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Membrane cholesterol depletion as a trigger of Nav1.9 channel-mediated inflammatory pain

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1st Editorial Decision 23 June 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.

REFEREE REPORTS

Referee #1:

In this study Muriel Amsalem and colleagues described modulation of nociceptive neuron specific sodium channel Nav1.9 by cholesterol. They showed that Nav1.9 localizes to the lipid raft membrane fraction in DRG and that cholesterol depletion disrupts this localization which, in turn, results in Nav1.9 potentiation, increased excitability and hyperalgesia. Moreover they also provided some data to suggest that tissue inflammation results in reduction of plasma membrane cholesterol levels, which could be a causative factor in the development of inflammatory pain. In support to this hypothesis, in vivo local cholesterol depletion produces hyperalgesia, which was absent in Nav1.9 KO mice. Finally, local skin delivery of cholesterol via topical gel was analgesic in acute and chronic inflammatory pain models. This is a very interesting study reporting a novel and potentially very important phenomenon. Experiments are robust and the manuscript is very well written. The in vivo cholesterol delivery experiments carry a very high translational potential and should be published promptly. Yet, in my opinion, there are some gaps in the authors' concept and more work will be needed in order to describe the phenomenon explicitly.

Major

The data are clearly show that 1) cholesterol depletion in DRG neurons upregulates Nav1.9 and 2) that delivery of cholesterol to skin is analgesic. Yet, what is still tentative is that upregulation of Nav1.9 by cholesterol depletion in DRG neurons is indeed a major factor in inflammatory pain. Some of the presented experiments are broadly consistent with the suggested hypothesis, these include an observation that MβCD-induced pain hypersensitivity is reduced in Nav1.9 KO mice and the body of in vivo cholesterol delivery experiments. Yet, these are not in any way a proof.

Moreover, some of the data lowers my enthusiasm for this hypothesis. Particularly - the ibuprofen experiments; authors do not discuss these much, just merely acknowledge the effects, but I would urge them to provide a proper explanation. To me, the facts that 1) ibuprofen is entirely ineffective to alleviate the MCβD-induced hypersensitivity (Fig 2B) and 2) that ibuprofen is fairly efficacious to alleviate carrageenan-induced hypersensitivity (Fig. 8A), suggest that these are two distinct mechanisms or, in other words, that carrageenan-induced hypersensitivity is not mediated by the cholesterol depletion. One could perhaps argue that only a fraction of carrageenan-induced hyperalgesia is mediated by Nav1.9-cholesterol pathway, this fraction of the effect would then be expected to be ibuprofen-insensitive. Yet, as follows from Fig 3C, it is the non-Nav1.9-dependent hypersensitivity which is ibuprofen-insensitive (p. 7: "Residual MβCD-induced allodynia in Nav1.9 KO mice was insensitive to intraperitoneal injection of ibuprofen"). Perhaps all the MβCD-induced hypersensitivity is ibuprofen-insensitive and a fraction of it depends on Nav1.9, which is, in turn, is the same fraction of the carrageenan-induced hypersensitivity, which is insensitive to ibuprofen. However, this would make Nav1.9-dependent mechanism only a relatively minor contributor to the carrageenan-induced pain. I'm baffled by this apparent conundrum and would welcome a clarification.

In relation to the same question, it is not clear how robust the cholesterol depletion produced by inflammation in nociceptive nerve endings is. There is 10-20% decrease of total cholesterol in the skin after plantar injection of carrageenan or inflammatory mediator cocktail (Fig. 1C, E). Yet, nociceptive nerve endings represent only a minute fraction of total skin tissue, thus, the change of cholesterol content in the nerve terminals cannot be deduced from such data. There is also 16% reduction of total cholesterol content in DRG culture treated with the inflammatory cocktail. But again, DRG culture is a mix of neurons and glia, in 24h culture there will likely be more glial cells than neurons and since the total change in cholesterol level is small, it is impossible to say how much of the change is attributable to neurons. One way to get around this problem is to test the effect of inflammatory cocktail on the cholesterol level in purified neuronal DRG culture, this can be achieved by suppressing glial proliferation with cytosine arabinoside or by magnetic cell sorting.

According to Fig. 5A, treatment with inflammatory cocktail did not affect partitioning of caveolin and flotilin into cholesterol-rich membrane fractions, this further indicates that inflammatory treatment did not produce sufficient cholesterol depletion to destroy lipid rafts.

To summarize the above, I do not think that authors provided sufficient evidence to conclude that potentiation of Nav1.9 by inflammatory mediators (let alone the inflammatory excitability and hypersensitivity) are indeed mediated by cholesterol depletion. It is of note that potentiation of Nav1.9 by inflammatory mediators is a well-recognized effect that was attributed to GPCR modulation (e.g. Rush & Waxman Brain Research 2004; Ostman et al. J Physiol 2008; Vanoye et al. J Gen Physiol 2013). For instance, Nav1.9 current is potentiated during whole-cell patch clamp recording with intracellular dialysis of GTPγS. So, the inflammatory potentiation observed in the present study could be a GPCR-mediated effect or a combination of several factors. Indeed, in one of their previous studies the authors suggested that inflammatory Nav1.9 upregulation is a result of integration of several signaling cascades (Maingret et al. J Gen Physiol 2008). It also conceivable that the GPCR- or GTPγS-mediated Nav1.9 potentiation is in fact mediated by cholesterol depletion, but authors do not test this directly. Does cholesterol delivery prevent GTPγS-induced Na1.9 upregulation? This would be an important experiment as it would provide a better link with previous literature and also could give a clue for answering another important question: how inflammatory signalling causes cholesterol depletion. This is not addressed in the current version of the manuscript. Some hypotheses are discussed but not tested, even though at least some of these is fairly easy to test. For instance, the ROS hypothesis can be tested by checking if the cholesterol depletion or Nav1.9 upregulation in DRG neurons are abolished in the presence of an antioxidant or a reducing agent.

In my opinion this aspect of the study needs to be significantly strengthened in order to provide a more mechanistic insight into this very interesting phenomenon.

Specific comments.

1. At a few instances authors state that no one have studied a role of cholesterol or lipid rafts in inflammatory pain signaling before (e.g. page 2: "So far, no one has investigated the putative role of

cholesterol in inflammatory pain..."; page 14: "Although lipid mediators are well known to be part of the pain pathway...cholesterol has not yet been linked to pain processing."). This is not true, there is a number of studies on the effect of cholesterol or lipid rafts on nociceptive signaling; below are just a few examples but there are more.
• Gnanasekaran et al. Mol Pain 2011: cholesterol/lipid raft regulation of P2X3 channels in trigeminal nociceptors.
• Pristerà, Baker & Okuse PLoS One 2012: lipid rafts localization of Nav1.8
• Jin et al. Sci Signal 2013: role of cholesterol/lipid rafts in regulation of ANO1 channel in nociceptors.
• Sághy et al. Pharmacol Res 2015: lipid raft modulation of TRPV1 and TRPA1

2. Page 4: "Concomitant with the decrease in cholesterol level, we observed an overall increase in excitability of cultured DRG neurons exposed to the inflammatory cocktail..." There are multiple well known excitatory mechanisms triggered by inflammatory mediators, including P2X channel activation (there was ATP in the cocktail), TRP channel sensitization, KCNQ channel inhibition, Nav1.8 phosphorylation etc. If the authors want to evaluate a fraction of the inflammatory excitation, which is dependent on the cholesterol depletion, they would need to repeat these experiments in the presence of MβCD-cholesterol.

3. Suppl. Fig. 2: it would be good to test if Nav1.9 clustering disapers after carrageenan or inflammatory cocktail injections.

4. Did cholesterol depletion affect Nav1.9 inactivation kinetics or voltage-dependence?

5. Fig. 5: since inflammatory cocktail did not affect distribution of raft and non-raft markers, it would be good to include a MβCD control.

6. Chronic inflammation model (Fig. 8 and the corresponding Results section): local cholesterol levels in this model are not tested.

Minor

1. Dose cholesterol oxidase acts from the extracellular side of the membrane or is it taken up by the cells somehow? Please clarify in the text.

2. In Fig. 4F, left panel, why AP amplitude declines during recording? Also it is very hard to decipher the current clamp panels (Fig. 2G, Fig 4F), dotted lines are supposed to be 0 mV but it labelled in pA. Or the red pA number represents the current threshold value? Or a current injected to produce red voltage trace? In that case can you move it from the dotted line and provide a more explicit description in the legend?

Referee #2:

The paper presents an interesting story: Inflammation (possibly via ROS production) depletes cholesterol and translocates Nav1.9 from lipid rafts to non-raft domains. This leads to a shift in the voltage dependence of activation of Nav1.9 to more negative potentials and, accordingly, to neuronal hyperexcitability.

My main concern is the use of very high concentrations of MbetaCD (20 and 40 mM), which are known to be detrimental to neurons (Ottico et al., 2003). MbetaCD (10 mM, 2h) kills 50% of cerebellar neurons in culture (also producing changes in cellular morphology); also, 10 mM MbetaCD for 30 min leads to Trypan blue incorporation in 100% of cells. At such high concentrations it is quite likely that MbetaCD also depletes phospholipids such as sphingomyelin and phosphatidyl choline. It is true that the authors tried to control for this, and alpha-CD (which does not deplete cholesterol, but depletes phospholipids) did not reproduce the effects of MbetaCD. Moreover, MbetaCD-cholesterol also did not induce pain behavior. In addition, MbetaCD may alter the relative distribution of cholesterol between the plasma membrane and intracellular membranes.

Cholesterol oxidase (CholOxi) is likely to generate various oxysterols which are known to have

multiple biological (cytotoxic?) functions, including ROS production, so it may be that ROS play a role in the process (which the authors also suggest, but do not follow up). Perhaps it should be investigated whether antioxidants are able to prevent the CholOxi-induced alteration of Nav1.9 function. In addition, the effects of CholOxi were not measured in Nav1.8-/- mice.
The inflammatory cocktail has two important effects: It reduces cholesterol and also (while not disrupting rafts) redistributes Nav1.9. Are these effects related? Do they require oxidative stress?
I have a slight problem with the voltage clamp recordings, which are carried out in different populations of neurons (MbCD and CholOxi were pre-applied for 15 min), in contrast to current clamp recordings that are paired (MbCD and CholOxi were acutely applied for 15 min). It would have been more convincing to see in real time the shift in activation voltage (i.e. the increase in Nav1.9 current at say -35 or -40 mV) during cholesterol depletion.
There is merit in the paper, but some questions are left unanswered, particularly how do the inflammatory mediators interact with membrane cholesterol and lipid rafts in such a specific way as to only extract Nav1.9 from rafts.

Referee #3:

"Membrane cholesterol depletion as a trigger of Nav1.9-mediated inflammatory pain" by Amsalem et al reports some novel and interesting findings with basic neurophysiology as well as translational-medical relevance.

1. The authors should have the lipid:cholesterol ratio for DRGs and note how that ratio changed when they removed cholesterol. What does 18% reduction mean in molar ratios?

2. The authors are encouraged to test for reversibility after removing cholesterol as was done in the studies of Rosenbaum et al when they were assessing TRPV1.

3. Can cholesterol distribution in skin strata be determined ?

4. P.6 . In the current clamp experiments they report the effects of 2 of 6 or 7 responses - I think this is questionable to report on the responses of two cells although I believe the general result.

5. The activation results are strong and convincing. Did the authors conduct inactivation studies ? Did they measure any changes in resting potential? Were cell sizes taken into account ?

6. Re the cholesterol binding domains - the authors need to make clear the limitations of their claims, what is experimentally-based, what is enlightened reasoning.

7. Authors should make sure that p values have the correct number of significant digits (especially in figure legends).

1st Revision - authors' response 27 September 2017

Referee #1, (Remarks to the Authors):

Reviewer#1's comments were very helpful overall, and we are appreciative of such constructive feedback on our original submission.

Major points

1) MβCD-induced hypersensitivity and carrageenan-induced hypersensitivity.
To me, the facts that 1) ibuprofen is entirely ineffective to alleviate the MCβD-induced hypersensitivity (Fig 2B) and 2) that ibuprofen is fairly efficacious to alleviate carrageenan-induced hypersensitivity (Fig. 8A), suggest that these are two distinct mechanisms or, in other words, that carrageenan-induced hypersensitivity is not mediated by the cholesterol depletion. One could perhaps argue that only a fraction of carrageenan-induced hyperalgesia is mediated by Nav1.9-cholesterol pathway, this fraction of the effect would then be expected to be ibuprofen-insensitive. Yet, as follows from Fig 3C, it is the non-Nav1.9-dependent

hypersensitivity which is ibuprofen-insensitive (p. 7: "Residual MβCD-induced allodynia in Nav1.9 KO mice was insensitive to intraperitoneal injection of ibuprofen"). Perhaps all the MβCD-induced hypersensitivity is ibuprofen-insensitive and a fraction of it depends on Nav1.9, which is, in turn, is the same fraction of the carrageenan-induced hypersensitivity, which is insensitive to ibuprofen. However, this would make Nav1.9-dependent mechanism only a relatively minor contributor to the carrageenan-induced pain. I'm baffled by this apparent conundrum and would welcome a clarification.

We provided a new figure (Supplemental Fig. S11) that will help clarify the MβCD- and Carrageenan/inflammatory pathways that emerge from our study. MβCD causes depletion of membrane cholesterol and subsequent activation of Nav1.9. In vivo, MβCD generates pain, which is reduced by half in Nav1.9 KO mice. This indicates that an important component of MβCD-induced pain depends on Nav1.9 but that other, yet unknown, mechanisms also contribute to MβCD effects (dubbed other channels in the summary Fig. S11). Nav1.9-independent MβCD-induced pain components are insensitive to ibuprofen treatment (e.g. Fig. 3C). Meaning that MβCD does not trigger -Cox-related- inflammation. MβCD acts downstream to inflammatory mediator signaling and mimic cholesterol depletion observed upon inflammation (see Supplemental Fig. S11). Carrageenan-induced hypersensitivity has been shown to involve Nav1.9 (Lolignier et al., 2011; Priest et al., 2005; Amaya et al., 2006; cited in the ms). Nav1.9 is therefore an important - but not exclusive - contributor to this phenomenon. About the same level of inhibition (50 %) of the Carrageenan-induced hypersensitivity in WT mice is induced by soluble cholesterol or injection of ibuprofen (cf Fig. 8A). Therefore, the most logical explanation is that Carrageenan-induced hypersensitivity involved at least 3 different pathways, one that depends on cholesterol depletion and Nav1.9, one that results from cholesterol depletion but that is independent of Nav1.9, and a latter component(s) that is insensitive to ibuprofen and that recruit different types of ion channels (new Supplemental Fig. S11). In conclusion, we agree with the referee: the fraction of MβCD-induced hypersensitivity that is not dependent on Nav1.9 is ibuprofen-insensitive and is likely to be the same fraction than that of the ibuprofen-insensitive carrageenan-induced hypersensitivity. Because deletion of Nav1.9-dependent mechanism reduced carrageenan-induced pain by ~45 % it should be considered as an important – but certainly not unique - contributor to the associated pain.

2) Cholesterol depletion in DRG neurons.

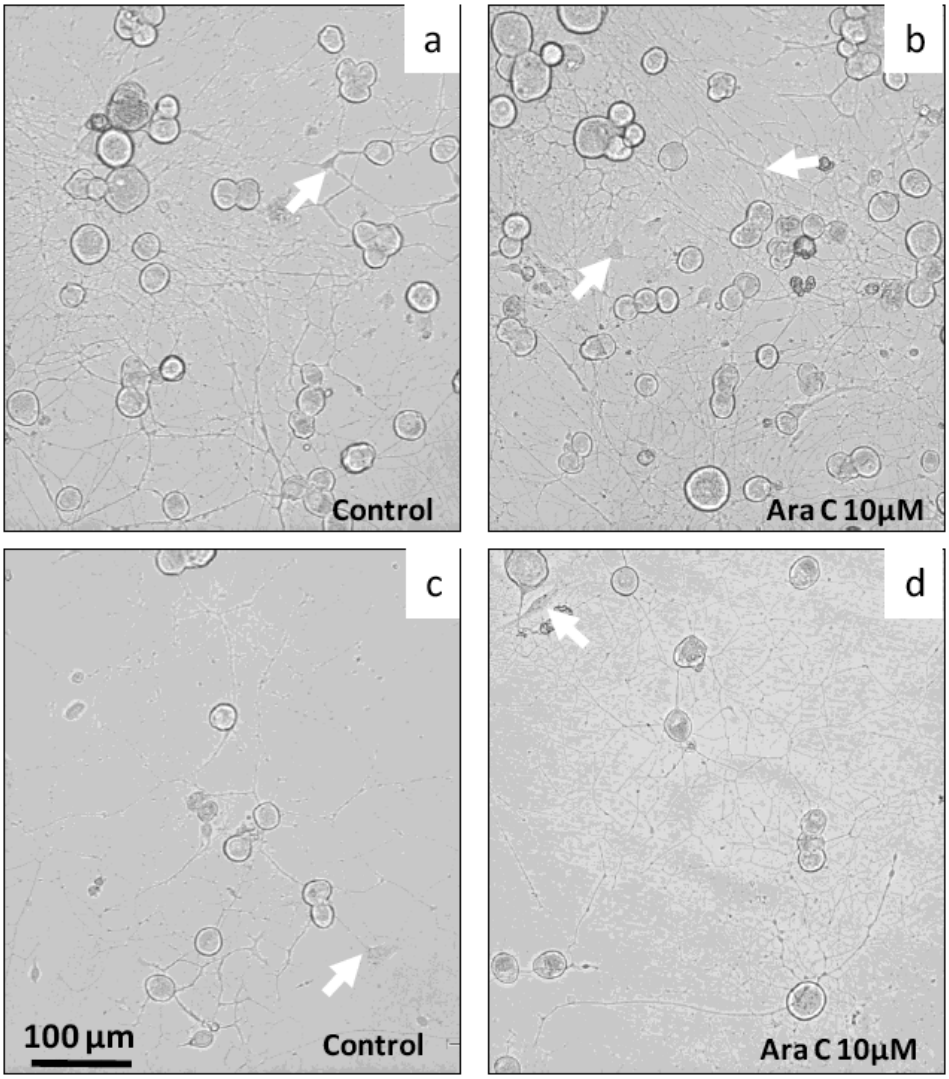
In relation to the same question, it is not clear how robust the cholesterol depletion produced by inflammation in nociceptive nerve endings is. There is 10-20% decrease of total cholesterol in the skin after plantar injection of carrageenan or inflammatory mediator cocktail (Fig. 1C, E). Yet, nociceptive nerve endings represent only a minute fraction of total skin tissue, thus, the change of cholesterol content in the nerve terminals cannot be deduced from such data. There is also 16% reduction of total cholesterol content in DRG culture treated with the inflammatory cocktail. But again, DRG culture is a mix of neurons and glia, in 24h culture there will likely be more glial cells than neurons and since the total change in cholesterol level is small, it is impossible to say how much of the change is attributable to neurons. One way to get around this problem is to test the effect of inflammatory cocktail on the cholesterol level in purified neuronal DRG culture, this can be achieved by suppressing glial proliferation with cytosine arabinoside or by magnetic cell sorting.

We agree that "in the skin nociceptive nerve endings represent only a minute fraction of total skin tissue", and this the reason why we highlighted this point in the discussion by the sentence "*The global decrease (~18 %) however that was observed in inflamed skin biopsies suggests that cholesterol level may be lowered in keratinocytes, which is the predominant cell type (~90%) in the epidermis."*

Thus, we measured cholesterol level in cultures of DRG cells exposed to the inflammatory cocktail. The DRG culture is clearly not a pure neuronal culture, but our experimental procedure that we detailed more in the revised manuscript leads us to get enriched proportion of neurons relative to glial cells. It now reads in the method section" *Thoraco-lumbar DRG ganglia were excised from intervertebral foramina, ventral and dorsal roots were then cut as close as possible and connective tissues were removed to minimize Schwann cell and fibroblast contamination*." Cholesterol dosage was performed 16h after plating, a relatively short period that minimizes proliferation of 'resident' glial cell. Bright field images below showing low (c) and high (a) density regions of our cultures, illustrate the low density of glial cells (white arrows) compared to neuronal density (round and phase bright cell bodies).

Therefore, we trust that the decrease in cholesterol that we detected in response to inflammatory cocktail exposure reflects changes in cholesterol in DRG neurons; however this is not a definite proof.

We tested referee's suggestion of getting a purified neuronal DRG culture by adding 10 µM of the anti-mitotic agent cytosine arabinoside (AraC) 3 hours after cell plating. After 16h in vitro, there was no difference in the amount of glial cells present in treated dished (b-d) compared to untreated dishes (a-b). This confirmed that our culture contains a low number of glial cells capable of DNA-replication in this short (16 h) period of time. This result, along with the fact that AraC is known to be toxic for postmitotic neurons (Wallace et al., 1989) and may adversely change neuronal features and fate, has detracted us from doing cholesterol dosage in these conditions.



We then considered the second suggestion of cell purification by magnetic cell sorting. This is a powerful technique but requires specific antibody directed against an extracellular epitope specific for the targeted cell population. Unfortunately, satellite cells which are potentially the most frequent glial cells encountered in DRG cultures express most receptors present in the neuronal population (Hanami, 2005).

Thus, because we could not improve our yield of DRG neurons in culture, we modified the text in the manuscript to reflect the fact that we cannot ascertain that the percentage of cholesterol depletion measured resulted from changes occurring exclusively in neurons. It reads in the introduction "*We show that cellular cholesterol content is reduced both in mouse skin tissue and DRG cultures during inflammation*" and in the discussion on page 16 "*The global decrease (~18 %) of cholesterol however that was observed in inflamed skin biopsies suggests that cholesterol level may be lowered in keratinocytes, which is the predominant cell type (~90%) in the epidermis. We provide evidence to suggest that cholesterol depletion also occurs in DRG cultures (~16 %) exposed to inflammatory mediators, and demonstrate that reducing membrane cholesterol level causes enhanced excitability*

of small-diameter pain-processing neurons. Although we cultured DRG cells under conditions that minimize the presence of fibroblasts and primary Schwann cells, such cultures contain heterogeneous populations of neurons and satellite cells, which makes difficult to ascertain the exact proportion of decrease of cholesterol to the neuronal population."

References:
Thomas L. Wallace' and Eugene M. Johnson, Jr. Cytosine Arabinoside Kills Postmitotic Neurons: Evidence That Deoxycytidine May Have a Role in Neuronal Survival That Is Independent of DNA Synthesis. The Journal of Neuroscience, January 1989, 9(l): 115-124.
Hanani, M. Satellite glial cells in sensory ganglia: from form to function. Brain Research Reviews 48 (2005) 457–476.

3) Inflammatory treatment did not produce sufficient cholesterol depletion to destroy lipid rafts
According to Fig. 5A, treatment with inflammatory cocktail did not affect partitioning of caveolin and flotilin into cholesterol-rich membrane fractions, this further indicates that inflammatory treatment did not produce sufficient cholesterol depletion to destroy lipid rafts.
Our data indicate that Nav1.9 channel activation induced by the inflammatory cocktail occurred in parallel with reduced levels of cholesterol. Yet, raft formation was not disrupted by the inflammatory cocktail, as assessed by detergent resistant membrane (DRM) fractions. From this, it can be suggested (as correctly stated by the referee) that the inflammatory treatment did not produce sufficient cholesterol depletion to disrupt lipid rafts. It follows that cholesterol depletion by the inflammatory cocktail may not be strong enough to alter the overall organization/formation of lipid rafts (as attested by the presence of flotilin and caveolin in fractions 1-4, cf Figure 5), but sufficient however to reduce the 'affinity' of Nav1.9 subunits to raft microdomains. The identification of several putative cholesterol binding domains in Nav1.9 aa sequence (see Fig. 6) suggests physical interaction between cholesterol molecules and Nav1.9.
To further evaluate the impact of cholesterol depletion on raft/caveola formation and Nav1.9 location, we now provide data using methyl-β cyclodextrin the most widely used /efficient agent to remove cholesterol from cell membrane. We now show in Supplemental Figure S7 that partial disruption of lipid rafts (as judged from the redistribution of flotilin in non-raft membranes) also caused a partial redistribution of Nav1.9 out of raft fractions.

4) Does cholesterol delivery prevent GTPγS-induced Na1.9 upregulation?
It is of note that potentiation of Nav1.9 by inflammatory mediators is a well-recognized effect that was attributed to GPCR modulation (e.g. Rush & Waxman Brain Research 2004; Ostman et al. J Physiol 2008; Vanoye et al. J Gen Physiol 2013). For instance, Nav1.9 current is potentiated during whole-cell patch clamp recording with intracellular dialysis of GTPγS. So, the inflammatory potentiation observed in the present study could be a GPCR-mediated effect or a combination of several factors. Indeed, in one of their previous studies the authors suggested that inflammatory Nav1.9 upregulation is a result of integration of several signaling cascades (Maingret et al. J Gen Physiol 2008). It also conceivable that the GPCR- or GTPγS-mediated Nav1.9 potentiation is in fact mediated by cholesterol depletion, but authors do not test this directly. Does cholesterol delivery prevent GTPγS-induced Na1.9 upregulation? This would be an important experiment as it would provide a better link with previous literature and also could give a clue for answering another important question: how inflammatory signalling causes cholesterol depletion. This is not addressed in the current version of the manuscript.
This is a very important point indeed. The inflammatory cocktail used in our study is likely to activate GCPRs and to produce – at least part of its effect – through activation of heteromeric G-protein signaling pathways as reported previously. To test whether G-protein activation modulates Nav1.9 via membrane cholesterol depletion, we recorded DRG neurons with GTPS added to the patch pipette solution, and determined the voltage-dependent properties of Nav1.9 with and without soluble cholesterol (MβCD-chol, 20 mM). As previously reported, GTPS caused a leftward shift of about 20 mV of the activation curve of Nav1.9 with a midpoint of -49.2 ± 0.8 mV (n = 8). MβCD-chol pre-treatment prevented the effects of internal GTPS with a V0.5 value for Nav1.9 activation of -27.4 ± 0.7 (n = 7). These new data are now presented in Supplemental Fig. S9. Together, they favor the view that delivery of cholesterol to plasma membrane can prevent the action of the inflammatory soup and its downstream G-protein-coupled membrane receptors on Nav1.9. This is not to say that

inflammatory mediators act only through cholesterol-dependent pathways and likewise that Nav1.9 is only modulated by cholesterol-dependent mechanisms.

5) Nav1.9 upregulation in the presence of an antioxidant or a reducing agent.
Some hypotheses are discussed but not tested, even though at least some of these is fairly easy to test. For instance, the ROS hypothesis can be tested by checking if the cholesterol depletion or Nav1.9 upregulation in DRG neurons are abolished in the presence of an antioxidant or a reducing agent.
As requested by the referee, we tested whether the inflammatory cocktail modulates Nav1.9 through production of increased reactive oxygen species (ROS). We now show in the new Supplemental Fig. S6A,B that ROS production was increased in DRG neurons exposed to the inflammatory cocktail and that this enhanced production of ROS was reversed by 4 mM of the ROS scavenger alpha Phenyl t-Butyl Nitrone (PBN). We also carried out a new series of experiments in which we tested the effects of another ROS scavenger (N-acetyl-cysteine, NAC), which is more suitable for stable electrophysiological recordings, on Nav1.9 activation by the inflammatory cocktail. The midpoint of activation curve for Nav1.9 was -35.02 ± 0.79 mV for cells exposed to the cocktail (n= 3, new cocktail cells) and -26.34 ± 1.5 mV (n=8) for cells bathed with the cocktail + NAC (new Supplemental Fig. S6C). Thus, this new set of data indicates that inflammatory mediators modulate Nav1.9 via an oxidative stress-mediated signaling mechanism.
We further confirmed that this mechanism is also at play in vivo by testing the effect of NAC on the mechanical hypersensitivity induced by intraplantar injection of the cocktail. We found that intraplantar co-injection of NAC with the inflammatory cocktail (n=9) strongly reduced the mechanical hypersensitivity typically provoked by the inflammatory cocktail injected alone (n=4) (new Supplemental Fig. S6D), demonstrating that ROS production is enhanced both *in vitro* and *in vivo*.

Specific comments

1) Reference to effects of cholesterol or lipid rafts on nociceptive signaling.
At a few instances authors state that no one have studied a role of cholesterol or lipid rafts in inflammatory pain signaling before (e.g. page 2: "So far, no one has investigated the putative role of cholesterol in inflammatory pain..."; page 14: "Although lipid mediators are well known to be part of the pain pathway...cholesterol has not yet been linked to pain processing."). This is not true, there is a number of studies on the effect of cholesterol or lipid rafts on nociceptive signaling; below are just a few examples but there are more.
• Gnanasekaran et al. Mol Pain 2011: cholesterol/lipid raft regulation of P2X3 channels in trigeminal nociceptors.
• Pristerà, Baker & Okuse PLoS One 2012: lipid rafts localization of Nav1.8
• Jin et al. Sci Signal 2013: role of cholesterol/lipid rafts in regulation of ANO1 channel in nociceptors.
• Sághy et al. Pharmacol Res 2015: lipid raft modulation of TRPV1 and TRPA1
Good point. We have modified the introduction on page 2 as follows: "*Growing evidence suggests that pain sensation caused by tissue damage and inflammation is, in part, regulated by the pro- or anti-nociceptive actions of lipid mediators, such as arachidonic acid or anandamide, on neural pathways (Piomelli and Sasso, 2014). Cholesterol/lipid rafts also have been shown to be involved in regulating nociceptive ion channels in DRGs (Gnanasekaran et al., 2011; Pristerà et al., 2012; Jin et al., 2013; Sághy et al., 2015). Although these studies highlight interaction between cholesterol/lipid rafts and nociceptive signaling, they provide little information about whether change in cholesterol homeostasis contributes to inflammatory hyperalgesia. Accordingly, we investigated whether change in plasma membrane cholesterol may contribute to the mechanisms of inflammatory nociceptor hypersensitization.*"
The statement on page 16 (first paragraph of the discussion) has been deleted.

2) Inflammatory excitation in the presence of MβCD-cholesterol.
Page 4: "Concomitant with the decrease in cholesterol level, we observed an overall increase in excitability of cultured DRG neurons exposed to the inflammatory cocktail..." There are multiple well known excitatory mechanisms triggered by inflammatory mediators, including P2X channel activation (there was ATP in the cocktail), TRP channel sensitization, KCNQ channel inhibition, Nav1.8 phosphorylation etc. If the authors want to evaluate a fraction of

the inflammatory excitation, which is dependent on the cholesterol depletion, they would need to repeat these experiments in the presence of MβCD-cholesterol.
We agree. We made additional experiments in which we tested whether MβCD-chol could inhibit the effects of the inflammatory cocktail on the increase in excitability typically evoked by the inflammatory cocktail.
Exposure of DRG neurons to the inflammatory cocktail reduced the current threshold necessary to elicit an action potential by 32 ± 14% (Supplemental Fig. S1A, B) and increased the mean firing rate of DRG neurons by 2-3-fold (Supplemental Fig. S1D). Pretreatment with MβCD-chol complex (20 mM) for 10 min prevented any significant changes of current threshold for AP and firing in DRG neurons exposed to the inflammatory cocktail compared to vehicle control neurons (Supplemental Fig. 1SA,C,E). It should be noted that the value of the current threshold for AP in DRG neurons (with similar Cm, 15-37 pF) pre-treated with MβCD-chol is smaller than that of control DRG neurons treated with the vehicle, most likely indicating that the inflammatory cocktail is also acting on DRG neuron excitability via non-cholesterol-dependent regulations; however the small number of neurons obtained during the revision round makes this difference not statistically significant. These data are now depicted in the new Supplemental Fig. S1 and included in the result section.

3) Nav1.9 clustering .
Suppl. Fig. 2: it would be good to test if Nav1.9 clustering disapers after carrageenan or inflammatory cocktail injections.
This is an interesting question to address but that requires specific tools which are not available right now. As stated in our manuscript, Nav1.9 clustering along axons is reminiscent of microdomain clustering of Nav1.8 along DRG neuron fibers (Pristera et al., 2012; cited in the ms). To test for Nav1.9 cluster redistribution along peripheral nerve terminals upon inflammation, confocal microscopy is not appropriate because it has a 200 nm resolution in x and y axis while typical size of raft domains in neurons are considered to be far below this limit. Therefore, we have tried to address this question with nanoscale imaging technique like STORM (Stochastic Optical Reconstruction Microscopy). Unfortunately our Nav1.9 antibody that only works on frozen tissues was not compatible with this approach, which requires fixed tissues (a prerequisite for the study of fine-structural distribution of proteins).
Please, note also that because confocal images of Nav1.9 clusters along peripheral axons is not a proof of its presence in raft domains, we rephrased the sentence on page 7 to read " *Nav1.9 is distributed in clusters along the peripheral fibers of trigeminal neurons (Padilla et al., 2007) and DRG sensory fibers of skin territories (Supplemental Fig. S2), which bears a resemblance to the distribution of Nav1.8 in supposed lipid rafts (Pristera et al., 2012)*."

4) Did cholesterol depletion affect Nav1.9 inactivation kinetics or voltage-dependence?
Good point. We made a new series of experiments in which we tested how cholesterol depletion using MβCD and ChOxi impacts Nav1.9 channel inactivation. We have to point out that we looked at the 'fast' inactivation of Nav1.9 rather than at the steady-state/slow inactivation because we could not manage to get long duration recordings (which are a prerequisite for studying Nav1.9 slow inactivation - more than 1 min at each potential) upon adding MβCD (20 mM). As seen with the activation, we found that V0.5 values for 'fast' inactivation were also negatively shifted from -25.17 ± 1.14 mV in control DRG neurons to -34.8 ± 1.1 and -41.54 ± 0.38 mV in neurons pretreated with MβCD and ChOxi, respectively. These data are now illustrated in the new Supplemental Fig. S5.

5) Fig. 5: since inflammatory cocktail did not affect distribution of raft and non-raft markers, it would be good to include a MβCD control.
We now included a MβCD control in the new Supplemental Figure S7. It shows that short (15 min) treatment with MβCD (same conditions than those of electrophysiological experiments and cholesterol dosage) induces partial disruption of lipid rafts as judged from the redistribution of flotilin in non-raft membranes. These experiments also confirmed that lipid raft disruption, although incomplete, following cholesterol depletion provokes a redistribution of Nav1.9 out of raft fractions.

6) Chronic inflammation model (Fig. 8 and the corresponding Results section): local cholesterol levels in this model are not tested.
This is indeed an important question but that we failed to address. Actually, we found very difficult to get a 3.5 mm punch biopsy specimen from the mouse ankle near the tibio-tarsal joint where CFA was injected.

Minor points

1) Dose cholesterol oxidase acts from the extracellular side of the membrane or is it taken up by the cells somehow? Please clarify in the text.
Cholesterol oxidase is a flavoenzyme that catalyzes the oxidation and isomerization of cholesterol to cholest-4-en-3-one. The enzyme is extracellular and binds transiently to the membrane surface during catalysis (the general principle of interfacial enzymes). The rate of cholesterol desorption (getting out of the membrane and diffuse through the aqueous phase) is about 5 orders of magnitude slower than the catalytic turnover rate of ChOxi measured with a variety of membranes (Vrielink, 2010). Therefore, the enzyme does not capture free cholesterol from aqueous solution but rather binds cholesterol from the extra cellular side of the membrane.
This is now clarified in the Materials and method section.

Reference:
Vrielink A. (2010) Cholesterol Oxidase: Structure and Function. In: Harris J. (eds) Cholesterol Binding and Cholesterol Transport Proteins:. Subcellular Biochemistry, vol 51. Springer, Dordrecht

2) In Fig. 4F, left panel, why AP amplitude declines during recording?
We did not investigate the reason for the apparent decrease in spike overshoot and widening of APs during ChOxi superfusion, but this was observed in all Nav1.9 DRG cells tested. We think this may be due to the activation/increase of Kv1.5 as this conductance, as many Kv1 isoforms, has been shown to be inhibited by cholesterol (see Levitan et al., 2010). Martens and colleagues (Martens et al. 2001), for example showed that cholesterol depletion resulted in a hyperpolarizing shift in the voltage dependence of both activation and inactivation of Kv1.5 current, which would be expected to have a significant impact on the duration of action potentials that are controlled by Kv1.5 channels. We may expect this modulation to be present in wt DRG neurons, however with fewer incidences on AP waveform due to the enhanced depolarizing drive induced by potentiated Nav1.9. More investigation is however needed to decipher the mechanisms of this phenomenon.

Reference:
Levitan, I., Fang, Y., Rosenhouse-Dantsker, A., Romanenko, V. (2010) Cholesterol and Ion Channels. Subcell Biochem. 2010; 51: 509–549.
Martens JR, Sakamoto N, Sullivan SA, Grobaski TD, Tamkun MM. Isoform-specific Localization of Voltage-gated K+ Channels to Distinct Lipid Raft Populations. Targeting of Kv1.5 to caveolae. J. Biol. Chem. 2001;276:8409–8414.

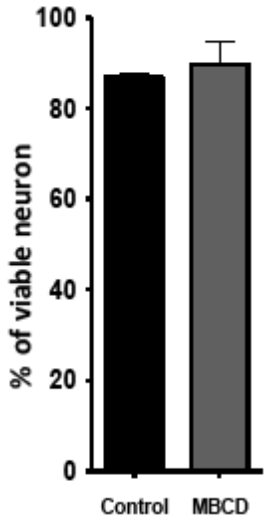
3) Also it is very hard to decipher the current clamp panels (Fig. 2G, Fig 4F), dotted lines are supposed to be 0 mV but it labelled in pA. Or the red pA number represents the current threshold value? Or a current injected to produce red voltage trace? In that case can you move it from the dotted line and provide a more explicit description in the legend?
It was confusing indeed. The dashed line certainly refers to 0 mV baseline and the value in pA indicates the amplitude of the minimum injected current that evokes an AP at t = 12 min (red voltage traces). In the revised figures, the red pA number was moved down close to the protocol; we now provided a more precise description of what is shown in the corresponding legends of Fig. 2G, Fig. 4F and Supplemental Fig. S1 B,C .

Referee #2, (Remarks to the Authors):

We thank Referee #2 for his/her useful comments. We endeavored to improve the paper by incorporating comments from the referee. We hope the referee will view our revision attempt positively.

1) MβCD concentration.
My main concern is the use of very high concentrations of MbetaCD (20 and 40 mM), which are known to be detrimental to neurons (Ottico et al., 2003). MbetaCD (10 mM, 2h) kills 50% of cerebellar neurons in culture (also producing changes in cellular morphology); also, 10 mM MbetaCD for 30 min leads to Trypan blue incorporation in 100% of cells. At such high concentrations it is quite likely that MbetaCD also depletes phospholipids such as sphingomyelin and phosphatidyl choline.

It is true that high concentrations of MβCD are detrimental to neurons as stated by the referee. We were well aware of this difficulty in our study, so we used a specific incubation protocol and many controls in our study. From the literature and our own experience, it appears it is the duration of incubation of MβCD that is important for the viability of cells, this is why we used relatively short pre-incubation time (15 min) with MβCD (10-20 mM). In electrophysiological experiments, this protocol allowed to record viable neurons. In addition, we carried out new experiments to test the viability of DRG cells after 15 min incubation with MβCD (20 mM), see figure below. This short treatment did not change cell viability as the percentage of live DRG neurons using the trypan blue exclusion test was similar to that measured in control DRG cultures (89.6 ± 4.8% for MCD, n=2 cultures / 4859 neurons; versus 86.8 ± 0.9 % for control, n = 3 cultures / 7857 neurons).



The result of this viability test is now included in the result section on p6.

2) Control experiments.
It is true that the authors tried to control for this, and alpha-CD (which does not deplete cholesterol, but depletes phospholipids) did not reproduce the effects of MbetaCD. Moreover, MbetaCD-cholesterol also did not induce pain behavior. In addition, MbetaCD may alter the relative distribution of cholesterol between the plasma membrane and intracellular membranes.
As acknowledged by the referee, we made at lot of efforts to provide in *vitro* and *in vivo* control experiments, including tests with α-cyclodextrin (up to 20-40 mM, tests for the lipid specificity), tests with MβCD saturated with cholesterol (tests for the non specific effects of MβCD) and tests with CholOxi (tests for cholesterol depletion using enzymatic catalysis). We are confident that the effects we observed resulted from depletion of membrane cholesterol.

3) Antioxidants on CholOxi-induced alteration of Nav1.9 function.
Cholesterol oxidase (CholOxi) is likely to generate various oxysterols which are known to have multiple biological (cytotoxic?) functions, including ROS production, so it may be that ROS play a role in the process (which the authors also suggest, but do not follow up). Perhaps it should be investigated whether antioxidants are able to prevent the CholOxi-induced alteration of Nav1.9 function.
The involvement of ROS in Nav1.9 modulation has now been tested. However, ROS involvement was assessed on the effects of the inflammatory cocktail both *in vitro* and *in vivo*, rather than on those of CholOxi because they appear more physiologically relevant. In addition, we reasoned that because CholOxi produced the same effects than MβCD, which extracts cholesterol from membranes, depletion of membrane cholesterol is probably the trigger mechanism of Nav1.9 activation.
We tested whether the inflammatory cocktail modulates Nav1.9 through production of increased reactive oxygen species. We now show in the new Supplemental Fig. S6A,B that ROS production was increased in DRG neurons exposed to the inflammatory cocktail and that this enhanced production of ROS was reversed by 4 mM of the ROS scavenger alpha Phenyl t-Butyl Nitrone. We also carried out a new series of experiments in which we tested the effects of another ROS scavenger (N-acetyl-cysteine, NAC), which is more suitable for stable electrophysiological recordings, on Nav1.9 activation by the inflammatory cocktail. The midpoint of activation curve for

Nav1.9 was -35.02 ± 0.79 mV for cells exposed to the cocktail (n= 3, new cocktail cells) and -26.34 ± 1.5 mV (n=8) for cells bathed with the cocktail + NAC (new Supplemental Fig. S6C). Thus, this new set of data indicates that inflammatory mediators modulate Nav1.9 via an oxidative stress-mediated signaling mechanism.
We further confirmed that this mechanism is also at play *in vivo* by testing the effect of NAC on the mechanical hypersensitivity induced by intraplantar injection of the cocktail. We found that intraplantar co-injection of NAC with the inflammatory cocktail (n=9) strongly reduced the mechanical hypersensitivity typically provoked by the inflammatory cocktail injected alone (n=4) (new Supplemental Fig. S6D), demonstrating that ROS production is enhanced both *in vitro* and *in vivo*.

4) In addition, the effects of CholOxi were not measured in Nav1.8-/- mice.
We now tested whether hyperalgesia induced by intraplantar injection of cholesterol oxidase was reduced in Nav1.8 KO mice as seen in Nav1.9 KO mice. We found that CholOxi-induced hyperalgesia caused by intraplantar injection of CholOxi (4 U/ml) was similar in Nav1.8 KO mice (n = 8) compared with their wild-type littermates (n = 6). These data are now included in the result section and are illustrated in the new Supplemental Fig. S3.

5) Cholesterol depletion and Nav1.9 redistribution.
The inflammatory cocktail has two important effects: It reduces cholesterol and also (while not disrupting rafts) redistributes Nav1.9. Are these effects related? Do they require oxidative stress?
We think these effects to be functionally related. The new Supplemental Figure S7 was designed to evaluate the impact of strong cholesterol depletion (using methyl-β cyclodextrin) on raft/caveola formation and Nav1.9 location/function. It shows that partial disruption of lipid rafts (as judged from the redistribution of flotilin in non-raft membranes) also caused a partial redistribution of Nav1.9 out of raft fractions. This apparent redistribution is associated with Nav1.9 upregulation in electrophysiological studies. Because 13
MβCD, like CholOxi, shifts leftward the voltage-dependent properties of Nav1.9, it can be rationally hypothesized that it is the redeployment of Nav1.9 out of lipid raft-cholesterol rich domains that alters Nav1.9 properties. Although, we now provided evidence to suggest that the inflammatory cocktail produces ROS concomitantly with similar Nav1.9 modulation (see above and new Supplemental Fig. S6), it is the depletion of membrane cholesterol by oxidation, rather than the action of its oxidized products that seems to mediate modulation of Nav1.9. Having said that, these hypothesis need to be tested throughously in a new study.

6) Real time recording.
I have a slight problem with the voltage clamp recordings, which are carried out in different populations of neurons (MbCD and CholOxi were pre-applied for 15 min), in contrast to current clamp recordings that are paired (MbCD and CholOxi were acutely applied for 15 min). It would have been more convincing to see in real time the shift in activation voltage (i.e. the increase in Nav1.9 current at say -35 or -40 mV) during cholesterol depletion.
The reason for the difference in current clamp versus voltage clamp data was that it is difficult to maintain cells at -100 mV under voltage clamp during superfusion of MβCD, while it is less challenging under current clamp mode (the Vm ranging between -70 to -60 mV).
We followed the referee's suggestions and tested the effects of MβCD (10 and 20 mM), CD (20 mM) and the vehicle on the Nav1.9 current in paired experiments. Representative examples of such recordings are now illustrated in the new Supplemental Figure S4. These data show that MβCD shifts the activation voltage of Nav1.9 (i.e. increase Nav1.9 current at -30 or -40 mV) in about 6 min (to plateau), whereas no effects were seen with CD or the vehicle.

7) Specific extraction of Nav1.9 from rafts.
There is merit in the paper, but some questions are left unanswered, particularly how do the inflammatory mediators interact with membrane cholesterol and lipid rafts in such a specific way as to only extract Nav1.9 from rafts.
We now provide new evidence to suggest that inflammatory mediators interact with membrane cholesterol and lipid rafts via the activation of heteromeric G-proteins (new Supplemental Fig. S9) and the subsequent production of ROS (new Supplemental Fig. S6), the action of which decreases membrane cholesterol level. However from the new Supplemental Fig. S7, in which we tested the effects of MβCD incubation on lipid raft disruption and redistribution of Nav1.9, it can be seen that

depletion of membrane cholesterol by 15 min treatment with MβCD induces only a partial disruption of lipid rafts as judged from the limited redistribution of flotilin in non-raft membranes. It follows that cholesterol depletion by the inflammatory cocktail may not be strong enough to alter the overall organization/formation of lipid rafts (as attested by the presence of flotilin and caveolin in fractions 1-4, cf Figure 5), but sufficient however to reduce the 'affinity' of Nav1.9 subunits to raft microdomains. The identification of several putative cholesterol binding domains in Nav1.9 aa sequence (see Fig. 6) suggests physical interaction between cholesterol molecules and Nav1.9; additional experiments using site directed mutagenesis will be required to test this hypothesis further.

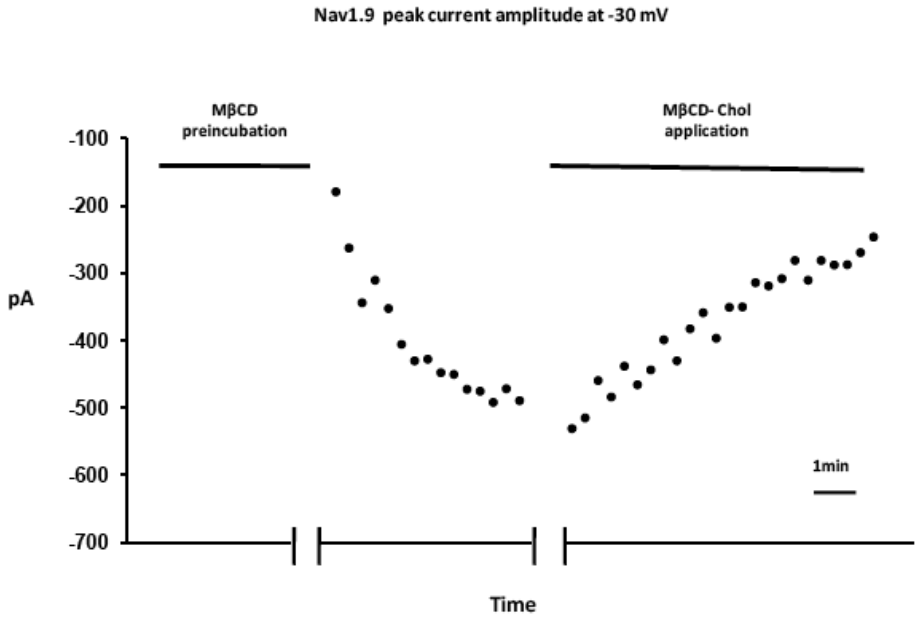
Referee #3, (Remarks to the Authors):

We thank Referee #3 for the positive comments on the paper. We have attempted to strengthen the paper by incorporating comments from the referee when possible. We hope the referee will view our revision attempt positively.

1) The authors should have the lipid:cholesterol ratio for DRGs and note how that ratio changed when they removed cholesterol. What does 18% reduction mean in molar ratios?
This is indeed a good point that may help to address the question of whether other lipids than cholesterol are extracted by MβCD and whether the inflammatory cocktail also downregulates other membrane lipid constituents. In addition, a difference in the cholesterol to phospholipid ratio may affect the fluidity of the lipid layers.
The lipid composition of neuronal somata of cultured root ganglia have been previously determined (Calderon et al., 1995). Neuronal soma contained 37% of dry weight as lipid (15.4% cholesterol, 4.8% galactolipid, and 57.1% phospholipid). The major phospholipids were phosphatidylcholine and phosphatidyl ethanolamine. Galactolipids consisted of cerebroside and sulfatide in molar ratio 2:1. The neuronal soma contained tetrasialo-, disialo-, and monosialoganglioside.
It has been also shown that higher cholesterol content is present in neurites reflecting the higher percentage of plasma membrane in this neuronal compartment and possibly its distinctive functions (Calderon et al., 1995).
Measuring the lipid:cholesterol ratio for DRGs under our experimental conditions therefore would require an entire new study and also to benefit from a pure culture of DRG neurons, which is not the case at present. In addition, because we are not very confident in our biochemical competences, we put all our efforts on other, more attainable, experiments in the short time (3 months) allowed to revise the ms. We apologize for not reaching the referee expectations.

Reference:
Calderon R, Attema B, DeVries GH (1995). Lipid composition of neuronal cell bodies and neurites from cultured dorsal root ganglia. J Neurochem. 1995 Jan;64(1):424-9.

2) The authors are encouraged to test for reversibility after removing cholesterol as was done in the studies of Rosenbaum et al when they were assessing TRPV1.
We made a new series of experiments specifically testing whether i) MβCD-Chol may reverse the effects of the inflammatory cocktail on DRG neuron firing and whether ii) MβCD-Chol may reverse the effects of MβCD cholesterol extraction on Nav1.9.
Exposure of DRG neurons to the inflammatory cocktail reduced the current threshold necessary to elicit an action potential by 32 ± 14% (Supplemental Fig. S1A, B) and increased the mean firing rate of DRG neurons by 2-3-fold (Supplemental Fig. S1D). Pretreatment with MβCD-chol complex (20 mM) for 10 min prevented any significant changes of current threshold for AP and firing in DRG neurons exposed to the inflammatory cocktail compared to vehicle control neurons (Supplemental Fig. 1SA,C,E). These data are now depicted in the new Supplemental Fig.1S and included in the result section.
Cholesterol reloading has also been tested on Nav1.9 previously activated by MβCD. These experiments are tricky because they require maintaining stable voltage-clamp recording while bathing the MβCD-pretreated DRG neurons with MβCD-Chol. In four DRG neurons where this protocol could be successfully achieved without increase in leak currents at -100 mV, we could see a decrease of 59 ± 6.5 % of Nav1.9 current amplitude at -30mV after acute (6 to 15 min) MβCD-Chol treatment on neuron treated with MβCD. Such a recording is illustrated below, however because of the small number of DRG neurons recorded we choose not to include these data in the ms.



3) Can cholesterol distribution in skin strata be determined ?
It is possible to separate stratum corneum, epidermis and dermis strata from relatively large human skin sample. Technically stratum corneum is removed by successive tape-strapping (with D-Squam tape) with a monitoring of the transepidermal water loss (TEWL) to achieve complete removal of stratum corneum (TEWL > 20g/m2/h, measured with specialized apparatus). Epidermis and dermis are separated with short (30s) thermal heat at 65°C. We mandated a specialized company to perform cholesterol dosage in the 3 layers of human skin from plastic surgery samples upon application of cholesterol cream in a Frantz cell chamber. This study revealed the transcutaneous delivery of cholesterol up to the dermis. Unfortunately, these techniques are not currently adapted for mouse paw biopsy.

4) Increasing n number.
P.6 . In the current clamp experiments they report the effects of 2 of 6 or 7 responses - I think this is questionable to report on the responses of two cells although I believe the general result.
We agree. We made additional experiments with CholOxi for which the n number was quite low. We recorded now from 14 DRG neurons treated with cholesterol oxidase (2 U/ml). Although reduction of the current threshold for AP was almost systematic we observed a clear increase of firing rate in 5 out of the 14 neurons tested. These new data are now included in result section and illustrated in Figure 2I.

5) Inactivation and resting potential.
The activation results are strong and convincing. Did the authors conduct inactivation studies ? Did they measure any changes in resting potential? Were cell sizes taken into account ?
We made a new series of experiments in which we tested how cholesterol depletion using MβCD and ChOxi impacts Nav1.9 channel inactivation. We have to point out that we looked at the 'fast' inactivation of Nav1.9 rather than at the steady-state/slow inactivation because we could not manage to get long duration recordings (which are a prerequisite for studying Nav1.9 slow inactivation - more than 1 min at each potential) upon adding MβCD (20 mM). As seen with the activation, we found that V0.5 values for 'fast' inactivation were also negatively shifted from -25.17 ± 1.14 mV in control DRG neurons to -34.8 ± 1.1 and -41.54 ± 0.38 mV in neurons pretreated with MβCD or ChOxi, respectively. These data are now illustrated in the new Supplemental Fig. S5.
We typically recorded from relatively small DRG neurons (Cm ranging from 15 to 37 pF) to increase the likelihood of getting neurons expressing Nav1.9. When let unclamped, minor variations in resting membrane potential could be seen with MβCD or ChOxi, however we cannot ascribed them to Nav1.9 modulation. We do not wish to speculate too much regarding these effects.

6) Cholesterol binding domains - the authors need to make clear the limitations of their claims, what is experimentally-based, what is enlightened reasoning.
We identified 3 cholesterol binding domains that efficiently bind cholesterol molecules using surface plasmon resonance technique (cf Fig. 6B-D), but the role of these domains in subcellular location and function of Nav1.9 remains to be addressed. To avoid confusion, we have rephrased the relevant text on page 3 in the introduction as follows :" We identify 3 peptide domains on the Nav1.9 channel subunit exhibiting cholesterol binding properties suggesting physical interaction. " and on pages 12-13 as follows: "*These results indicate that cholesterol directly binds to at least 3 domains of Nav1.9, however whether these docking sites play a role in Nav1.9 modulation remains to be investigated."*

7) Authors should make sure that p values have the correct number of significant digits (especially in figure legends).
Corrected in the legends of Fig.3, Fig 5 and main text ; eg : p =0.0357 became p = 0.04, etc.

2nd Editorial Decision 25 October 2017

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

As you can see the referees appreciate the introduced changes and support publication here. They raise a few remaining points that should be fairly easy to resolve.

REFEREE REPORTS

Referee #1:

The authors should be commended for their thorough approach to revision and for many additional experiments and improvements. In general, the revised manuscript addresses my main concerns well. The data are broadly consistent with the author's hypotheses. There are some minor discrepancies here and there that perhaps could be discussed and reflected better in the summary cartoon (which otherwise is very helpful) and the Discussion section. For instance, the lipid raft experiments still leave space for alternative interpretations: inflammatory mediators cause redistribution of Nav1.9 but do not destroy lipid rafts while cholesterol extraction does both - the Nav1.9 re-distribution and raft disruption. Given small-ish changes in cellular cholesterol induced by inflammatory mediators, and no obvious effect on lipid rafts integrity, can it be that ROS and cholesterol extraction produce mechanistically different effects which however both result in redistribution of Nav1.9? E.g. it could be some direct channel oxidation that causes a conformational change that reduces channel's retention at a raft (while cholesterol depletion would just get rid of rafts altogether)... This is something that authors seemingly suggested in their rebuttal letter:

"It follows that cholesterol depletion by the inflammatory cocktail may not be strong enough to alter the overall organization/formation of lipid rafts (as attested by the presence of flotilin and caveolin

in fractions 1-4, cf Figure 5), but sufficient however to reduce the 'affinity' of Nav1.9 subunits to raft microdomains."

Yet, in their cartoon and in Discussion it follows that ROS cause cholesterol depletion, which I don't think they have explicitly demonstrated.

Somewhat related to that, I still cannot not fully accept the way how the 'ibuprofen issue' is addressed in the manuscript. While cartoon is definitely very helpful, it is necessary (in this reviewer's opinion) to have a brief statement in the Discussion that would address the issue. Particularly, behavioural tests (Fig 2B and Fig. 8A specifically) suggest that there are ibuprofen-sensitive and ibuprofen-insensitive fractions in the carrageenan-induced hyperalgesia, with cholesterol-mediated fraction being ibuprofen-insensitive; the same is implied in the cartoon. Yet, in Fig. 8C, topical cholesterol apparently abolished both components, suggesting that the procedure is perhaps doing something less specific than just reverting the cholesterol depletion produced by carrageenan injection. This, in turn, cast some doubts over the mechanistic interpretation of cholesterol co-injection experiments.

I am fully aware that behavioural responses cannot be taken as direct correlates of intracellular signaling cascades in nociceptors, and so I do not suggest further experiments but I just think that authors could be a bit more comprehensive and inclusive in the way they interpret the results. To be fair, some alternative hypotheses are briefly mentioned in the Discussion, but I feel that both the cartoon and the Discussion could go a bit further in separating what is definitely shown, what is inferred and what alternative explanations could be put forward in addition to authors' main hypothesis.

Minor: is there a way to include the revised cartoon (Suppl Fig 11) into the main manuscript?

Referee #2:

The extensive revision of this paper has been performed in exemplary quality, all my previous major and minor concerns have been appropriately addressed, either by (many) additional experiments and convincing results or by adequate explanations in the rebuttal letter and amendments of the MS text. The paper is now certainly worth publishing.

Referee #3:

The authors have provided a well-rounded response to critique.
The paper is much improved now.

I have a few minor questions/issues that I encourage the authors to address.
1) The topical application with cholesterol-loaded HEC gels is an exciting component of the entire study. It clearly has a translational-medical direction. The authors need to provide more detail in Methods how the gels were fabricated, e.g. vendors of ingredients/components, temperatures, moisture-levels and other relevant conditions when generating the gels, also solutions used, so that other investigators can readily replicate this method in their own environments.
2) toxicity assay in human artificial skin is helpful, but we also want to see H&E of mouse paw of animals that have been treated with the cholesterol-loaded HEC gels.
3) do the authors have evidence that cholesterol-loaded HEC gels do not act simply as (low-potency) local anesthetics ? If not, then this possibility needs to be indicated in the ms.

3) minor issue: it's ANOVA, not ANNOVA. - Would not one-way ANOVA have been sufficient for some of the experiments where it was applied ?

Referee #1:

The authors should be commended for their thorough approach to revision and for many additional experiments and improvements. In general, the revised manuscript addresses my main concerns well. The data are broadly consistent with the author's hypotheses. There are some minor discrepancies here and there that perhaps could be discussed and reflected better in the summary cartoon (which otherwise is very helpful) and the Discussion section. For instance, the lipid raft experiments still leave space for alternative interpretations: inflammatory mediators cause redistribution of Nav1.9 but do not destroy lipid rafts while cholesterol extraction does both - the Nav1.9 re-distribution and raft disruption. Given small-ish changes in cellular cholesterol induced by inflammatory mediators, and no obvious effect on lipid rafts integrity, can it be that ROS and cholesterol extraction produce mechanistically different effects which however both result in redistribution of Nav1.9? E.g. it could be some direct channel oxidation that causes a conformational change that reduces channel's retention at a raft (while cholesterol depletion would just get rid of rafts altogether)... This is something that authors seemingly suggested in their rebuttal letter:
"It follows that cholesterol depletion by the inflammatory cocktail may not be strong enough to alter the overall organization/formation of lipid rafts (as attested by the presence of flotilin and caveolin in fractions 1-4, cf Figure 5), but sufficient however to reduce the 'affinity' of Nav1.9 subunits to raft microdomains."
Yet, in their cartoon and in Discussion it follows that ROS cause cholesterol depletion, which I don't think they have explicitly demonstrated.
Reviewer#1's comments were very helpful overall, and we are appreciative of such constructive feedback on our original/revised submission.
The referee is right. Although plausible, we did not provide direct evidence that ROS cause cholesterol oxidation/depletion. Thus, direct oxidation of Nav1.9 channels may also be a mechanism that modulates Nav1.9 properties by altering channel's retention at rafts. We have modified the cartoon (old supplemental Fig. S11, now Fig 9) to explicitly reflect this possibility. We also amended the discussion on page 18 to read:
"*Thus, the overall picture that emerges from our study is that cholesterol oxidation caused by inflammation/ROS production may lead to decreased levels of membrane cholesterol in the vicinity of Nav1.9 channels, promoting Nav.1.9 channel relocation/opening, hyperexcitability and pain signaling (Fig 9). However, we cannot rule out a direct oxidation of Nav1.9 channels by ROS, which may reduce channel's retention at rafts. Inflammation-triggered cholesterol depletion pathway should be seen as one of the multiple pathways that concurrently lead to nociceptor sensitization during inflammation (Fig 9)*."

Somewhat related to that, I still cannot not fully accept the way how the 'ibuprofen issue' is addressed in the manuscript. While cartoon is definitely very helpful, it is necessary (in this reviewer's opinion) to have a brief statement in the Discussion that would address the issue. Particularly, behavioural tests (Fig 2B and Fig. 8A specifically) suggest that there are ibuprofen-sensitive and ibuprofen-insensitive fractions in the carrageenan-induced hyperalgesia, with cholesterol-mediated fraction being ibuprofen-insensitive; the same is implied in the cartoon.
The referee is right when he/she says that the carrageenan-induced hyperalgesia has both ibuprofen-sensitive and ibuprofen-insensitive components (from Fig. 8A), but Fig. 2B does not provide evidence to suggest that in the carrageenan response the 'cholesterol depletion-mediated fraction is ibuprofen-insensitive' – we actually don't know that. What emerges from Fig. 2B is that MβCD, which acts downstream to inflammatory mediators, does not cause pain through local (ibuprofen-sensitive) inflammation, which would have generated pain regardless of the effect of MβCD on cholesterol level.
To sum up, one component of the carrageenan response that causes cholesterol depletion (and Nav1.9 activation) may well be ibuprofen insensitive, but we have not tested this specifically. This may be the subject of another study.

Yet, in Fig. 8C, topical cholesterol apparently abolished both components, suggesting that the procedure is perhaps doing something less specific than just reverting the cholesterol depletion produced by carrageenan injection.
In Fig. 8C topical cholesterol causes a strong reduction of the carrageenan-induced mechanical hypersensitivity. The referee is therefore wondering whether the cholesterol gel represses the component of the carrageenan hypersensitivity that may result from the ibuprofen-insensitive pathway, suggesting that topical cholesterol has additional effects that may be interpreted as nonspecific. However, as abovementioned, it is not known from our experiments whether the ibuprofen-sensitive fraction and the cholesterol depletion-mediated fraction are part of the same signaling pathway and conversely whether the ibuprofen-insensitive pathway overlaps or not with the cholesterol one. Thus, cholesterol delivery may well reduce both components, but it is presently unknown.
To address the possibility that cholesterol delivery may have additional effects, we tested in the new expanded view Figure EV5C, whether cholesterol gel had potential anesthetic effect. We found that the gel supplemented with 28 mM cholesterol applied for 1h30 to the hind paw of naïve mice, did not alter the mechanical withdrawal threshold, suggesting that cholesterol gel has no detectable anesthetic effect on the mechanosensory response.

This, in turn, cast some doubts over the mechanistic interpretation of cholesterol co-injection experiments.
If we understand correctly, the referee is asking about the apparent difference in efficiency between the cholesterol gel (Fig. 8C) and cholesterol intraplantar injection (Fig. 8A) in reducing carrageenan hypersensitivity. We would like to point out that in Fig. 8C we did not phenotype the animal in the first 2 hours due to the gel application time lapse. For the later time points (3, 4 and 5 h) after carrageenan injection, both cholesterol gel and intraplantar cholesterol injection gave very similar reduction in mechanical hypersensitivity compared to respective controls. To facilitate comparison, the table below gives the numerical values of the mean ± SEM of the normalized withdrawal mechanical threshold ratio from Fig. 8A and Fig. 8C.

	Co-injection		Gel	
Time	Carra	Carra + Cholesterol	Carra	Carra + Cholesterol
3 h	0.2 ± 0.05	0.7 ± 0.08	0.3 ± 0.05	0.8 ± 0.13
4h	0.3 ± 0.06	0.9 ± 0.08	0.3 ± 0.06	1 ± 0.12
5h	0.4 ± 0.11	0.9 ±0.08	0.4 ± 0.09	0.9 ± 0.11

In addition, the procedure for cholesterol delivery being quite different in the 2 set of experiments (one single 20 µl intraplantar injection in Fig. 8A versus 100 µl gel cutaneous application for 1.5 hour in Fig. 8C), one should be cautious in comparing the efficiency of the 2 methods of cholesterol delivery.

I am fully aware that behavioural responses cannot be taken as direct correlates of intracellular signaling cascades in nociceptors, and so I do not suggest further experiments but I just think that authors could be a bit more comprehensive and inclusive in the way they interpret the results. To be fair, some alternative hypotheses are briefly mentioned in the Discussion, but I feel that both the cartoon and the Discussion could go a bit further in separating what is definitely shown, what is inferred and what alternative explanations could be put forward in addition to authors' main hypothesis.
We have amended the cartoon to better reflect the different signaling pathways, those demonstrated in our study and those more hypothetic. We also modified the discussion to refer to the possibility of direct action of ROS on ion channels. However, because we have not studied the ibuprofen-sensitive/insensitive components of the carrageenan response specifically, and their link with cholesterol, we think too premature to discuss this point in the discussion.

Minor: is there a way to include the revised cartoon (Suppl Fig 11) into the main manuscript?
The revised cartoon is now included into the main text as Figure 9.

Referee #2:

The extensive revision of this paper has been performed in exemplary quality, all my previous major and minor concerns have been appropriately addressed, either by (many) additional experiments and convincing results or by adequate explanations in the rebuttal letter and amendments of the MS text. The paper is now certainly worth publishing.
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I have a few minor questions/issues that I encourage the authors to address.
1) The topical application with cholesterol-loaded HEC gels is an exciting component of the entire study. It clearly has a translational-medical direction. The authors need to provide more detail in Methods how the gels were fabricated, e.g. vendors of ingredients/components, temperatures, moisture-levels and other relevant conditions when generating the gels, also solutions used, so that other investigators can readily replicate this method in their own environments.
We added more details as requested in the materials and method section on page 28: " *For transderma delivery, 2% hydroxyethyl cellulose (Sigma-Aldrich) dissolved in pure water, was supplemented with cholesterol-water soluble (5.6 or 28 mM based on cholesterol concentration; Sigma-Aldrich, or MP bio) and let polymerise for 1 to 1h30 at room temperature under 360° vertical rotation at 30 rpm. Small amount of gel, typically 1 ml, was prepared daily just prior utilization. 100 µL of gel was applied on mouse hindpaw for carrageenan experiment or on the mouse hind paw and ankle for the CFA experiment, under 2 % isoflurane anesthesia for 1.5 h. Skin paw was then carefully cleaned with saline solution, and recovery from anesthesia was allowed for 30 min before assessment of mechanical thresholds*."

2) Toxicity assay in human artificial skin is helpful, but we also want to see H&E of mouse paw of animals that have been treated with the cholesterol-loaded HEC gels.
We now provided images of H&E staining of ipsilateral skin paw treated with 28 mM-cholesterol cream together with the control controlateral paw (new Fig. EV5B). These stainings indicate that cream application did do disturb the structure of the skin layers.

3) Do the authors have evidence that cholesterol-loaded HEC gels do not act simply as (low-potency) local anesthetics? If not, then this possibility needs to be indicated in the ms.
This is a good point. In the previous version of the manuscript, we provided evidence that intraplantar injection of cholesterol did not produce anesthetic effects in Nav1.9 KO mice.
This is now further addressed in the new supplemental Figure EV5C, in which we tested the potential anesthetic effect of the cholesterol gel. We found that the gel supplemented with 28 mM cholesterol applied for 1h30 to the hind paw of naïve (WT) mice, did not alter the mechanical withdrawal threshold, suggesting that the cholesterol gel has no detectable anesthetic effect on mechanosensory fibers.

3) Minor issue: it's ANOVA, not ANNOVA. - Would not one-way ANOVA have been sufficient for some of the experiments where it was applied?
We corrected the figure legends. We used two-way ANOVA to compare the effect of two variables (time and treatment) on a third quantitative variable (animal threshold

Accepted 22 December 2017

Thank you for submitting your revised manuscript to The EMBO Journal. I have now had a chance to take a look at everything and I appreciate the introduced changes. I am therefore very pleased to accept the manuscript for publication here.

There are just a few minor things to take care off before we can send the manuscript to our publisher.

Congratulations on a nice paper!

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: patrick delmas
Journal Submitted to: EMBO journal
Manuscript Number: EMBOJ-2017-97349R

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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?	Size sample was chosen after evaluating the variance of each test experiment
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The sample for animal studies contains a minimum of 6 animals, except for some control (saline) experiments which were done many times in the lab and published previously
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Criteria were pre-established indeed; we only used males, mice 6 to 7 weeks old
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.	For the experiments of skin cholesterol quantification, since the contralateral paw served as control for the paw in which the drug was applied, no randomization was used. When different substances were tested in vivo, groups of animals from the same cage were randomly injected with drugs or vehicle, so variation in mean baseline sensory thresholds was minimized.
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	Blind tests were used for each new series of experiment (if applicable) but not for routine experiments.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Results for a given test experiment were performed by at least 2 independent experimentators.
5. For every figure, are statistical tests justified as appropriate?	Yes, the statistical tests were chosen accordingly to the data set
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We used non parametric, two-tailed P values with 95 % confidence limits
Is there an estimate of variation within each group of data?	Data are presented as mean +/- sem
Is the variance similar between the groups that are being statistically compared?	Yes but not systematically included in the ms. Variance may differ between control (non painful) and test (hyperalgesic) mice. This may depend on the von Frey technique.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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).	Padilla et al., (2007) MCN Vol 35, P138-152. Cited in the ms.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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

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.	We used 6 to 7 weeks old males from C57bl6, Nav1.8-null, Nav1.9-null genetic strains and their respective wild type littermates
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Procedures were in accordance with the directives of the French Ministry of Agriculture and Fisheries and the European Communities Council (86/609/EEC). Behavioral tests and pain models used in this study were all approved by the ethics committee of Région PACA (FRANCE).
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.	OK

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