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Guanylate cyclase-G is an alarm pheromone receptor in mice

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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.)

1st Editorial Decision 29 May 2017

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see, the referees find the analysis interesting and timely. They raise a number of different concerns that I anticipate you should be able to respond to in a good manner. Given the input from the referees I would like to invite you to submit a suitably revised manuscript for our consideration. I should add that it is EMBO Journal policy to allow only a major single round of revision and that it is therefore important to address the raised concerns at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

The manuscript entitled "Guanylate cyclase-G is an alarm pheromone receptor in mice"

by Chao et al. describes carefully performed, very elegant molecular, functional and imaging studies in vitro/in vivo which demonstrate for the first time the activation of a mammalian membrane guanylyl cyclase receptor by a non-peptide ligand, the alarm pheromone SBT, and the role of this signaling pathway in fear behavior and stress-induced neurohumoral responses of rodents.

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 peptide hormones (ANP, BNP, CNP, guanylins), the others remained 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.

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. In a previous study published in the EMBO J, the authors of the present manuscript described 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, they demonstrated that the cGMP-synthesizing activity of GC-G is directly activated by low temperatures (15oC). These previous studies also showed that 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 (EMBO J. 2015 Feb 3;34(3):294-306).

In the present study the authors followed the hypothesis that GC-G participates in the chemosensation of alarm pheromones such as SBT, a volatile small molecule released by rodents during danger, to warn conspecifics. This hypothesis was indeed challenging, since all previously known extracellular ligands activating particulate GCs to cGMP production are small peptides. By fine biochemical and imaging studies in overexpressing HEK cells, Chao et al. demonstrate that SBT activates GC-G to cGMP production, that this activation requires the extracellular domain of the receptor, and that this results in cGMP-dependent opening of CNGA3 channels and increases of intracellular calcium levels. To assess the functional, physiological relevance of this signaling pathway, the authors carefully performed comparative molecular, behavioral and biochemical studies in GC-G-gene knockout mice and their wildtype littermates. These studies demonstrate that 1) SBT induces calcium signaling in specific GC-G expressing neurons of the grüneberg ganglion (GG) in situ; and 2) such GC-G-induced cGMP and calcium increases are critically involved in the fear- and stress-associated responses of mice to SBT (walking distance, serum corticosterone levels, blood pressure). Taken together these studies demonstrate for the first time the activation of the extracellular domain of a particulate GC, GC-G, by a small molecule, the alarm pheromone SBT, and the relevance of this signaling pathway in chemosensation, specifically in the fear responses of mice.

Although GC-G is not expressed in higher mammals like humans, this is a novel, original and exciting study. 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:

- The introduction should explain more clearly how SBT is synthesized and excreted. Is it in urine, feces or sweat? Is it released acutely or chronically? Is it volatile (how will it reach the GG in the nose of conspecifics?)
- Are the concentrations of SBT used in vitro (Fig. 1 and 2) similar to the "natural" concentrations which mediate communication between conspecifics?
- Which sequences within the ECD of GC-G are distinct from respective regions in other pGCs and could account for this selective responsiveness of GC-G to the non-peptide molecule SBT?

- The experiments with the deletion constructs lead to the valid hypothesis that SBT activates the ECD. However, the authors have to demonstrate that the mutated GC-G protein (lacking the ECD) is still functional, e.g. capable of stimulated synthesis of cGMP (Fig. 1).
- On page 7, Para 2, artificial cerebrospinal fluid was used as control. Why is this an appropriate negative control for studies of SBT in vivo?
- Is GC-G activated by other small alarm pheromones?
- 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.
- Minor: On several pages the word "manor" has to be replaced by "manner"
- The IUPHAR recommends to use the nomenclature "guanylyl" (not guanylate) for this receptor family

Referee #2:

In this study, Chao, Fleischer and Yang provide evidence for a novel role for the mouse transmembrane guanylyl cyclase-G (GC-G). Specifically they show that this membrane protein expressed in the neurons of the olfactory Grueneberg ganglion is the first alarm pheromone (AP) receptor identified in mice.
This is both a very interesting and awaited discovery. Indeed the search for the AP receptor(s) started in 2008 with the discovery of alarm pheromones being detected by the neurons of the olfactory Grueneberg ganglion in mice. The first mouse alarm pheromone, SBT, has been identified in 2013. The authors show that this alarm pheromone binds to the GC-G enzyme increasing its activity. After heterologous expression, they verified that the cells responded indeed to SBT. Finally, they convincingly use a transgenic mouse they devopped previously to demonstrate that this protein is involved in this fundamental chemosensing event. The experiments have been comprehensively described and executed. The authors mostly use previously published experimental strategies. The conclusions are justified. This will be an important contribution for the olfactory field and for the guanylyl cyclase community as well as for others interested in animal communication and survival, in evolution as well as in fear signaling and anxiety.

My minor concerns as recommandations for improvement and questions regarding this manuscript are the following:

1)The authors claim that they have deorphanized GC-G as an AP receptor as they show, after heterologous expression, activation with 2-sec-butyl-4,5 dihydrothiazole (SBT), the only mouse AP identified so far. Previous studies have demonstrated that GG neurons not only respond to SBT but to a family of ligands sharing a similar chemical structure. Could the authors please either reformulate and moderate their discussion arguments on GC-G deorphanization and focus only on GC-G as a SBT receptor or apply their readily available techniques to determine the GC-G receptor selectivity and sensitivity in a true deorphanizing process. They could test, for example, its activation by TMT (2,3,5-trimethyl-3-thiazoline), a fox emmited chemical, widely used to induce fear. TMT shares a very similar chemical structure with SBT. This would greatly improve the importance of the study as TMT is used as a common fear inducing substance in many labs trying to understand, for example, the brain regions implicated in fear and anxiety.

2)The authors have modified the name of the protein they already study/publish for years (Guanylyl cyclase-G → Guanylate cyclase-G) ? Is there any scientific intent ?

3)The experimental protocols used in this manuscript have been established and previously published either by the authors themselves or by others in the field. This earlier literature should be quoted more fairly.

4)(Figure 3). Butyric acid, 2-heptanone or menthol, known chemicals that do not activate the GG should be used as typical and appropriate control in these fundamental experiments. It would reinforce the results and claims of the paper.

5)(Figure 4). The authors have previously demonstrated that the temperature strongly influences the recorded GG responsiveness observed in mice pups (Mamasuew et al., 2011) ; Chao et al. 2015,

EMBO J.). Thus why do they perform their calcium imaging experiments at 37{degree sign}C, a temperature at which they have shown that chemical responses are strongly inhibited ? Could parallel signaling pathways for SBT and for other GG ligands differentially influenced by temperature coexist? Furthermore, why is IBMX used during these sets of experiments ?

6)(Figure 4). The authors still recorded SBT-induced responses in calcium imaging experiments on GG neurons from KO mice (6/32). They decided not to show these responses (data not shown). This is unfortunate as analysis of these responses would greatly help the understanding of the molecular pathway(s), or parallel pathway(s) ? involved in the detection of GG ligands.

7)(Figure 4). Ratio calcium imaging should be expressed as Delta R not Delta F.

8)The authors should state and describe in more details the importance of the results found in Figure 5. The description of the phenotype observed in the KO mice is very important. These animals do not freeze anymore although they still smell SBT. The authors should add/comment on the statistics/significance in between the conditions shown.

9)The authors used a different physiological solution for presenting the alarm pheromone as a chemical cue in their behavioral experiments (ACSF, Figure 5) as in their calcium imaging experiments (Figure 4). The same solution should be used as control. SBT should be diluted in ACSF or the diluting agent used, DMSO, should be tested for its putative freezing properties.

10)The alarm pheromone SBT has been shown to activate neurons of different olfactory systems, as mentioned by the authors. The work of Matsuo et al., (2015, PNAS) should be mentioned at appropriate places.

11)The authors used the word "axiotomy" and should instead use the word "axotomy" (word derived from axo- (=axon) and -tomy (=surgery)) as it is the cutting or otherwise severing of an axon, that they are performing. A surgical procedure widely used.

12)Please use the same nomenclature for GC-G in all the figures.

13)Page 5, the word "manor" should be replaced by "manner".

Referee #3:

Chao et al. show show in this manuscript that heterologous expression of the GCG receptor in HEK cells renders them responsive to SBT, an alarm pheromone in mice. Moreover, they point to a role played by the extracellular domain of the receptor in this interaction. Finally, they show that mice lacking the GCG receptor exhibit altered responses to SBT.

This study is very interesting, and timely. I however have a few questions, some of which need to be answered for me to be convinced of the story.

I have three main problems.

1) First, the authors use a variety of controls, but always aimed at controlling the specificity of the receptor, and never at controlling the ligand SBT they are testing. I do not understand that. For example:
a) in Figure 1, how is the specificity of the ligand tested? I see no GCG-FL transfected HEK on which another molecule (a non-GCG agonist) is added.
b) on Figure 3. Is there a control with a non-binding compound?

2) Second, since it is not mentioned, I am afraid that the wt and GCG-ko mice that are compared could be from separate colonies, and not littermates. They should be littermates. Even if there were 15 backcrosses of the null allele with C57BL6, the KO mice cannot be compared with wt C57BL6. As we know today, there are many problems that may arise by comparing mice that are not littermates, that range from epigenetic modulations to differences that may result from being raised by GCG-ko versus wt parents. So I really hope the authors will respond that the mice were

littermates. Otherwise, these data should not be published.

3) Third, the authors somehow evacuate the fact that multiple neurons still respond to SBT when lacking GCG. In Figure 3D for example, why is the SBT-induced deltaF induced in the 6 responsive GCG-KO cells not shown when the one corresponding to the 24 GCG+ is shown? This is a critical question. To me, GCG seems to favor a response that may anyway occur even in its absence.

Other questions:

4) Mice were exposed to either SBT or to the control ACSF. SBT is dissolved in DMSO apparently. I guess that the corresponding amount of DMSO was added to the control condition?

5) Figure 2. I could not find any indication on the number of plates tested, on the number of replicates or on the number of separate experiments. Do the graphs correspond to a mean between different readings? I hope so.

6) The authors indicate "OMP-GFP" mice as a genotype. This is not good enough, in particular because I believe that this OMP allele corresponds to a null allele. So if the mice are homozygous for the mutant allele, they are OMP-null mice. This may not be of major importance, but since Grueneberg neurons express OMP, it should at least be indicated.

7) Figure 3B and C is problematic because one only sees a chosen neuron. Why not showing the 30 and 32 traces? Just using thin lines and colors would do it. I would be very interested in seeing the trace of the 6 responsive GCG-KO cells. Do they respond as well as the GCG+ cells?

8) On Figure 5 C, the upper right panel does not seem representative to me when I compare it to 5B.

1st Revision - authors' response 04 August 2017

To Referee #1:

(1)The introduction should explain more clearly how SBT is synthesized and excreted. Is it in urine, feces or sweat? Is it released acutely or chronically? Is it volatile (how will it reach the GG in the nose of conspecifics?)
Response: Following the referee's advice, we have included information about the release and volatility of SBT in the Introduction section of the revised manuscript (page 3). SBT is a volatile substance; according to ChemSpider (http:// www.chemspider.com/ Chemical-Structure.142398.html), its vapor pressure is 0.7 ± 0.4 mmHg (at 25 °C).

(2) Are the concentrations of SBT used in vitro (Fig. 1 and 2) similar to the "natural" concentrations which mediate communication between conspecifics?
Response: In a recent study, the estimated SBT concentration released during different alarm conditions was in the micromolar range (Brechbuhl et al. 2013, PNAS, 110:4762-4767). For the in vitro experiments depicted in Fig. 1 and Fig. 2, we used SBT concentrations up to 500 nM. Thus, the SBT concentrations utilized in these approaches are even lower than the "natural" concentrations used for intraspecific communication.

(3) Which sequences within the ECD of GC-G are distinct from respective regions in other pGCs and could account for this selective responsiveness of GC-G to the non-peptide molecule SBT?
Response: Based on a protein sequence alignment, the ECD of GC-G shares limited sequence identify (less than 20%) with the ECD of other receptor GCs. Although a few stretches of amino acid sequences seem to be conserved between mouse and rat GC-G (see the alignment below), it remains difficult to assess whether these sequences within the ECD of GC-G are functionally unique and could be responsible for the selective responsiveness of GC-G to SBT. Further experiments are required to verify whether these sequences are indeed involved in the ligand selectivity of GC-G.

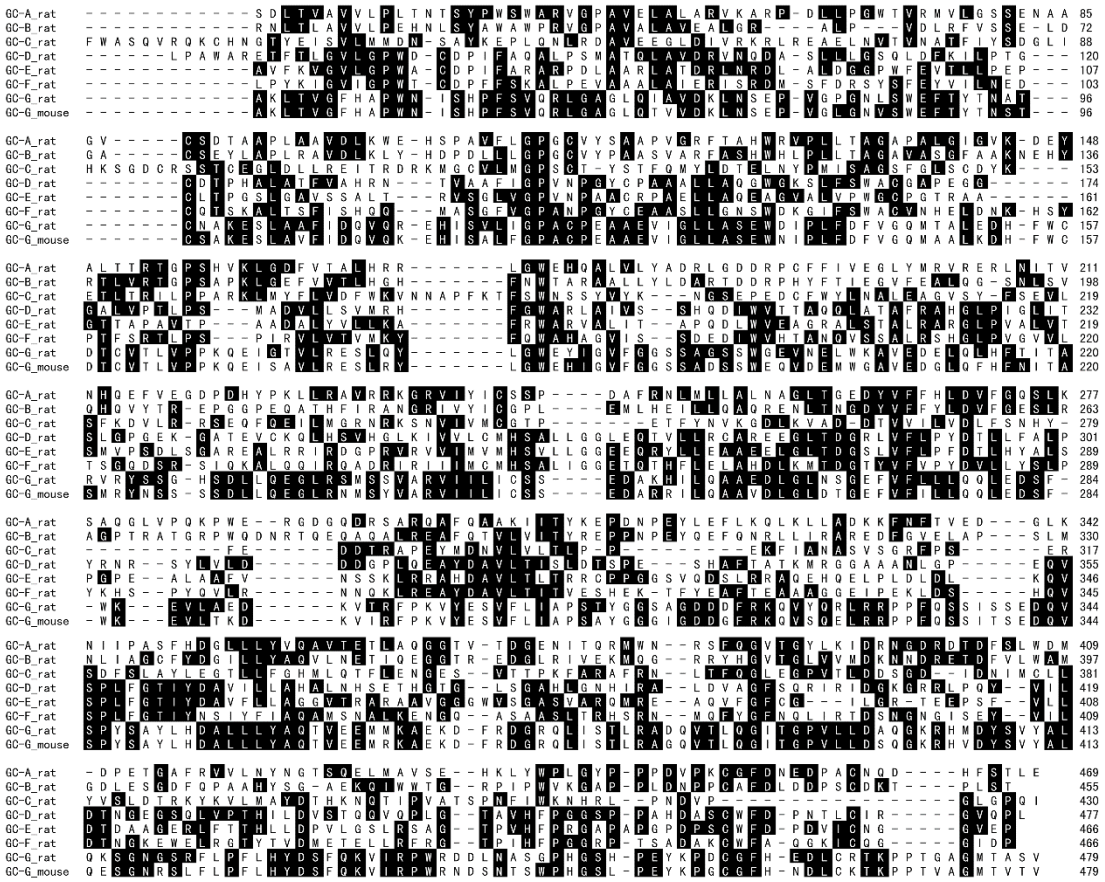

Fig.: Sequence alignment of the extracellular domains (ECDs) of transmembrane GCs.

(4) The experiments with the deletion constructs lead to the valid hypothesis that SBT activates the ECD. However, the authors have to demonstrate that the mutated GC-G protein (lacking the ECD) is still functional, e.g. capable of stimulated synthesis of cGMP (Fig. 1).
Response: We have performed additional experiments to verify that the mutated GC-G protein (lacking the ECD) remains capable of stimulated cGMP synthesis. In this context, it has been reported previously that similar to other receptor GCs, Mn^{2+} or Mn^{2+} and Triton X-100 increased the enzymatic activity of full-length (FL) GC-G (Kuhn et al. 2004, Biochem J, 379:385-393). As shown in Supplementary Fig. S2A of the revised manuscript, the cyclase activity of the mutant GC-G protein lacking the ECD is clearly stimulated in the presence of Mn^{2+} or Mn^{2+} and Triton X-100. In addition to activation by Mn^{2+} or Mn^{2+} and Triton X-100, GC-G enzymatic activity was recently found to be stimulated by cool temperatures (Chao et al. 2015, EMBO J 34:294-306). Similar to full-length (FL) GC-G, cGMP accumulation in HEK cells expressing the truncated isoform of the GC-G protein lacking the ECD was largely increased at a cool ambient temperature (Supplementary Fig. S2B of the revised manuscript), indicating that this mutant protein can be stimulated to produce cGMP by coolness.

(5) On page 7, Para 2, artificial cerebrospinal fluid was used as control. Why is this an appropriate negative control for studies of SBT in vivo?
Response: Artificial cerebrospinal fluid (ACSF) is frequently used as a vehicle solution for testing chemical agents in neuroscientific studies. Moreover, ACSF has also been used as a control in the first study investigating the role of SBT as an alarm pheromone in mice (Brechbuhl et al. 2013, PNAS, 110:4762-4767).

(6) Is GC-G activated by other small alarm pheromones?
Response: To our knowledge, no other small-molecule alarm pheromones have been identified in mice. Therefore, it is currently unknown whether GC-G can be activated by other alarm pheromones in mice.

(7) 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: The present results indicate that GC-G is activated in GG neurons by the small-molecule ligand SBT. These findings raise the possibility that chemically related or unrelated small-molecule ligands might stimulate the enzymatic activity of GC-G in other organs/tissues as well, including kidney and sperm cells. This aspect has been added to the Discussion section.
Another known mechanism to regulate GC-G activity is apparently ligand-independent since GC-G is activated by coolness (Chao et al. 2015, EMBO J 34:294-306). This aspect is also mentioned and discussed in the Introduction and the Discussion section. Moreover, it is shown in Supplementary Fig. S2B.

Minor:
(8) On several pages the word "manor" has to be replaced by "manner"
Response: We are grateful for the reviewer's correction. The word "manor" has been replaced by "manner" throughout the revised manuscript.

(9) The IUPHAR recommends to use the nomenclature "guanylyl" (not guanylate) for this receptor family
Response: We are thankful for the reviewer's suggestion. The word "guanylate" has been changed to "guanylyl" throughout the manuscript.

To Referee #2:

(1)The authors claim that they have deorphanized GC-G as an AP receptor as they show, after heterologous expression, activation with 2-sec-butyl-4,5 dihydrothiazole (SBT), the only mouse AP identified so far. Previous studies have demonstrated that GG neurons not only respond to SBT but to a family of ligands sharing a similar chemical structure. Could the authors please either reformulate and moderate their discussion arguments on GC-G deorphanization and focus only on GC-G as a SBT receptor or apply their readily available techniques to determine the GC-G receptor selectivity and sensitivity in a true deorphanizing process. They could test, for example, its activation by TMT (2,3,5-trimethyl-3-thiazoline), a fox emmited chemical, widely used to induce fear. TMT shares a very similar chemical structure with SBT. This would greatly improve the importance of the study as TMT is used as a common fear inducing substance in many labs trying to understand, for example, the brain regions implicated in fear and anxiety.
Response: The referee proposes to focus only on GC-G as a SBT receptor. Following this suggestion, we have reformulated and moderated our discussion on GC-G deorphanization. Accordingly, we have replaced the word(s) "alarm pheromone" or "alarm pheromones" by "alarm pheromone substance SBT".
Unfortunately, we could not test further alarm pheromone (AP) substances regarding a potential activation of GC-G since there are no other APs known for mice (as already mentioned by the referee). TMT (2,3,5-trimethyl-3-thiazoline) is not an AP but (as also mentioned by the reviewer) a kairomone released by foxes. Consequently, TMT is beyond the focus of the present manuscript that concentrates on the activation of GC-G by the AP substance SBT. Moreover, in this context, the findings of a recent report (Pérez-Gómez et al. 2015, Curr Biol, 25:1340-1346) suggest that the responses of GG neurons to TMT do not rely on cGMP signaling, an observation that does not support the notion that GC-G serves as a receptor for TMT.

(2)The authors have modified the name of the protein they already study/publish for years (Guanylyl cyclase-G → Guanylate cyclase-G) ? Is there any scientific intent?
Response: We are grateful for the reviewer's suggestion. The word "guanylate" has been changed to "guanylyl" throughout the revised manuscript.

(3)The experimental protocols used in this manuscript have been established and previously published either by the authors themselves or by others in the field. This earlier literature should be quoted more fairly.
Response: In response to the reviewer's suggestions, we have now cited additional references for SPR binding, innate fear behavior test, and serum corticosterone assays in the Materials and Methods section (page 14 and page16-17).

(4)(Figure 3). Butyric acid, 2-heptanone or menthol, known chemicals that do not activate the GG should be used as typical and appropriate control in these fundamental experiments. It would reinforce the results and claims of the paper.
Response: Following the referee's suggestion, we have conducted SPR binding assays (similar to that depicted in Fig. 3) with 2-heptanone and butyric acid. The results of these approaches are shown in Supplementary Fig. S3.

(5)(Figure 4). The authors have previously demonstrated that the temperature strongly influences the recorded GG responsiveness observed in mice pups (Mamasuew et al., 2011); Chao et al. 2015, EMBO J.). Thus why do they perform their calcium imaging experiments at 37{degree sign}C, a temperature at which they have shown that chemical responses are strongly inhibited? Could parallel signaling pathways for SBT and for other GG ligands differentially influenced by temperature coexist? Furthermore, why is IBMX used during these sets of experiments?
Response: The referee asks why we have performed calcium imaging experiments at 37 °C, a temperature at which we have shown that chemical responses are strongly inhibited. In fact, we have not shown previously that a temperature of 37 °C inhibits chemosensory responses in the GG. We have previously only described that warm temperatures enhance adaptational processes in GG neurons upon long-term exposures (up to several hours) to appropriate odorants [Mamasuew et al. 2011, Neurosignals, 19:198-209]. For the calcium imaging experiments of the present study, however, we only used short-term exposures (of a few minutes) to SBT. Moreover, cool temperatures enhance chemosensory responses in the GG [Mamasuew et al. 2011, Chem Senses, 36:271-82]; potentially in a GC-G-dependent manner since GC-G is crucial for GG responses to cool temperatures (Chao et al. 2015, EMBO J, 34:294-306). Therefore, we decided to use a clearly warm temperature of 37 °C for the calcium imaging experiments of this study.
The reviewer wonders whether parallel signaling pathways for SBT and/or other GG ligands that are differentially influenced by temperature might co-exist. So far, it is unknown whether parallel signaling cascades in GG neurons mediating the activation by a given ligand exist. Consequently, it is impossible to assess whether such potential parallel signaling pathways could be differentially influenced by temperature. In fact, in GC-G-deficient mice, responsiveness of GG neurons to SBT was abolished in the overwhelming majority of these cells (Fig. 4). These observations do not support the notion that a parallel (and GC-G-independent) signaling pathway might substantially contribute to SBT responsiveness. Yet (as mentioned in the Results section), in a small portion of GG neurons from GC-G-deficient mice, responsiveness to SBT was detectable, indicating that a GC-G-independent signaling cascade accounting for SBT responses exists in these cells.
The referee also asks why IBMX was used during calcium imaging experiments. As a phosphodiesterase inhibitor IBMX (3-isobutyl-1-methylxanthine) prevents the rapid degradation of cytosolic cGMP. Therefore, we used IBMX to sensitize calcium imaging approaches by increasing the cGMP-dependent calcium influx via the CNGA3 channel expressed in GG neurons and in our HEK cells.

(6)(Figure 4). The authors still recorded SBT-induced responses in calcium imaging experiments on GG neurons from KO mice (6/32). They decided not to show these responses (data not shown). This is unfortunate as analysis of these responses would greatly help the understanding of the molecular pathway(s), or parallel pathway(s)? involved in the detection of GG ligands.
Response: Following the reviewer's suggestion, the SBT-evoked calcium signals of the 6 SBT-responsive GG neurons from GC-G-KO pups are depicted in Supplementary Fig. S4 of the revised manuscript. For comparison, we also included in this novel figure results from GG neurons of animals endowed with GC-G.

(7)(Figure 4). Ratio calcium imaging should be expressed as Delta R not Delta F.
Response: According to the reviewer's suggestion, we have replaced ΔF by ΔR throughout the revised manuscript.
(8)The authors should state and describe in more details the importance of the results found in Figure 5. The description of the phenotype observed in the KO mice is very important. These animals do not freeze anymore although they still smell SBT. The authors should add/comment on the statistics/significance in between the conditions shown.
Response: Following the reviewer's suggestion, we have stated and described in more detail the significances of the results depicted in Fig. 5 by adding to the Results section the statistics and p-

values concerning walking distance and freezing time comparing WT and GC-G-KO mice (page 8 and page 9).

(9)The authors used a different physiological solution for presenting the alarm pheromone as a chemical cue in their behavioral experiments (ACSF, Figure 5) as in their calcium imaging experiments (Figure 4). The same solution should be used as control. SBT should be diluted in ACSF or the diluting agent used, DMSO, should be tested for its putative freezing properties.
Response: In Fig. 4 (calcium imaging experiments) we compare the responses of GG neurons from OMP/GFP animals with that of OMP-GFP/GC-G-KO mice upon exposure to SBT. For both mouse strains, SBT was dissolved in DMSO and then diluted in recording buffer. For the behavioral experiments depicted in Fig. 5, (wild type) mice were either exposed to ACSF (control) or to SBT dissolved in DMSO. Importantly, as documented in Supplementary Fig. S6 of the revised manuscript, DMSO does not significantly affect walking distance and freezing behavior in comparison to ACSF.

(10)The alarm pheromone SBT has been shown to activate neurons of different olfactory systems, as mentioned by the authors. The work of Matsuo et al., (2015, PNAS) should be mentioned at appropriate places.
Response: According to the reviewer's suggestion, the work by Matsuo and colleagues (2015, PNAS) is cited in the Introduction and in the Discussion section of the revised manuscript (page 3 and page 12).

(11)The authors used the word "axiotomy" and should instead use the word "axotomy" (word derived from axo- (=axon) and -tomy (=surgery)) as it is the cutting or otherwise severing of an axon, that they are performing. A surgical procedure widely used.
Response: We are very grateful to the referee for mentioning this spelling mistake. The word "axiotomy" has been changed to "axotomy" throughout the manuscript.

(12)Please use the same nomenclature for GC-G in all the figures.
Response: We have corrected the typo in Fig. 1B and used the same nomenclature for GC-G in all figures. We are very thankful for this hint.

(13)Page 5, the word "manor" should be replaced by "manner".
Response: We are grateful for the reviewer's correction. The word "manor" has been replaced by "manner" throughout the manuscript.

To Referee #3:

(1) First, the authors use a variety of controls, but always aimed at controlling the specificity of the receptor, and never at controlling the ligand SBT they are testing. I do not understand that. For example:
a) in Figure 1, how is the specificity of the ligand tested? I see no GCG-FL transfected HEK on which another molecule (a non-GCG agonist) is added.
Response: To assess the specificity of GC-G activation by SBT, two other odorous compounds (butyric acid and 2-heptanone) that do not activate GG neurons (Mamasuew et al. 2011, Chem Senses, 36: 271-28; Brechbuhl et al. 2013, PNAS, 110: 4762-4767) were tested for their potential to stimulate GC-G enzymatic activity. In contrast to SBT, these two compounds did not affect the intracellular cGMP accumulation (Supplementary Fig. S1).

b) on Figure 3. Is there a control with a non-binding compound?
Response: Control experiments for SPR binding assays (Fig. 3) have been performed with two non-binding compounds (butyric acid and 2-heptanone). The results are shown in Supplementary Fig. S3 of the revised manuscript.

(2) Second, since it is not mentioned, I am afraid that the wt and GCG-ko mice that are compared could be from separate colonies, and not littermates. They should be littermates. Even if there were 15 backcrosses of the null allele with C57BL6, the KO mice cannot be compared with wt C57BL6. As we know today, there are many problems that may arise by comparing mice that are not littermates, that range from epigenetic modulations to differences that may result from being raised

by GCG-ko versus wt parents. So I really hope the authors will respond that the mice were littermates. Otherwise, these data should not be published.
Response: We thank the reviewer for reiterating the importance of comparing littermates. We are aware of these concerns. Indeed, throughout this study, the WT and GC-G-KO animals compared are littermates derived from heterozygous intercrosses. The use of littermates has now been clearly stated in the Materials and Methods section (page 15).

(3) Third, the authors somehow evacuate the fact that multiple neurons still respond to SBT when lacking GCG. In Figure 3C for example, why is the SBT-induced deltaF induced in the 6 responsive GCG-KO cells not shown when the one corresponding to the 24 GCG+ is shown? This is a critical question. To me, GCG seems to favor a response that may anyway occur even in its absence.
Response: According to the reviewer's question, in the revised manuscript, the SBT-evoked calcium signals of the 6 SBT-responsive GG neurons from GC-G-KO pups are depicted in Supplementary Fig. S4. For comparison, we also included in this figure results from GG neurons of animals endowed with GC-G.
Based on our data, from our point of view, GC-G does not favor a response to SBT that may occur even in the absence of GC-G since in the overwhelming majority of GG neurons from GC-G-deficient animals, we could not observe a response to SBT.

Other questions:

(4) Mice were exposed to either SBT or to the control ACSF. SBT is dissolved in DMSO apparently. I guess that the corresponding amount of DMSO was added to the control condition?
Response: Comparing wild type mice either exposed to DMSO or ACSF, DMSO had no significant effect on freezing behavior and walking distance (Supplementary Fig. S6).

(5) Figure 1. I could not find any indication on the number of plates tested, on the number of replicates or on the number of separate experiments. Do the graphs correspond to a mean between different readings? I hope so.
Response: As stated in the corresponding figure legend, the results shown in Fig. 1 are mean ± SD from three experiments in triplicate.

(6) The authors indicate "OMP-GFP" mice as a genotype. This is not good enough, in particular because I believe that this OMP allele corresponds to a null allele. So if the mice are homozygous for the mutant allele, they are OMP-null mice. This may not be of major importance, but since Grueneberg neurons express OMP, it should at least be indicated.
Response: According to the reviewer's suggestion, it is described in the Material and Methods section of the revised manuscript that in OMP-GFP mice (page 15), the coding sequence and a portion of the 3' untranslated region of the OMP gene is replaced by a sequence encoding GFP; thus the targeted mutation results in a knockout.

(7) Figure 4B and C is problematic because one only sees a chosen neuron. Why not showing the 30 and 32 traces? Just using thin lines and colors would do it. I would be very interested in seeing the trace of the 6 responsive GCG-KO cells. Do they respond as well as the GCG+ cells?
Response: Regarding the reviewer's suggestion (and as already mentioned in our response to question #3 of the referee), additional representative ratiometric calcium traces following exposure to SBT in GFP-positive GG neurons from OMP-GFP as well as OMP-GFP/GC-G-KO pups are shown in Supplementary Fig. S4 of the revised manuscript. Responses to SBT in the 6 SBT-reactive GG neurons from GC-G-KO mice seem to be weaker and slower as compared to that in GG neurons endowed with GC-G.

(8) On Figure 5 C, the upper right panel does not seem representative to me when I compare it to 5B.
Response: To clarify the reviewer's concern, the walking traces upon exposure to ACSF or SBT from all wild type and GC-G-KO animals analyzed are shown in Supplementary. Fig. S5.

2nd Editorial Decision 18 August 2017

Thank you for submitting your revised manuscript to The EMBO journal. Your study has now been seen by referee #1 and 3 and their comments are provided below.

As you can see, both referees appreciate the introduced changes and support publication here. I am therefore very pleased to accept the manuscript.

There are just a few last things that need to be sorted out. You can submit the files using the link below

- Please upload individual figure files

- The Appendix figures are incorrectly labeled plus it needs a TOC. Please see http://emboj.embopress.org/authorguide#expandedview

- We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

- We also need a summary figure for the synopsis. The size should be 550 wide by 400 high (pixels). You can also use something from the figures if that is easier.

That should be all - once we get the last things sorted out then I will send you the formal acceptance letter.

REFEREE REPORTS

Referee #1:

The manuscript entitled "Guanylate cyclase-G is an alarm pheromone receptor in mice" by Chao et al. describes carefully performed, very elegant molecular, functional and imaging studies in vitro/in vivo which demonstrate for the first time the activation of a mammalian membrane guanylyl cyclase receptor by a non-peptide ligand, the alarm pheromone SBT, and the role of this signaling pathway in fear behavior and stress-induced neurohumoral responses of rodents. 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 peptide hormones (ANP, BNP, CNP, guanylins), the others remained 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.

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. In a previous study published in the EMBO J, the authors of the present manuscript described 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, they demonstrated that the cGMP-synthesizing activity of GC-G is directly activated by low temperatures (15oC). These previous studies also showed that 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 (EMBO J. 2015 Feb 3;34(3):294-306).

In the present study the authors followed the hypothesis that GC-G participates in the chemosensation of alarm pheromones such as SBT, a volatile small molecule released by rodents during danger, to warn conspecifics. This hypothesis was indeed challenging, since all previously

known extracellular ligands activating particulate GCs to cGMP production are small peptides. By fine biochemical and imaging studies in overexpressing HEK cells, Chao et al. demonstrate that SBT activates GC-G to cGMP production, that this activation requires the extracellular domain of the receptor, and that this results in cGMP-dependent opening of CNGA3 channels and increases of intracellular calcium levels. To assess the functional, physiological relevance of this signaling pathway, the authors carefully performed comparative molecular, behavioral and biochemical studies in GC-G-gene knockout mice and their wildtype littermates. These studies demonstrate that 1) SBT induces calcium signaling in specific GC-G expressing neurons of the grüneberg ganglion (GG) in situ; and 2) such GC-G-induced cGMP and calcium increases are critically involved in the fear- and stress-associated responses of mice to SBT (walking distance, serum corticosterone levels, blood pressure). Taken together these studies demonstrate for the first time the activation of the extracellular domain of a particulate GC, GC-G, by a small molecule, the alarm pheromone SBT, and the relevance of this signaling pathway in chemosensation, specifically in the fear responses of mice.

Although GC-G is not expressed in higher mammals like humans, this is a novel, original and exciting study. With fine biochemistry and physiology, the authors demonstrate the role of GC-G in behaviour and, for the first time, the activation mode by a volatile, non-peptidergic ligand. The experiments are well conducted and clearly described. The manuscript is written in clear and comprehensive way. My previous questions and criticisms have been carefully addressed in the revised manuscript. The study will be clearly very interesting and stimulating for other scientists working in the cGMP field or in neuroscience.

Referee #3:

The authors responded adequately to my questions.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Ruey-Bing Yang
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2017-97155R

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For well-established techniques in the lab, all experiments were done at least three independent times.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The number of mice analyzed per genotype is specified for each experiment (see figure legends, page 22 to 25).
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	Mice from different breeding pairs were included in the experiments to increase randomization
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	We did not perform blinding test.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	We used Anti-FLAG M2 monoclonal antibodies as well as Anti-FLAG M2 covalently attached to agarose were also purchased from Sigma-Aldrich. References are provided in the respective Company's webpages.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK-293T cells utilized in these experiments were acquired from ATCC. No STR profiling and mycoplasma contamination were tested.

* for all hyperlinks, please see the table at the top right of the document

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Methods page 15 to 17.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Methods page 15 to 17. All animal experimenal procedures were approved by the Institute Animal Care and Utilization Committee at Academia Sinica (Taiwan).
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confirm complience with ARRIVE guildlines

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA