures (in the absence of their "warming mothers") stimulates the emission of ultrasound calls by the neonates, to recruit maternal-care behavior.
Although GC-G is not expressed in higher mammals like humans, this is a novel, original and exciting (really cool!) study. This is the first report of a temperature sensitive pGC and, to my knowledge, of temperature-dependent synthesis of cGMP in mammals. With fine biochemistry and physiology, the authors demonstrate the role of GC-G in behaviour. The experiments are well conducted and clearly described. The manuscript is written in clear and comprehensive way. This fascinating study will be clearly very interesting and stimulating for other scientists working in the cGMP field or in neuroscience.
The authors have addressed all my questions and concerns. They performed additional experiments indicating that low temperatures facilitate the formation of GC-G homodimers (which is essential for guanylyl cyclase activity). I have no additional questions.

Referee #3:

The authors have addressed my concerns well and I support publication as is.

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