THE EMBO JOURNAL

Manuscript EMBO-2011-80173

Ciguatoxins activate specific cold pain pathways to elicit burning pain from cooling

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Review timeline:

Submission date:	17 November 2011
Editorial Decision:	17 January 2012
Additional Correspondence:	27 January 2012
Editorial Decision:	23 March 2012
Revision received:	26 March 2012
Editorial Decision:	17 May 2012
Additional Correspondence:	23 June 2012
Revision received:	27 June 2012
Accepted:	28 June 2012

Transaction Report:

(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 17 January 2012

Thank you for submitting your manuscript to the EMBO Journal. I have now heard back from the three referees who have evaluated your study and their comments are provided below.

As you can see, the referees find the study interesting and appreciate the insights gained into the pain pathways involved in ciguatoxin-induced cold allodynia. However, a major issue highlighted by referee 3 concerns the data based on human subjects. We have discussed the issues raised with an expert in medical ethics and we share these concerns. We cannot proceed with the publication of this manuscript in light of these issues, which are outlined in more detail below.

Specifically, the issues are as follows:

1) the use of self-experimentation - 6 of the authors were injected with P-CTX-1. This is problematic as the consent of lab personnel/authors might be compromised due to conflict of interests

2) The information provided for how P-CTX-1 was prepared is not sufficient and it remains unclear if it follows clinically approved protocol to be used on human subjects.

3) There is insufficient information provided for how the human study was carried out. Was there formal approval for the procedure and the protocol from an IRB? Finally, it is not clear that these experiments were essential in the first place.

All these issues raised are important as ciguatoxin elicits severe neuropathic pain and as ciguatoxin may affect the central nervous system, and may cause other side effects. We therefore have to apply the same stringent ethical standards as we would apply to a clinically focussed study.

Given the concerns raised regarding the human experimentation, I am afraid that we cannot consider publication in the EMBO Journal.

I am very sorry that I can't be more positive on this occasion.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

The data presented by the authors provides a molecular explanation to the pathophysiology of Ciguatoxin evoked cold allodynia. The authors first demonstrate the painful reaction exacerbated by cold induced by Cinguatoxin skin injection in humans (performing these experiments on them!). By creating a mouse model of this painful toxic effect prevalent the pacific area in humans following eating certain fish, they showed the involvement of primary afferent sensory C and A fibers, as well as the identification of the brain structures mediating cold allodynia. The findings are totally novel and thrilling not only for the sensory neurons field but more broadly to the large community of physiologists and pharmacologists. The experiments are convincing and technically well done. In conclusion the manuscript is relatively clear and provides the reader with the background necessary for a good understanding of the results. I have anyway some question that would need to be addressed.

Specific points:

1) Ciguatoxin toxicity develops in human after eating poisoned fish, why not reproducing this in mice by giving diluted amounts of the toxin per os? This would be a clinically relevant model. Although I understand that intraplantar injection is experimentally much easier, it reflects only in part the clinical situation. This should be at least discussed.

2) At the molecular level, a great part of the neuronal population that is affected by the toxin involves DRG neurons expressing NaV1.8 TTX resistant sodium channels as well as TRPA1 channels. Both of these channels have been implicated in mechanosensation. Accordingly, the genetic ablation of the NaV1.8 expressing neurons results in a phenotype of deficient mechanical and cold perception. This has been commonly retrieved with almost all the conditional knock outs using the NaV1.8-Cre mice. Furthermore, the TRPA1 channel, presented here as a major cold sensor, is also reported in the literature as a mechanically activated channel. In addition, for the experiments using the skin nerve preparation in this study, the responsiveness of isolated fibers is first probed by mechanical stimulation before cold stimulation. Thus the effect of Cinguatoxin on the mechanical thresholds should be studied both in vitro and in vivo. In addition, it would be fair to cite the literature evidencing a role of TRPA1 in mechanotransduction.

3) Cinguatoxin mediated cold allodynia involves NaV1.8 / TRPA1 expressing C fibers and A fibers expressing TTX sensitive sodium channels. This demonstrated in vitro and in vivo on behavioral test. Anyway, the final level of functional exploration, using fMRI analysis of brain regions mediating cold allodynia, only concentrates on the role of TRPA1 expressing fibers and subsequent neuronal circuitry in the pain matrix. This latter part, to be consistent with the other parts of the study would benefit from the exploration of TTX-sensitive afferent fibers by using TTX in the paw

and subsequent effect on fMRI signal. Similarly using TTX in combination with HC030031 would be of interest to completely abolish the cold allodynia and monitor it at the fMRI level.

Minor point:

1) TRPM8 positive neurons seem to be responsive to Cinguatoxin in vitro, but the TRPM8 KO shows no alteration of cold allodynia! Please provide an explanation. Is it possible that the antibody used is non specific...

Referee #2

Overall, this is a very interesting manuscript explaining cold allodynia induced by ciguatoxin, which involves TRPA1 and voltage-gated sodium currents. The manuscript is, however, somewhat confusing in many instances, and often lacks a clear description of what is shown in the figures, as outlined below. Resolving these issues would make this a strong paper of broad interest.
Specific points:
*Page 5: What is meant with "that overlapped sensory effects in humans."
*Legend to Figure 1: F should be G and vice versa.
*Page 6: it would be informative to have some references for the statement that the toxin is "the most potent pro-algesic compound known." Maybe some effective doses of other potent pro-algesics.
*There is no good rationale to express Fura-2 data as deltaRatio/Ratio (e.g. in Figure 3H, I, K-N). This way the advantage of a ratiometric dye is lost by normalisation, and responses of equal amplitude will yield higher values for cells with a lower basal calcium. I strongly advise to use deltaRatio or, ideally, absolute calcium values.
*It is fully unclear to me what is exactly shown in Figure 3I,J. How is TTX-sensitivity defined in this context. Description in text and legend is insufficient.
*In Figure 3K-N I miss examples of what happens when wt and TRPA1-/- neurons are stimulated twice with cold, in the absence of P-CTX-1. As presented now, there is no evidence that the sensitisation to cold is due to the toxin, and not due to another form of P-CTX-1-independent, TRPA1-dependent sensitisation.
*In Figure 4A it seems that cold is inhibiting P-CTX-1-induced APs. Is this a consistent observation?
*FIgure4F: it would be good to see experimental examples of the P-CTX-1-induced membrane potential changes.
*Figure 4g: What was the dose of p-CTX-1? WHy was this performed in different cells than Figure 4D? Is the response also fully blocked by a NaV1.3 blocker? What is the resting membrane potential of NaV1.3-TRPA1-expressing HEK293 cells? The answers to these questions are important to support the proposed mechanism of P-CTX-1-induced, TRPA1-dependent responses.
* Page 10: The senior author recently published that TRPC5 is a candidate cold sensor, in addition to TRPM8 and TRPA1. Shouldn't that be mentioned here?
* Is the initial pain response dependent on TRPA1?
* Page 15: Note that Gentry et al. (Mol Pain 2010) also find altered cold sensitivity in vivo.
* Page 16: It is unclear whether the cold responses in TRPA1-/- neurons are purely TRPM8. Are these neurons menthol-sensitive?
* Other TRP channels expressed in the sensory neurons are also voltage-gated (e.g. TRPV1), and could thus potentially be activated by the P-CTX-1-induced depolarisation. It would therefore be interesting to know whether there is also heat-hyperalgesia after P-CTX-1 injections.

Referee #3

Despite some advances discussed below, this paper reports results from human experimentation that have been conducted in a questionable manner, as it appears to this reviewer (see below).
Excluding human experiments, overall, this paper presents interesting and novel findings, gathered by a group of experimentally well-accomplished scholars. The role of the Trpa1 / TRPA1 gene and ion channel protein in altered neural transduction caused by ciguetera toxin is examined, and evidence is presented in favor of a critical role for TRPA1.

A couple of caveats will have to be heeded by the authors.

Re the abstract: are all claims from the abstract valid ?
Also, the abstract needs to be rewritten for a general audience, as is it is targeting a more specialized neurophysiology audience.

Re the ciguatera clinical complex beyond the acute intoxication. Sensory disturbances are very disturbing to patients, especially as they involve cold allodynia, but perhaps they are not the most disturbing clinical features that can occur. CNS neuropsychiatric manifestations and especially asthenia and a chronic-fatigue-like syndrome have also been reported. In case these symptoms are part of chronic ciguatera, then it is hard to see that they not become the clinically dominating feature re quality of life of the affected patient.

Intraplantar injection is not only targeting sensory neurons' peripheral projections, TRPA1 is also expressed in keratinocytes where it might function in a modulatory role (J Neurosci. 2009 Apr 15;29(15):4808-19). Having established another specific tool will enable the investigators to test this particular hypothesis. Intraplantar injection is certainly not a valid model for ciguatera, rather it is an approach to better understand issues of peripheral neural transduction problems that can happen in ciguatera.
In human disease, how does the toxin reach sensory neurons and their peripheral projections after initially being absorbed from the intestine ? This process happens in the human intoxication but has not been modeled in the described animal experiments.

fMRI CNS signals are also a result of sensory transduction, but certainly also driven by intra-CNS processing (see Nature Neuroscience 15, 70-80, 2012, referring to a possible role for TRPA1 in astrocytic modulation of neural transmission). In Trpa1 pan-null mice, that is in the complete absence of Trpa1 in all cells at all developmental changes, intra CNS changes are not necessarily caused by TRPA1-expressing DRG neurons.

Finally, since the ms. is not concise, it can certainly benefit from organizing it with subheadings.

Re the human experimentation, the most questionable part of the entire study:
This reviewer has doubts about the conducted human experimentation, their appropriateness and whether they have been conducted in an ethically sound manner, despite mentioning of approval from the local university.
Specifically:
Insufficient information is given about the human study or how the compound was prepared for administration to humans, about the review of the institutional human experimentation protocol process, and the process of informed consent. After all, as reported, severe neuropathic pain was elicited in human subjects (!).
At least as troubling is an apparent violation to the Belmont Report regarding human research. This set of guidelines explicitly prohibits lab personnel from being human subjects because their consent might be compromised, that is they may not feel free to refuse participation without adverse consequences or other bias. Furthermore, was the possibility of remote effects of the injected ciguatoxin revealed to participants in writing, including the possibility of effects on the central nervous system and sexual transmission (Clin Toxicol 1989;27:193-197) ?

Additional Correspondence 27 January 2012

The authors submitted full documentation (IRB approval and informed consent forms) to The EMBO Journal.

1st Editorial Decision 23 March 2012

Thank you for your patience in this case. As indicated to you previously, we involved an additional ethical advisor to look at the raised ethical issues and the IRB approval. I have now received input from the advisor, which is provided below. The advisor finds that the consent documentation and the IRB approval in order. Also s/he is of the opinion that appropriate actions were taken to limit the risks and to avoid compromising the consent of the lab personnel that took part in the study.

Given that appropriate measures were taken concerning the human experimentation aspect of the study, we can therefore offer to consider a revised manuscript. As the advisor also suggests below, it would be good to integrate the ethic considerations, as provided in your response, into the revised manuscript. You can incorporate it into the materials and method section.

Yours sincerely

Editor
The EMBO Journal

Advisor:

After having read a) the text, b) the answers to the reviewers and c) the ethical consent documentation, I personally am of the opinion that the manuscript should be published (from an ethical perspective) provided that the biomedical elements of the text are convincing. Deleting just the sections about self-experimentation is no option. The experiments exist.

Although it is true that self-experiments like the one described are always in danger of including "vulnerable" probands whose informed consent might be "enforced" by dependencies or gratifications, in this case the authors' argumentation AND their documentation that they tried to avoid this situation are convincing.

They took several actions to limit the risks and to obtain autonomous probands. The Independent Ethical Review Board (IRB) in Erlangen was fully aware of what they were approving. They made clear in the second paragraph of their approval that only probands should be chosen and included who are not in any way in a relationship of dependence with the researchers conducting the study. I personally think that the authors could clarify in their answer to the reviewers that they had this warning in mind when they chose the probands. Additionally the application to the IRB has several sections in which the authors state their sensibility regarding this ethically difficult issue. They clarify the dose of the substance used, offered a risk-benefit ratio etc. Maybe they should have performed a test for pregnancy before the experiments but at least they asked the probands whether they think they might be pregnant. The informed consent forms are clear, state every risk.

The only inconsistency is that in the informed consent form the pain induced is compared to that of bloodletting while in the manuscript the scale went up to "10 maximal imaginable pain" (only 8 was reached, see fig. 1).

Regarding the questions whether the experiments were necessary, the arguments the authors give for having conducted the experiments seem to be reasonable as well.

Finally, I would not think that in this case the risks taken were inappropriate or that the experiments would encourage others to perform dangerous self-experiments.

Whether self-experiments are a suitable tool for research or whether they always cause selection biases etc. is not so much an ethical but a methodological question.

All in all, I would recommend or encourage the authors to add their detailed ethical considerations, which they specified in their response to the manuscript (it could be integrated in the method section). This would clarify that self-experimentation is not just for fun and that ethical requirements must be taken into consideration and that the authors considered these BEFORE the experiments.

1st Revision - authors' response 26 March 2012

Referee #1:

The data presented by the authors provides a molecular explanation to the pathophysiology of Ciguatoxin evoked cold allodynia. The authors first demonstrate the painful reaction exacerbated by cold induced by Cinguatoxin skin injection in humans (performing these experiments on them!). By creating a mouse model of this painful toxic effect prevalent the pacific area in humans following eating certain fish, they showed the involvement of primary afferent sensory C and A fibers, as well as the identification of the brain structures mediating cold allodynia. The findings are totally novel and thrilling not only for the sensory neurons field but more broadly to the large community of physiologists and pharmacologists. The experiments are convincing and technically well done. In conclusion the manuscript is relatively clear and provides the reader with the background necessary for a good understanding of the results. I have anyway some question that would need to be addressed.

Specific points:
1) Ciguatoxin toxicity develops in human after eating poisoned fish, why not reproducing this in mice by giving diluted amounts of the toxin per os? This would be a clinically relevant model. Although I understand that intraplantar injection is experimentally much easier, it reflects only in part the clinical situation. This should be at least discussed.

Ciguatera presents with complex gastrointestinal, cardiac and neuronal pathology which is dominated by peripheral sensory disturbances. We were specifically interested in the pathogonomic symptom of CTX-induced cold allodynia, and in particular the actions of CTX on peripheral sensory neurons. Previous studies using orally or intraperitoneally administered CTX report diverse symptoms, some of which are similar to human ciguatera intoxication (Hoffman, Granade et al. 1983). Specifically, intraperitoneal administration of CTX is associated with diarrhoea, hypothermia, salivation, lacrimation, muscle weakness, decreased motor activity and cyanosis. Importantly, systemic administration of ciguatoxin causes decreased nerve conduction velocity and decreased corneal and nociceptive withdrawal reflexes, precluding evaluation of nociceptive thresholds in mice treated with systemic ciguatoxin. In addition, purified P-CTX-1 is in extremely limited supply. Systemic treatment with CTX would have required doses up to 1000-fold higher than those we administered. For these reasons, we chose to administer CTX purely by the intraplantar route to isolate the actions of CTX on peripheral sensory neurons and to avoid any potentially interfering systemic effects. In addition, this approach has the advantage of presenting control responses in the contralateral paw for direct comparison. **We have now modified the manuscript to expand on the effects of systemic administration of CTX (Results, page 6, line 10).**

2) At the molecular level, a great part of the neuronal population that is affected by the toxin involves DRG neurons expressing NaV1.8 TTX resistant sodium channels as well as TRPA1 channels. Both of these channels have been implicated in mechanosensation. Accordingly, the genetic ablation of the NaV1.8 expressing neurons results in a phenotype of deficient mechanical and cold perception. This has been commonly retrieved with almost all the conditional knock outs using the NaV1.8-Cre mice. Furthermore, the TRPA1 channel, presented here as a major cold sensor, is also reported in the literature as a mechanically activated channel. In addition, for the experiments using the skin nerve preparation in this study, the responsiveness of isolated fibers is first probed by mechanical stimulation before cold stimulation. Thus the effect of Cinguatoxin on the mechanical thresholds should be studied both in vitro and in vivo. In addition, it would be fair to cite the literature evidencing a role of TRPA1 in mechanotransduction.

The reviewer raises an interesting point.
Clinically, ciguatera is not associated with mechanical allodynia, nor did we note mechanical allodynia in our human experiments. In addition, we have performed extensive assessment of the mechanical von Frey threshold in the rat skin-nerve preparation which we now present in the manuscript. We found no alteration in mechanical threshold after treatment with 0.1 nM P-CTX-1,

with 13 fibres maintaining the pre-treatment value; 6 fibres appeared sensitized and the remaining 7 fibres were desensitized and their threshold increased (Supplementary Fig. 1).
A net increase in mechanical threshold occurred after treatment with 1 nM P-CTX-1, with 16 fibers being completely desensitized. These fibers were only excitable by applying strong pressure with a glass rod which already led to deformation of the corium. The threshold of 1 fiber was unaffected and 4 fibers appeared sensitized in comparison to the pretreatment value (Supplementary Fig. 1). Due to these negative results and the emergence of spontaneous action potential firing after treatment with CTX in the mouse skin we abandoned further evaluation of mechanical threshold.

While TRPA1 has been implicated as a mechanosensor, it is not absolutely required for mechanical sensitivity of afferent nerve terminals per se, as mechanically sensitive cutaneous fibres are present in normal numbers in TRPA1-deficient mice (Kwan, Glazer et al. 2009). In addition, deficits of TRAP1 KO mice to mechanical force were only observed in response to intense mechanical stimulation (Kwan, Glazer et al. 2009). However, slowly adapting low-threshold A-fiber mechanoreceptors from TRPA1-KO mice have been shown to have reduced action potential firing, suggesting a role of TRPA1 in mechanosensation in these fibers. It is plausible that TRPA1-mediated mechanosensation in these fibres contributes to ciguatoxin-induced sensory disturbances other than mechanical allodynia, such as the tingling and pricking sensations which are commonly described by ciguatera victims, but which are difficult to assess in this murine model. **We have now modified the manuscript to extend discussion of these interesting points (Results, page 5, line 15; Results, page 6, line 23 and Discussion, page 21, line 4) and have included additional data illustrating unaltered von Frey thresholds in the rodent skin nerve preparation and the absence of mechanical allodynia in our murine behavioural model in Supplementary Fig. 1.**

3) Ciguatoxin mediated cold allodynia involves NaV1.8 / TRPA1 expressing C fibers and A fibers expressing TTX sensitive sodium channels. This demonstrated in vitro and in vivo on behavioral test. Anyway, the final level of functional exploration, using fMRI analysis of brain regions mediating cold allodynia, only concentrates on the role of TRPA1 expressing fibers and subsequent neuronal circuitry in the pain matrix. This latter part, to be consistent with the other parts of the study would benefit from the exploration of TTX-sensitive afferent fibers by using TTX in the paw and subsequent effect on fMRI signal. Similarly using TTX in combination with HC030031 would be of interest to completely abolish the cold allodynia and monitor it at the fMRI level.

While this suggestion provides certainly an interesting approach it is unfortunately not technically feasible. This arises because in our behavioural experiments the apparent duration of action of TTX is less than 30 mins when injected by the intraplantar route. Unfortunately, fMRI measurements usually take at least 90 min because a detailed anatomical set of images needs to be acquired before starting the functional measurement, thus precluding studies on transient effects such as those of TTX. We only succeeded with our fMRI study design because CTX causes cold allodynia lasting for more than 2 hours (see Fig. 2).

The fMRI experiments we describe were conducted specifically to shed light on the role of TRPA1 in noxious and non-noxious cold detection and perception. Indeed, the role of TRPA1 in cold sensing has been controversial, with several studies reporting a lack of behavioural effects in TRPA1 knockout animals, leading the authors to dispute the function of TRPA1 as a cold sensor *in vivo*. We would not expect additional mechanistic insight from assessing fMRI signals using the pharmacological modulators which were already evaluated in the behavioural studies. Our fMRI data clearly show for the first time that TRPA1 contributes to temperature sensing and not only in the noxious but also in the innocuous range and may mediate a more subtle perception of cooling than previously thought.

Minor point:
1) TRPM8 positive neurons seem to be responsive to Ciguatoxin in vitro, but the TRPM8 KO shows no alteration of cold allodynia! Please provide an explanation. Is it possible that the antibody used is non specific...

The reviewer raises a valid point. This specific antibody was tested in knockout mice and was previously used in another study (Zimmermann, Lennerz et al. 2011). Nevertheless, it is plausible that low levels of non-specific fluorescence may inadvertently be classified as TRPM8-positive neurons. For this reason, the contribution of TRPM8 to ciguatoxin-induced cold allodynia was

rigorously assessed in behavioural studies. It is likely that the proportion of TRPM8-positive neurons which responded to CTX may represent only the relatively small proportion of neurons which express both TRPA1 and TRPM8. Alternatively, TRPM8-positive neurons sensitive to CTX may mediate sensory effects other than cold allodynia. As indicated in the manuscript, since TRPM8 has been shown to be involved in tear production in mice (Parra, Madrid et al. 2010), it is plausible that TRPM8-expressing neurons contribute to ciguatoxin-induced lachrymation (Lewis and Sellin 1993). Importantly, to further confirm the lack of contribution of TRPM8 to CTX-induced cold allodynia, **we now show that treatment with the TRPM8-specific antagonist, AMTB, did not affect cold allodynia, confirming the lack of effect seen in the TRPM8 KO animals and include this additional data in Fig 5 L.**

Referee #2:

Overall, this is a very interesting manuscript explaining cold allodynia induced by ciguatoxin, which involves TRPA1 and voltage-gated sodium currents. The manuscript is, however, somewhat confusing in many instances, and often lacks a clear description of what is shown in the figures, as outlined below. Resolving these issues would make this a strong paper of broad interest.
Specific points:
**Page 5: What is meant with "that overlapped sensory effects in humans."*

We apologise for being unclear. We refer to the observation that concentrations of CTX below the EC_{50} for CGRP release elicited only itch in the human experiments, while concentrations above the EC_{50} for CGRP release caused pain and cold allodynia. Thus, the amount of CGRP release – and hence peripheral neuron activation – in rat skin paralleled the intensity and nature of sensations elicited by intradermal injection of CTX in humans, providing evidence that CTX effects on peripheral sensory neurons is equipotent in human and rat skin. **To clarify this point, we have now altered the manuscript (Results, page 5, line 21) and modified Fig. 1G, including the Figure legend.**

Legend to Figure 1: F should be G and vice versa.

We apologise for this error which has now been rectified.

Page 6: it would be informative to have some references for the statement that the toxin is "the most potent pro-algesic compound known." Maybe some effective doses of other potent pro-algesics.

The reviewer raises a valid point. Compounds which are known to elicit nociceptive responses after intradermal or intraplantar injection include capsaicin, nerve growth factor, prostaglandin E2 and bradykinin (Amaya, Wang et al. 2006). All of these compounds require injection of doses 100-1000-fold higher than the doses of CTX used by us to elicit comparable nocifensive responses. Specifically, to elicit nocifensive responses, capsaicin is generally administered at doses 2500 ng/paw, nerve growth factor at 50 ng/paw, PGE2 at 100 ng/paw, and bradykinin at 300 ng/paw (Amaya, Wang et al. 2006). In contrast, we injected 5 – 500 pg of ciguatoxin and observed potent pro-algesic effects at these doses. **This has now been included in the manuscript (Results, page 7, line 6).**

There is no good rationale to express Fura-2 data as deltaRatio/Ratio (e.g. in Figure 3H, I, K-N). This way the advantage of a ratiometric dye is lost by normalisation, and responses of equal amplitude will yield higher values for cells with a lower basal calcium. I strongly advise to use deltaRatio or, ideally, absolute calcium values.

We thank the reviewer for this suggestion. To clarify the magnitude of calcium responses, we have calibrated our system and have now modified these figures accordingly (Fig. 3 K-N are now presented in Fig. 4). Figure 3HI is now presented as deltaR, because the data were acquired on another calcium imaging setup which is not accessible anymore, and for which we are thus unable to access Fura-2 calibration data. The previous Fig 3H was removed in the revised manuscript, as we noticed that intracellular calcium after application of CTX did not always return to baseline after washout and therefore meaningful quantification of the magnitude of calcium responses to AITC was not possible when expressed as deltaRatio or intracellular Ca^{2+}.

We now present the data as the relative increase in intracellular calcium concentration for each of the two cold stimuli in Fig. 4.

It is fully unclear to me what is exactly shown in Figure 3I,J. How is TTX-sensitivity defined in this context. Description in text and legend is insufficient.

We thank the reviewer for identifying our lack of clarity on this important point. The Venn diagram in Fig. 3J (now Fig. 3 I) illustrates the characteristics of the 57 individual cells that showed a calcium increase upon CTX application: the green population are the 10 AITC-sensitive cells. The diagram also illustrates whether the calcium increase could be blocked by application of TTX (we used a minimum reduction of 50% of the calcium signal as a criterion for TTX-sensitivity), which was the case for most of the neurons. **We have now clarified these aspects in the text, figure legend and methods (Results, page 8, line 13; Methods p. 32, line 18).**

In Figure 3K-N I miss examples of what happens when wt and TRPA1-/- neurons are stimulated twice with cold, in the absence of P-CTX-1. As presented now, there is no evidence that the sensitisation to cold is due to the toxin, and not due to another form of P-CTX-1-independent, TRPA1-dependent sensitisation.

We apologise for this omission. **We now show additional data confirming that consecutive cold stimuli increase the proportion of cold-sensitive cells only minimally in the absence of CTX. This data is presented in Fig. 4 E.**

In Figure 4A it seems that cold is inhibiting P-CTX-1-induced APs. Is this a consistent observation?

We consistently observed that C-Fibers treated with CTX either lost their initial cold response or that ongoing activity induced by CTX at skin temperature ceased when the temperature was lowered. This is consistent with a shift of the working temperature range of CTX-modified C fibers, similar to what we have previously reported for C fibres treated with menthol (Zimmermann, Lennerz et al. 2011). Profound cooling, consistent with inactivation of TTX-s channels (Zimmermann, Leffler et al. 2007), eventually leads to quiescent C fibres. This is also consistent with the clinical picture of cold allodynia, where mild cooling rather than profound cooling leads to pain. **We now discuss this observation in the manuscript (Results, page 9, line 15).**

Figure 4F: it would be good to see experimental examples of the P-CTX-1-induced membrane potential changes.

We now include experimental examples of the P-CTX-1-induced membrane potential changes as well as membrane oscillations that are followed by series of action potentials in Fig. 5 F-H.

Figure 4g: What was the dose of p-CTX-1? Why was this performed in different cells than Figure 4D? Is the response also fully blocked by a NaV1.3 blocker? What is the resting membrane potential of NaV1.3-TRPA1-expressing HEK293 cells? The answers to these questions are important to support the proposed mechanism of P-CTX-1-induced, TRPA1-dependent responses.

We assessed Ca^{2+} responses to stimulation with a range of P-CTX-1 concentrations (5 – 0.03 nM) in HEK cells expressing $Na_v1.3$ and TRPA1. We used HEK cells for these co-expression studies because we have found that these cells are easier to transfect with $Na_v1.3$ and display robust $Na_v1.3$ responses.
As no selective $Na_v1.3$ antagonists are available, and we are using the TTXr $Na_v1.3$, we performed control experiments in untransfected HEK293 cells, showing that the presence of $Na_v1.3$ was essential to elicit TRPA1-mediated Ca^{2+} responses. The resting membrane potential of these HEK cells is approximately -35 mV. We have used CHO cells in other studies for technical reasons, as these cells are much more firmly adherent and thus easier to handle for FLIPR experiments in 384-well plates. **We have now clarified the manuscript by providing additional data showing that tetracaine blocks the TRPA1-mediated Ca^{2+} response, see Supplementary Fig. 5 and manuscript page 10 line 14.**

Page 10: The senior author recently published that TRPC5 is a candidate cold sensor, in addition to TRPM8 and TRPA1. Shouldn't that be mentioned here?

The reviewer is correct, since submission of this paper we have reported that TRPC5 is a candidate cold sensor. We have indeed tested the TRPC5 knockout mice in our behavioural model and also recorded several fibers in the skin-nerve preparations. We were not able to identify a contribution of TRPC5 to the CTX-induced cold-allodynia. **These results are now mentioned in the paper (Results, page 10, line 18).**

Is the initial pain response dependent on TRPA1?

The reviewer raises an interesting point. While we did not specifically quantify the contribution of TRPA1 to spontaneous pain, no gross reduction in spontaneous pain was apparent, suggesting the involvement of pathways not involving TRPA1 in this response. Interestingly, none of the knockouts tested showed any apparent reduction in spontaneous pain, including CGRP knockouts. **We now clarify the contribution of TRPA1 to spontaneous pain in the manuscript (Results, page 10, line 21).**

Page 15: Note that Gentry et al. (Mol Pain 2010) also find altered cold sensitivity in vivo.

We thank the reviewer for pointing out this reference and **we have now included this in the third paragraph of the discussion (page 18, line 16).**

Page 16: It is unclear whether the cold responses in TRPA1-/- neurons are purely TRPM8. Are these neurons menthol-sensitive?

As described in several studies in the literature, cold responses in cultured DRG neurons are mediated predominantly by TRPM8, although residual cold responses not mediated by either TRPA1 or TRPM8 have been described (Bautista, Siemens et al. 2007; Munns, AlQatari et al. 2007). While we did not specifically confirm that the cold responses we observed were mediated by TRPM8, we determined that they were not mediated by TRPA1, as no significant change (consistent with previous publications) in the cold-sensitive responses was observed in TRPA1 KO mice. In distinct contrast, the CTX-mediated cold sensitization was mediated exclusively through TRPA1. **We have now clarified this point in the manuscript (page 8, line 22)**

Other TRP channels expressed in the sensory neurons are also voltage-gated (e.g. TRPV1), and could thus potentially be activated by the P-CTX-1-induced depolarisation. It would therefore be interesting to know whether there is also heat-hyperalgesia after P-CTX-1 injections.

The reviewer is correct to point out that TRPV1 has been suggested to be allosterically gated by temperature, voltage and agonists such as capsaicin. However, heat allodynia is neither observed clinically(Cameron and Capra 1993), nor was it observed in our animal model. The precise reasons for the lack of heat hyperalgesia remains to be determined, however, it is likely that differential co-expression with CTX-sensitive Na_v and K_v channels contribute to this effect. **We have now expanded on this point in the discussion (page 17, line 18).**

Referee #3:

Despite some advances discussed below, this paper reports results from human experimentation that have been conducted in a questionable manner, as it appears to this reviewer (see below).
Excluding human experiments, overall, this paper presents interesting and novel findings, gathered by a group of experimentally well-accomplished scholars. The role of the Trpa1 / TRPA1 gene and ion channel protein in altered neural transduction caused by ciguaetera toxin is examined, and evidence is presented in favor of a critical role for TRPA1.

A couple of caveats will have to be heeded by the authors.

Re the abstract are all claims from the abstract valid

Also, the abstract needs to be rewritten for a general audience, as is it is targeting a more specialized neurophysiology audience.

We apologise for this oversight. **We have now revised statements in the abstract, referring to a large reduction of cold allodynia in TRPA1 deficient mice, consistent with our findings demonstrating a 60% reduction in cold allodynia. We have also reworded aspects of the abstract to make it more appropriate for a general audience. (Abstract, page 3, lines 4, 7 and 12)**

Re the ciguatera clinical complex beyond the acute intoxication Sensory disturbances are very disturbing to patients, especially as they involve cold allodynia, but perhaps they are not the most disturbing clinical features that can occur. CNS neuropsychiatric manifestations and especially asthenia and a chronic-fatigue-like syndrome have also been reported. In case these symptoms are part of chronic ciguatera, then it is hard to see that they not become the clinically dominating feature re quality of life of the affected patient.

We agree with the reviewer that this statement may be misinterpreted. We did not intend to suggest that only sensory disturbances are distressing to patients.
Ciguatera is a complex clinical syndrome and the individual symptoms which are most troubling to patients will vary significantly. While in some patients, ciguatera symptoms can persist long-term and may involve chronic fatigue-like symptoms, this fortunately occurs in a minority of patients, and perhaps only in the most severe poisonings. We referred to the peripheral sensory disturbances as particularly distressing in this context only, and with reference to more transient gastrointestinal symptoms.**To clarify this point, we have amended the manuscript to read: However, subjectively amongst the most distressing symptoms were neurological disturbances affecting the central nervous system, and also peripheral sensory disturbances incuding paresthesias, localized intense pruritus and several painful dysesthesias. (Introduction, page 4, line 6)**

Intraplantar injection is not only targeting sensory neurons' peripheral projections, TRPA1 is also expressed in keratinocytes where it might function in a modulatory role (J Neurosci. 2009 Apr 15;29(15):4808-19). Having established another specific tool will enable the investigators to test this particular hypothesis. Intraplantar injection is certainly not a valid model for ciguatera, rather it is an approach to better understand issues of peripheral neural transduction problems that can happen in ciguatera.

We thank the reviewer for drawing our attention to this reference. The reviewer is correct to state the intraplantar administration of CTX is not a valid model for ciguatera. However, our aim was to establish a model of CTX-induced cold allodynia which would allow us to dissect the mechanisms involved in peripheral sensory disturbances elicited by ciguatoxin, in particular cold allodynia and to gain new insight into the molecular mechanism of cold allodynia which may have relevance to other neuropathies. We also wanted to avoid the large array of sytemic side effects caused by i.p. injections of the toxin. **We now include this reference and extend our discussion to the putative role of TRPA1 in keratinocytes (Discussion, page 19, line 16)**

In human disease, how does the toxin reach sensory neurons and their peripheral projections after initially being absorbed from the intestine; This process happens in the human intoxication but has not been modeled in the described animal experiments.

The reviewer refers to an area which has only recently been addressed (Bottein, Wang et al. 2011). The toxicokinetics of ciguatoxin are complex and likely contribute to the clinical presentation of ciguatera. After oral administration in mice, ciguatoxin is rapidly absorbed from the gastrointestinal tract, where it exerts local action to elicit gastrointesintal symptoms such as diarrhoea and abdominal pain. The clearance of ciguatoxin involves a biexponential elimination best fit using a two-compartment model. This probably occurs due to accumulation of CTX in adipose tissue or even lipophilic neuronal membranes, which contributes to a long terminal elimination half-life of approximately 4 days. The slow elimination of CTX likely contributes to the prolonged duration of neurological effects, and renal excretion of ciguatoxin may contribute to urinary symptoms such as dysuria, although the majority of ciguatoxin is excreted in the faeces. **We have now included discussion of the toxicokinetics of ciguatoxin in our manuscript to clarify this point. (Discussion, page 16, line 19)**

fMRI CNS signals are also a result of sensory transduction, but certainly also driven by intra-CNS processing (see Nature Neuroscience 15, 70-80, 2012, referring to a possible role for TRPA1 in astrocytic modulation of neural transmission). In Trpa1 pan-null mice, that is in the complete absence of Trpa1 in all cells at all developmental changes, intra CNS changes are not necessarily caused by TRPA1-expressing DRG neurons.

The reviewer raises a valid point. The interesting paper the reviewer refers to was published since submission of this manuscript and highlights additional non-neuronal roles of TRPA1. Such roles may contribute to the altered physiology observed in global knockout animals. These limitations are an inherent drawback of transgenic animals and apply to many, if not all, KO model studies, where compensatory changes and developmental alterations can account for observed differences.
For this reason, we have verified our observations through extensive *in vivo* experiments involved knockout animals as well as pharmacological modulation which are strongly supported by *ex vivo* findings in the isolated skin-nerve preparation. **We have now included additional discussion and references to highlight these issues. (Discussion, page 20, line 17)**

Finally, since the ms. is not concise, it can certainly benefit from organizing it with subheadings.

We have now included further subheadings to improve the readability and clarity of the manuscript.

Re the human experimentation, the most questionable part of the entire study:
This reviewer has doubts about the conducted human experimentation, their appropriateness and whether they have been conducted in an ethically sound manner, despite mentioning of approval from the local university

We thank the reviewer for his/her concern. We are convinced that we have conducted all human and animal experiments in an ethically sound manner. We have now substantially expanded our manuscript to provide greater detail on the human experiments carried out. **We address specific concerns regarding the human experiments in detail below.**

Specifically:
Insufficient information is given about the human study or how the compound was prepared for administration to humans, ……

In detail, ciguatoxin was isolated from moray eel and purified to > 95% purity by HPLC using good laboratory practice (Lewis, Sellin et al. 1991). The lyophilized non-pyrogenic material was reconstituted in sterile medical grade Ringer solution for intradermal injection, as indicated in the manuscript. The procedure of the conducted experiment is most similar to common clinical practice allergy tests (or a tuberculin test). There, however, in many instances pyrogenic and crude natural materials (dust, mites) are intentionally introduced into the skin. **We apologize for the lack of detail and have modified the manuscript accordingly (Materials and Methods, page 26, line 8)**

……about the review of the institutional human experimentation protocol process, and the process of informed consent. After all, as reported, severe neuropathic pain was elicited in human subjects (!).

According to the requirements of the Nuremberg Code, the “no risk” determination was in fact made by an independent ethics committee, and the ethical approval (IRB approval) obtained met all legal requirements for recruitment of human subjects, including information about possible risks involved. Documentation was submitted to the editorial board. Formal approval for the procedure was obtained before other investigators were involved in the self-experiments. **Thus our process is in full accordance with German law and the Nuremberg Code**.

At least as troubling is an apparent violation to the Belmont Report regarding human research. This set of guidelines explicitly prohibits lab personnel from being human subjects because their consent might be compromised, that is they may not feel free to refuse participation without adverse consequences or other bias.

We believe there might be a misunderstanding about the contents and purpose of the *Belmont protocol*. „Lab personnel“ -as we explain below- was not involved in the human study and is to our knowledge not mentioned in the Belmont Report. The Belmont Protocol seeks to clarify the ethical issues inherent in research involving human subjects and to identify basic principles how research involving human subjects should be conducted following the reported abuses of human subjects in biomedical experiments, especially during the Second World War. **In the following, we would like to point out all principles/guidelines raised by the Belmont Protocol and relate it to our self experiment:**

Part B -Basic ethical Principles. Point 1: Respect for Persons*...implies* ***“the requirement to acknowledge autonomy and the requirement to protect those with diminished autonomy”***
All of our subjects were autonomous and free to act, and all of our subjects entered into the research voluntarily and no information about potential risks of the experiment was withheld from them (see below). Furthermore as a matter of course, any person could have refused participation without any explanatory statement and without suffering any derogation. In particular, the self-testing was NOT a condition for coauthorship on the paper and no contributor was expelled from the author list for refusing participation in the experiment.

Part B. Point 2: Beneficence…*implies to adopt* ***“the Hippocratic maxim “do not harm”*** *and that* ***“In the case of particular projects, investigators and members of their institutions are obliged to give forethought to the maximization of benefits and the reduction of risk that might occur from the research investigation”.***
Peter Reeh and myself were the first subjects to perform self experiment for which we conducted a personal risk assessment. I would like to emphasize that I would never have written an ethics proposal, if I was not convinced that the self-experiment was sufficiently safe and essential in the first place. To date there is no model for cold allodynia which would allow insight into its molecular pathology (in contrast, the molecular mechanism of heat hyperalgesia has been revealed more than ten years ago (Davis, Gray et al. 2000)and lead the way towards treatment options. Thus the availability of ciguatoxin as a model substance has offered great opportunities.
For pain scientists, self-experience constitutes reason and motivation to invest time and money in experiments which, without this knowledge, may turn out as waste of efforts. If CTX would not have induced (most impressive) cold allodynia in ourselves, we would have abandoned further studies.

Part B. Point 3: Justice*...in the sense of* ***“equals ought to be treated equally“***
This pertains largely to the selection of human subjects as research subjects. No particularly class (e.g. welfare patients, racial or ethnic minorities or institutionalized persons) was selected because of their compromised position, their easy availability or their manipulability. All of our subjects were autonomous and free to act as stated in Point 1. All of our subjects were involved in the research project and thus related with the problem being studied.

Part C -Applications.
Part C. Point 1: Informed Consent …“containing three elements: information, comprehension and voluntariness”
Our subjects were given a 6-page sheet of written informed consent with ***information*** *about the research procedure and risks entailed designed by Katharina Zimmermann and Irina Vetter, from the lab of Richard Lewis, the world-renowned expert on Ciguatoxins and Ciguatera. They were also given the opportunity to ask questions and to withdraw at any time from the research. All our subjects were academics and involved in theoretical research about ciguatera, therefore – being in essence a volunteer and an academic- they all had ample information available to them, they all were able to* ***comprehend*** *the information available to them, which is important, because they decided to undertake the risk gratuitously. Furthermore, the subjects were also given ample time to consider if they really wanted to participate in the experiment. Importantly, the experiment was conducted 3-4 days after obtaining signed informed consent from each of the participants. Lastly, the agreements to participate in research were valid consents, because they were given* ***voluntarily****. As stated earlier any person could have refused participation without suffering any derogation. Clearly, consents were not obtained under coercion or undue influence.*

Part C. Point 2: Assessment of risks and benefits*...requiring* ***“a careful arrayal of relevant data, “... “presenting both an opportunity and a responsibility to gather systematic and comprehensive information about proposed research”.***

An 12-page ethics proposal was written containing a detailed assessment of risks and benefits and submitted for review to the local ethics committee of the university of Erlangen-Nuremberg and approved in due course.
"The requirement that research be justified on the basis of a favourable risk/benefit assessment bears a close relation to the principle of beneficence, just as the moral requirement that informed consent be obtained is derived primarily from the principle of respect for persons. The term "risk" refers to a possibility that harm may occur".
we would like to refer therefore to our explanations given in Part B Points 1 and 2.

Part C. Point 3: Selection of Subjects *...imposes that* ***"the principle of justice gives rise to moral requirements that there be fair procedures and outcomes in the selection of research subjects****"...and* ***"Justice is relevant to the selection of subjects of research at two levels: the social and the individual."*** *meaning that researchers* ***"should not offer potentially beneficial research only to some patients who are in their favor or select only "undesirable" persons for risky research."*** *These aspects do not apply to our study design as they are more relevant when doing research studies on novel treatments for diseases. Furthermore no vulnerable subjects, such as racial minorities, economically disadvantaged, very sick or institutionalized persons were sought as research subjects.. The Belmont report points out their need for protections because of their* **"dependent status and their frequently compromised capacity for free consent"** *making them an easy subject* **"solely for administrative convenience, or because they are easy to manipulate as a result of their illness or socioeconomic condition".** *Again we would like to emphasize that all of our subjects were autonomous and free to act, and all of our subjects entered into the research voluntarily and no information about potential risks of the experiment was withheld from them.*

Therefore, based on this analysis, we cannot see any violation of the Belmont report. We would like to emphasize finally that at no point were "lab personnel" involved in these experiments or subjected to any coercion, all experiments were conducted by the authors of the paper themselves.

Furthermore, was the possibility of remote effects of the injected ciguatoxin revealed to participants in writing, including the possibility of effects on the central nervous system and sexual transmission (Clin Toxicol 1989;27:193-197) ?

The cited paper refers to a single case report. Given that each year more than 20,000 new cases of ciguatera occur, it is important to note that this 23-year old case story is the only evidence of sexual transmission of the toxin. Ciguatera is known to exhibit an incredibly wide range of symptoms according to the amount of toxin ingested, the susceptibility of the individual patient and the composition of subtypes of ciguatoxins ingested. Indeed, a very similar case of Ciguatera occurred in two patients in the Erlangen Internal Medicine Clinics. Both were affected with Ciguatera after eating fish during a vacation in Mexico. Both suffered from pain during sexual intercourse and many other ciguatera-associated symptoms, like muscle pain, cold allodynia, tooth pain and itch. Remarkably there was no ciguatoxin found in the semen sample of the affected male of this cited case story. In our opinion the evidence presented in the paper is not sufficient to make a strong case for a sexual transmission of ciguatera and it is the only published "evidence". Therefore this was not listed in our ethics proposal as a relevant risk; given the premise that the amount of toxin injected was well below the doses that are required to cause systemic ciguatera, as pointed out below.

According to Paracelsus everything is a poison and nothing is without poison ("*Dosis* Sola *Facit Venenum")*. The lethal dose of P-CTX-1 in mice was determined to be **0.25 microG per kg**. The main pacific ciguatoxin (P-CTX-1) causes ciguatera in humans at levels of **0.1 microG/kg** in the flesh of carnivorous fish. The threshold dose for the development of symptoms of ciguatera in humans is estimated to be **2 nanoG/kg** of orally ingested toxin. In contrast, we injected **10-100 picograms** of ciguatoxin, which is **one thousand to twelve thousand times less** than the dose which is required to cause any symptoms when ingested. We performed injections that specifically avoided any access to blood vessels. Thus we are convinced that our self experimentation was not associated with any likelihood of any systemic or CNS effects, or the chance of sexual transmission.

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2nd Editorial Decision 17 May 2012

Thank you for submitting your revised manuscript to the EMBO Journal. I have asked the three original referees to re-evaluate the revised version. I would like to apologize for the delay in getting back to you with a decision. Due to the nature of the experiments (i.e. the human self-experimentation analysis) the evaluation process was considerably more involved than usual. In particular, we required consultation with a number of additional ethics experts to complement the subject specific referee reports.

As you can see below, the three referees appreciate the revisions and report that they have strengthened the conclusions and findings reported. Indeed, the referees find the mechanistic insight provided into the mode of action of ciguatoxin exciting. The scientific aspects of the paper are not questioned, although referee 3 reiterates serious reservations about the ethical aspects of the human self-experimentation presented in figure1, questioning if they are in accordance with good ethical practice and international standards. As we noted before, we do not consider this data essential for the paper to be published in the EMBO Journal, and we would be happy to accept the paper without these data, as the mechanistic conclusions based on the mouse data stands alone. We do recognize that you have IRB approval for the experiments, but what the referee is reflecting upon is that what has been approved at your institute might not have been approved at other ones. After careful considerations, we have decided to allow publication of the paper as is. However, given the ambiguities concerning the ethics of the human experiments presented in figure 1, we would require the following editorial note to be published alongside the paper. We hope that the text is clear and acceptable to you and would like to emphasize that we are not in any way critical of the conclusions of the paper.

Please let us know if you chose to include the human data with the note, or if you would prefer to remove this data from the paper.

I thank you for your considerable patience during this unusual long process. I hope nevertheless that you find the process appropriate and fair.

Yours Sincerely

Editor
The EMBO Journal
.....

Editorial Note:

The authors have conducted human self-experimentation, which was approved by the local ethics committee (IRB) at the University of Erlangen-Nuremberg before submission of the manuscript on 8.6.2011, as described in the paper. A European ethics expert contacted by the EMBO Journal has reviewed the consent and IRB approval documentation submitted by the authors and found the documentation in accordance with local guidelines. See the review process document for further details [hyperlink]).

The EMBO Journal notes that authors are responsible for assuring that appropriate ethical guidelines and experimental protocol are followed that conform to international ethical and clinical standards or guidance, and that are in accordance with the relevant institutional approval processes. The EMBO Journal bears no responsibility for the human experimentation described in this paper. The publication of these data does not indicate endorsement of the experiments presented by the The EMBO Journal, EMBO, or NPG
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REFEREE REPORTS

Referee #1

The revision made to the manuscript is well done and the message is clear. Thanks to the authors for their answers to the comments. The model of cold hypersensitivity described here has both an interest for basic science as well as clinical implications. The findings are well described and compared to previous data on cold transduction in sensory neurons. In conclusion, I think that this study is technically well done and as a broad interest for the readers of EMBO journal.

Referee #2

The authors have adequately revised the manuscript. This is exciting work, of interest to a broad public.

Referee #3

The paper is now amended in its non-human sections, having addressed previous critique sufficiently. Re the human experimentation, the ms. provides more detail, yet as a bottom line, although ethics approval was obtained from the home institution of the lead author and lead-team, it is not clear to this reviewer that his study would have been approved by other comparable human ethics committees (i.e.in locations with a dedicated and professional science infrastructure). Indeed, this reviewer doubts this.

In view of this, this reviewer sees two possibilities - to separate human and animal experimentation, complement the conducted studies by properly approved studies in healthy volunteers, and make results from human experimentation public separately. As an alternative, together with the EMBO J, to pursue publication with an appropriate disclaimer by the Editorial Board, in case this body were agreeable to such a solution.

Additional Correspondence 23 June 2012

We apologize for the delay in adjudicating on the issue of the preparatory experiment involving self-experimentation included in your current manuscript entitled 'Ciguatoxins Activate Specific Cold Pain Pathways to Elicit Burning Pain from Cooling'. We did indeed discuss the matter in some depth with Drs. Schwilden, Vice-Chairman Ethical Committee, Friedrich-Alexander University Erlangen-Nürnberg. Indeed, Dr. Schwilden kindly sent a formal statement explaining the basis of the recommendation of your 'Ethik Kommission'.
As an international Journal we did not have a sufficiently refined understanding of medical ethics regulation in Germany and we therefore decided to consult further with our German ethics expert advisors. A key point is that in Germany an 'Ethik Kommission' does not pronounce rulings, but issues advice only.

As we noted previously, in our view you have submitted a very interesting and experimentally robust manuscript. We are certainly very happy to publish a modified version of the current manuscript. However, we have decided that we are not able to publish the human self experimentation depicted in Fig 1 of the current manuscript.

We would like to outline our reasoning below:
1) The EMBO Journal is an international scientific journal. As such, we must aim to make decisions on ethical aspects of manuscripts submitted for publication that approach an international consensus and which are in line with the ethical understanding of the majority of our readership. In the absence of detailed international guidelines beyond the Declaration of
Helsinki and the Belmont report, and rather divergent national regulation, the journal has to arrive at decisions which are not necessarily identical to the ethical guidelines of the country of origin of a manuscript. This is the case here.
2) After your most recent detailed explanation, we understand the rationale of your experiment on human subjects. We appreciate that it was of value (albeit likely not essential) in preparation for the mouse based experimentation. We appreciate that you took reasonable precautions, such as the presence of medical support during the procedure and initial self-experimentation restricted to the senior scientists of the study.
3) It is clear that the experiments themselves were carried out in accordance with German law and local ethics regulations as governed by the code of conduct of the Bayerische
Landes‰rztekammer {section sign}15. You have declared full responsibility for these experiments. We are not in any way insinuating that these experiments or your execution thereof are legally compromized. However, it is not clear that the advice of the 'Ethik Kommission' was followed adequately; the advice stated 'Die Ethikommission weist darauf hin, dass die Durchf¸hrung von Studien insbesondere mit Personen in mˆglichen Abh‰ngigkeitsverh‰ltnissen ethisch fraglich sein kann. Dies sollte bei der Auswahl der Probanden ber¸cksichtigt werden'. In spite of this advice, the experiment included subjects in dependency positions.
4) We appreciate that a detailed 'Einverst‰ndniserkl‰rung' was obtained from every subject included in the experiment. The subjects signed statements explaining the parameters of the experiment and confirmed that the experiment could be terminated at any point without any negative repercussions. You have documented that the financial support of the subjects was at least partially independent of the senior authors of the study. It is clear that in your view this addressed the advice of the 'Ethik Kommission'. However, in our view it does not - no subject in a positions that directly or indirectly depends on the senior authors should have been included to avoid any possible doubt on this most serious issue.
5) The data presented in fig. 1F and probably 1G are based on six subjects, which includes a subset of junior researchers who are linked to the senior authors. Even if the data for selected subjects is removed from the figure, as you have proposed, the experiments nevertheless happened. We have decided that this is therefore not an option.
6) The data presented in fig. 1 are not essential for the robustness of the dataset presented or the major conclusions drawn. Preparatory experiments do not have to be presented in a paper or they may be referred to in writing only as 'data not shown'. In our view a paper without this figure based on the murine data remains just as interesting and conclusive.
7) The experiments presented in fig. 1 of the current manuscript, in particular fig. 1F, will not be universally accepted by our international readership. In fact, there is a high risk that readers, or indeed the general press, might take issue with the experiments presented. We have decided that there is a significant risk that this may undermine the paper. The experiment as presented may also

undermine the reputation of the authors and indeed the journal, EMBO or the publisher.

Since the manuscript is undoubtedly publishable just as well without this figure and since, as outlined above, this data would pose a major risk, we have decided to ask you to remove either fig 1F and G, or indeed the whole of figure 1. We will be able to accommodate a textual description of these experiments, if you decide to retain it. We are certain that removal of these panels or indeed this entire figure will not put you at any sort of disadvantage or that it will in any way undermine the impact of the manuscript - quite the opposite.

We are sorry for the long delays, but we hope that it is clear that we felt that it was essential to take sufficient time to be able to consult broadly and to arrive at a fair and informed decision on the complex ethical parameters of the experiment in question. This has involved detailed consultation with ethics experts and a number of clinicians in Germany and the US, as well as legal advisors at our publishers, Nature Publishing Group. Throughout we certainly had both your interests and the journal's interests in mind.

We understand that you strongly favoured inclusion of this experiment and that this decision will disappoint you. Please note that with this editorial decision we are not intending to make any judgements at all on either your research project or indeed German regulations. We appreciate that you have explicitly taken full responsibility for the experiments presented in fig 1 and that you have been completely transparent in describing these experiments. However, it is in our view simply not justified to publish non-essential invasive experimentation on human subjects which may undermine the paper, the authors, the journal and the publisher to varying degrees. We hope this is understandable but if you wish to discuss the matter further, we are certainly available for a telephone call in the next couple of weeks.

Could you please briefly confirm that removal of either figure panel 1F and G, or figure 1, as well as appropriate edits to the text on page 5 and the methods section on page 23, is acceptable and send a revised version which addresses this issue both in the figure and the text. As soon as we have a revised manuscript we will proceed with fast tracked publication to make up for some of the delays.

Thank you for your understanding and patience in awaiting this decision. We thank you for selecting The EMBO Journal to publish your exciting findings.

Yours sincerely,

2nd Revision - authors' response 27 June 2012

Please find attached the modified version of our manuscript. We have made modifications in the Abstract, Results Methods and Discussion. The whole Figure 1 was removed and all other Figures were renumbered accordingly.

