Etv4 regulates nociception by controlling peptidergic sensory neuron development and peripheral tissue innervation

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I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPressand click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. Reviewers 2 and 3 have several suggestions for strengthening your conclusions and ask for clarifications in a number of places. I would also draw your attention to the questions about the statistical analyses. These should be addressed. Reviewer 1 would like a more extensive revision. While I agree with this referee that systematic assays for the role of Etv4 in the transcriptional regulation of sensory axon development would be of interest to the field, unless this is already available to you, it would require substantial additional work. One way of addressing these points would be to be explicit in your discussion about the limitations of the present study and the need for further work.

If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to

discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The manuscript by Rios et al describes the in vivo function of Etv4, a transcriptional factor, in mediating the development of peripheral innervation of a subpopulation of sensory neurons that are involved in pain. An early in vitro study by the same group showed that Etv4 is upregulated by NGF and required for axonal growth of TrkA+ neurons. The current study extends the finding and demonstrates the physiological function of Etv4 in vivo. Consistent with the in vitro data and Etv4 expression, the study nicely showed that peptidergic axons, a subpopulaton of TrkA neurons that normally express Etv4, have reduced innervation of in the peripheral targets, but no reduction of TrkA neuron survival in the Etv4 knockout mice. Moreover, the knockout mice exhibited reduced pain sensitivity to noxious heat, formalin, and capsaicin, but not to cold or mechanical stimuli. To understand the underlying molecular mechanism, the authors examined two groups of genes, metalloproteinases and channels, and showed reduced expression of Mmp3, 10, 12, and Upar as well as TrpV1. Most of the in vivo studies are well executed and provide convincing data of the phenotypes using the knockout mice. However, the mechanistic insights obtained are somewhat incremental, largely confirming the previous conclusion in the in vivo model. Nonetheless, the significance might be enhanced by including additional in vivo and/or RNAseq analysis (see below).

Comments for the author

Major Points:
1) The reduced expression of multiple metalloproteinases in the Etv4 knockout neurons suggest that they are responsible for the innervation defect. However, the function of the metalloproteinases was only tested in culture using the inhibitor GM6100. To support this conclusion, it would be important to test if overexpression could rescue the Etv4 knockout phenotype either in culture and/or in vivo.
2) Metalloprotinases are likely one of many targets regulated by Etv4 that are critical to nerve growth and target innervation. RNAseq analysis comparing wild type and Etv4 mutant neurons would provide additional insights into the transcriptional regulation of sensory axon development.
3) The pain phenotype of the Etv4 knockout mouse is interesting, but it is not clear whether it results from the lack of innervation or the reduced TrpV1 expression, or both. Rescue with TrpV1 expression might distinguish these possibilities.
Minor Points:
1) What is the phenotype of non-peptidergic axons in the Etv4 knockout mice?
2) The reduced staining of the central peptidergic projection in the spinal cord complicates the interpretation of the pain phenotype, which may not be solely attributed to the lack of peripheral innervation.
3) The section title “Etv4 knockout mice do not show defects in TrkA+ neurons” is not precise. The section mainly described data related to cell survival, and the previous section does suggest that TrkA+ neurons have innervation defects.
4) The expression level of Nav1.8 shown in the bar graph of Fig. 7A seems quite different, but the p value shows “not significant”. This raises the question of whether the sufficient sample number was used for this analysis (especially for the wild type).

Reviewer 2

Advance summary and potential significance to field

The manuscript by Rios et al., explores the control of sensory neuron development by the Etv4 transcription factor.
The modalities of sensory perception are established sequentially during development, through acquisition of specific properties distinguishing various subtypes of sensory neurons. The present study focuses on a subgroup of nociceptive neurons, defined by their dependence on NGF (nerve growth factor), mediated by the TrkA receptor, and by expression of several neuropeptides (CGRP, Substance P), thus identifying them as peptidergic nociceptors. NGF/TrkA signaling is known to ensure survival, but also to build their peripheral innervation and to promote establishment of the modalities of pain sensitivity. The authors have previously found that NGF signaling induces expression of the transcription factor Etv4, also known to control fate specification of other neuronal types, such as motor neurons. In this manuscript, the authors combine in vitro and in vivo approaches using control and Etv4 mutant mice, to explore how Etv4 deletion impacts nociceptive neuron development. They provide evidence that Etv4 is required for peripheral tissue innervation in response to NGF, and for establishing the capacity of these neurons to sense noxious temperatures by controlling expression of the heat receptor Trpv1, a sensory modality necessary for the withdrawal/avoidance behaviour.
The manuscript is clearly written, the hypotheses and approaches are systematically outlined, and experiments are carefully conducted, with solid and convincing data, fully supporting the proposed conclusion. By linking a developmental transcription factor to the acquisition of a functional modality of the adult sensory neurons, this work will be of interest to a broad range of scientists interested in neuronal development.

Comments for the author

I only have a few minor concerns about text issues (how certain concepts are expressed), and my main concern is related to some of the statistical analysis of some of the data in cultured neurons from Etv4-mutants and control embryos (explained below). Overall I believe the work should be published, after these concerns can be carefully addressed (which I believe may only require reanalyzing the available data).

Main comment:

I'm disturbed by the use of statistics in Fig. 4 and Fig. 5, when reporting in vitro results. The viability experiment shows that there are 3 dots per genotype, implying that the authors performed 3 independent experiments. However, for all the other panels in Fig.4, which report different parameters studied in culture (neurite length, etc), the plots show single dots corresponding to individual neurites (likely from these three independent experiments), and show 4 stars, to suggest that the result is strongly statistically significant. It seems that the authors conveniently consider that n= the number of neurites/growth cone/neurons when they show a statistically significant result, but consider n= the number of independent experiments when they show a non-significant result. For such experiments when one culture contains hundreds of neurons/cells, it is not appropriate to pool the number of units observed in each experiment to pretend having a large sample size (while sample size is the number of independent cultures for each genotype). For statistical analysis of this kind of experiments, one should calculate for each independent experiment, a distribution (percentage of neurons in a given measurement range (size, area, etc)), so that analysis can be done in a way that integrates the experimental variability in an adequate manner. For all parameter analyzed (where the observed parameter is a numerical value ranging across a given scale), the scale can be divided in subranges (0-10, 10-20, etc) across the numerical scale, and for each range, each culture/genotype gives a proportion. As a result, each independent experiment gives one data point (hence three data points for three experiments), a mean/median proportion can be calculated, and appropriate statistics tests can be done with a sample size corresponding to the number of independent experiments (per genotype). This way should be used for statistics tests in Figures 4&5. I'm not presuming how the results will affects the statistics tests, it is likely that the message and conclusions will not change, but significance will be calculated in an appropriate manner (although some of the results may fall below the significance threshold, or may become significant with one more set of independent cultures). On the opposite, the in vivo

analyses of mRNA expression of metalloproteases by RT-PCR are convincing because the stats are done in an appropriate manner.

Minor comments:
1- The authors find fewer and shorter peripheral (Figure 1) but not central (Figure 2) CGRP+ axons in Etv4-/- P0 and adult mice. It would have been nice to have a counterstaining with a general axon marker, to distinguish between lowered CGRP expression, and a reduction in the number of axon terminals inserted in the skin. If not possible, then I would prefer seeing the two hypotheses written, also because in Figure 2, they do measure a twofold reduction in intensity of central projections (thus there might be no difference between peripheral and central projections). When they measure a reduced fiber length, it would be helpful if they could normalize this by measuring skin thickness (which is not expected to be reduced).
2- In figure 1F-G, it would be more convincing to show the Etv4 channel alone (as done with TrkA chanel shown in green). Also, it would be helpful to show a view of an entire DRG, to see if there are any Etv4+/TrkA-negative neurons as detected in the trigeminal ganglion, or if Etv4 expression is fully coincinding with TrkA expression, both at E15.5 and after TrkA extinction in non-peptidergic neurons. Also it would be good to add some quantification of the distribution of Etv4+ neurons (percentage of the TrakA+ population, percentage of the Ret+, and eventually, percentage of other sensory neurons).
3- Related to comment 2, The Etv4 antibody staining doesn't seem exclusively nuclear. As this is a commercial antibody, potentially not validated in knockout tissue, it would have been good to have confirmation of staining specificity with another method (and useful to others to include a validation on knockouts). This could be either in situ hybridization, or with lacZ/anti-beta-galactosidase staining in Etv4-heterozygous, as I believe the authors use Etv4-NLZ mice. This would allow precisely matching Etv4 expression with respect to TrkA and Ret expression.
4- In FigS2, it would be helpful to comment that all of the TrkA+ neurons were Etv4+, and that there were no TrkA+/Etv4-, whereas an Etv4+/TrkA- was detected. In fact, given the presence of this other Etv4+ population, it would have been useful to identify (or discuss) if they match with Ret+ neurons or other sensory subtypes. This point is important in particular when considering the results later in the paper, documenting changes in expression of channels such as Trpv1, restricted to peptidergic neurons, but not of other channels expressed in other subtypes.
5- Throughout Figure 6 (functional analysis of sensory modalities in adult mice), I believe that it would make sense to present the latency scale in a uniform manner rather than changing the scale according to the temperature tested. Thus, when the latency is 20 seconds, it should be seen as lower than a latency of 40s. In this difference, the shorter the latency, the more noxious a temperature is. Same comment for Fig. 6B.
6- Since noxious cold sensing is not abolished and Trpm8 expression is preserved in Etv4-/- mice, it would make sense to discuss if these neurons are Etv4-negative (thus not expected to depend on Etv4 for their specification), or if any other modality matches with a Etv4+/TrkA-negative subgroup, thus behaving in an Etv4-independent manner (as in Figure S1, although this refers to the trigeminal ganglion).
Text issues:
Line 87: neurons extinguish the expression of TrkA expression: remove one of the two "expression"
Line 90: in adult DRG sensory neurons, TrkA is predominantly expressed by peptidergic nociceptors: I would suggest: "TrkA expression exclusively persists in adult peptidergic neurons Line 118: the role … remains Line 166: "no significant difference … suggesting that Etv4 is dispensable for the survival of DRG neurons". I wouldn't conclude that Etv4 is dispensable for survival, but that the reduction in peripheral density is not associated with, and therefore cot a consequence of a change in neuronal viability. This allows a missing logical connection between data and conclusion.
Line 170: "we asked whether the altered epidermal innervation in Etv4 KO mice could be explained by changes in the density of nociceptive neurons. Density is a relative measure. While in the skin, the density of axon terminals is normalized to the skin area, in the DRG, the density would be normalized to other sensory neurons. Given the lack of difference in the total number of sensory neurons (and size of ganglia), it would be more accurate to ask if the proportion of nociceptive neurons was reduced compared to other neuronal types. Same in the conclusion (line 179), replace density by proportion.
Line 177-179: The conclusion needs to include the "positive" finding, which is the reduction in peripheral arborization density. This works indicates that whereas Etv4 ablation does not impact the number of TrkA+ nociceptive neurons, nor expression of the major neurotrophin receptors (TrkA, Ret, GFRa1), it leads to a loss of peripheral but not central arborization.

Line 247: noxious heat above 43°: it sounds a bit excessive to describe any temperature above 43°C as noxious. The authors could say that temperatures above 43 are considered noxious for mice because they elicit a withdrawal response in a hotplate test, and this response is known to be mediated by heat receptors.
Line 256: Here the figure indicates 55°C, not 50°C as written in the text.
Figure 6C should indicate formalin somewhere in the figure, not just in the legend.

Reviewer 3

Advance summary and potential significance to field

Etv4 is a transcription factor expressed in developing TrkA+ dorsal root ganglia neurons that is known to be involved in NGF mediated target innervation. Here the authors report that the loss of Etv4 affects more significantly peripheral peptidergic neuron projections than their central projections, and is dispensable for DRG neuron survival. They show that Etv4 is required for NGF dependent axonal growth of sensory neurons and for the expression of ECM remodeling proteins, which likely explains the observed deficit of peripheral nerve innervation. They found that Etv4-null mice are less sensitive to noxious heat stimuli, formalin and capscaicin, and have reduced number of TRPV1+ DRG neurons. Overall, the study provides further evidence for a role of Etv4 in the maturation of nociceptive neurons. It is interesting as it provides insights into its mechanism of action.
The data are in overall of high quality and the conclusions are mostly convincing.

Comments for the author

A few issues we think could be addressed to strengthen what is already a nice study.
1. In figure 1, the authors show that the number of CGRP-expressing free nerve endings is reduced in the epidermis of Etv4 KO mice. Whether the loss of Etv4 affects specifically peripheral peptidergic neuron projections or whether it is also required for the outgrowth of other subtypes of nociceptive fibers is not investigated. This could be addressed by performing double immunostaining of the glabrous skin of the hindpaw of Etv4 KO and WT mice with a more general marker of nerve fibers such as BIII-Tubulin together with CGRP, counting double positive fibers and fibers that are only positive for BIII-Tubulin.
2. Figure 2 shows the consequences of Etv4 knock-out on spinal cord innervation by CGRP+ fibers. The data in panel C show that CGRP fluorescence intensity is decreased in the mutants by about 50%. As a control for this experiment, markers of mechanoreceptive or proprioceptive fibers, which are likely not modified in Etv4 mutants, could be tested. Could the reduced fluorescence be interpreted as a reduced density of CGRP-expressing fibers? These data should be discussed in the manuscript.
3. In figure 5E, the authors show that Etv4 is required for the expression of MM3/10/13. The authors should better explain why these particular MMPs have been investigated and not others. As the activity of MMPs is regulated by endogenous inhibitors (TIMPS), it would have been interesting to examine their expression in Etv4 KO.
4. The requirement of Etv4 for NGF induced expression of Upar is not convincing. The authors suggest that the downregulation of ECM remodeling proteins observed in DRG of Etv4 kO mice likely explains the observed axonal growth defects. This hypothesis could be tested in rescue experiments in PC12 cells.

Minor:
-Line 121. Please correct the sentence: “We also demonstrated that Etv4 mutant mice are more susceptible to painful thermal and chemical stimuli”.
-Line 245 – It is indicated that the tail-flick test has been performed at 50°C.
However, in figure 6B and in the legend of this figure, a temperature of 55°C. is indicated.
-In the abstract, line 39 it is indicated that Etv4 is required for the function of peptidergic neurons. Line 123, it is concluded that Etv4 is essential for the development of nociceptors. It is not to me clear from the data whether the loss of Etv4 affects the development of Trpv1+ nociceptors or its expression in mature nociceptors. This point should be raised in the discussion.
-The recent review on somatosensory neuron diversity and development by Meltzer et al., 2021 (10.1016/j.neuron.2021.09.004) could be added to the references.

-Statistics: All along the study, the authors use parametric tests to assess statistical significance of the data collected. These tests require that the normal distribution of the data is evaluated and validated before being applied as indeed stated by the authors in their material and methods section. However some datasets are constituted of 3 to 4 biological replicates per experimental conditions and a normal distribution of the data cannot be determined with such small datasets. The authors should thus avoid the use of student's t-tests for these specific datasets and instead consider using non-parametric tests (Mann-Whitney test) to assess statistical significance.

First revision

Author response to reviewers' comments

We sincerely thank to the reviewers for their careful and critical review of our manuscript entitled: "Etv4 regulates nociception by controlling peptidergic sensory neuron development and peripheral tissue innervation" by De Rios et al et al.

Reviewer#1

1) The reviewer mentions *"The reduced expression of multiple metalloproteinases in the Etv4 knockout neurons suggest that they are responsible for the innervation defect. However, the function of the metalloproteinases was only tested in culture using the inhibitor GM6100. To support this conclusion, it would be important to test if overexpression could rescue the Etv4 knockout phenotype either in culture and/or in vivo"*

Regarding to this point in the new version of the manuscript we have included a new experiment in which DRG neurons from WT and Etv4$^{-/-}$ mice were incubated in the presence of conditioned medium of HEK-293 cells transfected with plasmids overexpressing two of the main metalloproteinases regulated by Etv4: MMP3 and MMP13. We have included a new panel in Figure 5 showing these results. As the quantification of the experiment shows, the incubation of DRG sensory neurons with medium containing MMPs, reverts the reduced neurite outgrowth observed in Etv4 knockout neurons, indicating that these MMPs are important for the growth of these neurons in response to NGF.

2) The reviewer states *"RNAseq analysis comparing wild type and Etv4 mutant neurons would provide additional insights into the transcriptional regulation of sensory axon development"*.

We agree with the reviewer that it would be very interesting to make an RNA seq analysis comparing WT and Etv4 mutant neurons, but we could not include this type of analysis at the moment. It would be very interesting to find additional target genes for Etv4 which could be involved in peptidergic neuron innervation and pain sensation. In the present manuscript, our main objective was to analyze the physiological role of Etv4 in sensory neuron development, its contribution to nociceptive behavior and additionally to study the molecular mechanism that contribute to its function.

3) The reviewer says: *"the pain phenotype of Etv4 mice is the consequence of the two conditions, lack of a correct innervation and reduced expression of TrpV1 expression, or both. Rescue with TrpV1 expression might distinguish these possibilities"*.

It could be interesting to perform the experiment suggested by the reviewer, but this kind of *in vivo* experiment is technically difficult. We find that the only possibility to perform this would be to overexpress TrpV1 under a peptidergic driver in the DRG of Etv4-deficient mice and evaluate the pain behaviors in adult stages. So far, we didn´t succeed by doing *in utero* electroporation of DRG neurons in embryonic mice.
Because of this, in the discussion of our manuscript we stated that the deficits in pain sensation detected in Etv4 KO mice might be the consequence of the two conditions: lack of a correct innervation and/or reduced expression of TrpV channels.
Minor comments

1) In the new version of the manuscript we have analyzed the phenotype of non-peptidergic axons in the epidermis of Etv4-KO mice using antibodies against GFRα1/GFRα2 receptors, which labeled non-peptidergic DRG sensory fibers (Sakai et al 2017, De Vincenti et al 2021). Quantification of these fibers shows that the epidermal density of GFRa1/2-positive nerve fibers was unchanged between WT and Etv4-deficient mice. We have included this analysis in new panels of Figure 1.

2) In the new version of the manuscript, we have included a more complete analysis of the central innervation analyzing the central peptidergic fibers by TrkA staining. We didn´t find any difference neither in the area covered by TrkA positive fibers nor in the intensity of them in the spinal cord of WT and Etv4-KO mice. We have included this information in new panels of Figure 2. This new result support the absence of defects in central peptidergic innervation.

3) We agree with these observation of the reviewer and we have changed the title of the indicated section. We have change "Etv4 knockout mice do not show defects in $TrkA^+$ neurons" to "Etv4 is dispensable for DRG neuronal viability"

4) As the reviewer mentions it would be interesting to re-analyze the expression level of Nav1.8 including more animals, because the dispersion of the values obtained for mRNA expression Nav1.8 in WT animals, is high. However similar conditions and the same samples were used to evaluate the levels of the other channels shown in the present manuscript. In these conditions, our data indicates that Etv4 mediates only TrpV1/2 induction, which is in agreement with the reported induction of these channels in response to NGF and with the idea that Etv4 mediates the effects of NGF in DRG sensory neurons.

Reviewer #2

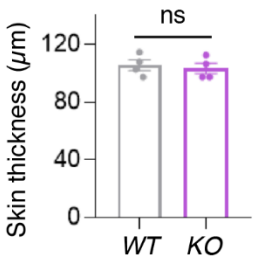
Main comment:

Regarding to the statistical analysis of Fig 4 and 5 we agree with the reviewer and we have changed the analysis as he/she suggested considering the number of independent experiments (n) and not the total number of cells analyzed. By changing the statistical analysis the results did not change in general terms (what was significant in the previous version remains significant in the new version) but the sample size (n) and the values of the significance (p value) have been modified.
We want to make clear that it was not our intention to conveniently consider a high number of *n* to get a high significance, it was performed that way to show in a graph all the individual measurements that we have obtained for each neuron in the different experiments, visualized as single dots.

Minor comments

1- In the new version of the manuscript, we have included the analysis of peripheral non-peptidergic innervation. We have performed staining of non-peptidergic fibers using anti-GFRα1 and GFRα2 antibodies. Our result indicates that there is no difference in non-peptidergic epidermal innervation. In the new version of the manuscript, we have included a new panel F in Figure 1 containing this information.

The reviewer mentions: "….they could normalize this by measuring skin thickness"
We have measured skin thickness between WT and Etv4-KO mice and we didn´t find differences between the different genotypes. We have included a sentence clarifying this point in the new version of the manuscript.



Comparison of Skin thickness between wild-type and Etv4-KO adult mice.

We have also analyzed the central peptidergic innervation using antibodies against TrkA and we didn´t find differences in the area of the spinal cord covered by TrkA-positive fibers, nor in the intensity of central projections between control and Etv4-KO mice. This information is included in new panels C and F in Figure 2 containing the analysis of $TrkA^+$ fibers innervating the spinal cord at postnatal stage. This new analysis of TrkA-positive spinal cord fibers supports the absence of defects in central innervation in Etv4-KO mice. However, we do not find explanation for the decrease in the CGRP intensity (but not in CGRP area) observed in the peptidergic fibers that innervate the spinal cord. Analysis of CGRP expression in DRGS by RT-PCR of WT and Etv4-KO mice did not show differences in their expression levels. As suggested by the reviewer, in the new version of the manuscript we mentioned the observed decrease in the intensity of CGRP.

2- *The reviewer mentions " In figure 1F-G, it would be more convincing to show the Etv4 channel alone (as done with TrkA channel shown in green). Also, it would be helpful to show a view of an entire DRG, to see if there are any Etv4+/TrkA-negative neurons as detected in the trigeminal ganglion"*
As the reviewer request we have replaced the images in Figure 1, showing the expression of Etv4 and TrkA in embryonic and postnatal DRG ganglia. In the new panel (Panel I) of figure 1 we have included images of an entire DRG ganglia at E14.5 and we show, separately and merged, the expression or Etv4 and TrkA respectively. We also include an image in higher magnification where it can be clearly observed that the majority of TrkA positive neurons express Etv4 in their nuclei. However, we indicated that the expression of Etv4 is not exclusively expressed in TrkA neurons. In a previous work we have described the percent of DRG neurons which express Etv4 during embryonic development (Fontanet et al 2013, J Neurosci). In the revised version of the manuscript we also replaced the image showing the expression of Etv4 and TrkA at postnatal stage. In agreement with the PCR results, the Etv4 protein expression decrease substantially but still there are some $TrkA^+$ cells which clearly express Etv4 as indicated in the new image.

3- *The reviewer mentions that "it would have been good to have confirmation of staining specificity with another method (and useful to others to include a validation on knockouts)".*
Following the suggestion of the reviewer, the specificity of the Etv4 antibody has been validated comparing the staining in tissue obtained from WT and Etv4-KO mice. We have included this control in a new supplementary figure (S1). We have also included a reference of our previous paper in which we controlled the specificity of the same anti-Etv4 antibody hippocampus of Etv4-KO mice (Fontanet et al 2018, Cerebral Cortex; Supplementary Figure 2). However, at postnatal stages (P20), we observed a non-specific staining of Etv4 that is indicated in the figure legend.

[NOTE: We have removed a figure which was provided for the referees in confidence.]

4- *The reviewer says "In FigS2, it would be helpful to comment that all of the TrkA+ neurons were Etv4+, and that there were no TrkA+/Etv4-, whereas an Etv4+/TrkA- was detected"*
In a previous work we have described that, at embryonic stages (E16.5-E17) 80.7± 1.5 % of TrkA-positive neurons expressed Etv4, and that aprox 80% of the Etv4 is localized in TrkA-positive neurons, supporting a relevant contribution of this factor to the biological response triggered by NGF during DRG development. Later in development (E17) we have also observed

expression of Etv4 in Ret-positive cells, this is also described in a previous paper (Fontanet et al 2013), indicating that the expression of Etv4 is not exclusive of TrkA-positive neurons.
In the present manuscript, we analyze the physiological consequences of Etv4 deficiency in peptidergic and non-peptidergic innervation and in the pain associated to these different types of innervation. We only found physiological phenotypes associated with peptidergic fiber development and we didn´t find any phenotype related with non-peptidergic neurons expressing Ret. This information has been included in the revised version of the manuscript.

5- *The reviewer suggests that " it would make sense to present the latency scale in a uniform manner rather than changing the scale according to the temperature tested"*
We don´t agree with the reviewer at this point. We consider it is better to maintain the scales of the different experiments as they are now because the differences or not between WT and KO animals in each experiment can be better visualized as they are. Thus, we prefer to maintain the scales relative to the values obtained in each experiment because the time of latency (in seconds) between the different conditions is quite different.

6- *The reviewer mentions "Since noxious cold sensing is not abolished and Trpm8 expression is preserved in Etv4$^{-/-}$ mice, it would make sense to discuss if these neurons are Etv4-negative"*
Regarding to this point, we have not analyzed in detail the expression or Etv4 in Ret^{+} DRG sensory cells. However, as the reviewer mentions the absence of cold-sensing defects in Etv4-deficient animals is in agreement with the absence of changes in Trpm8 expression in these mice. Moreover, in the new version of the manuscript we have analyzed the non- peptidergic innervation by staining with antibodies against GFR□ receptors which are expressed together with Ret in these fibers and we did not find differences between control and Etv4-KO animals. Altogether, these findings indicate that, at least in our analysis the absence of Etv4 do not impair non-peptidergic innervation and not affect mechanical pain transduction nor cold associated pain. In the new version of the manuscript we have discuss this point.

We went along the text issues mentioned by the reviewer and made the changes following the reviewer´s suggestion.

Reviewer #3

1) . *The reviewer says "Whether the loss of Etv4 affects specifically peripheral peptidergic neuron projections or whether it is also required for the outgrowth of other subtypes of nociceptive fibers is not investigated"* .
In order to analyze whether the loss of Etv4 affects specifically peripheral peptidergic neuron projections or whether it is also required for the outgrowth of other subtypes of nociceptive fibers, we have analyzed the non-pepetidergic innervation in control and Etv4 KO mice. As we mention previously, we have performed this analysis using antibodies against GFRa1 and GFRa2 receptors which are expressed in non-peptidergic fibers. Here, we didn´t find differences in non-peptidergic epidermal innervation between WT and Etv4 KO mice. In the new version of the manuscript we have included this results in the new figure 1.

2) *The reviewer states: "The data in panel C show that CGRP fluorescence intensity is decreased in the mutants by about 50%. As a control for this experiment, markers of mechanoreceptive or proprioceptive fibers, which are likely not modified in Etv4 mutants, could be tested. Could the reduced fluorescence be interpreted as a reduced density of CGRP-expressing fibers? These data should be discussed* in the manuscript".
As we previously mentioned, in the new version of the manuscript, we have reinforced the analysis of peptidergic central innervation using TrkA antibodies. We did not find difference in the area of the spinal cord covered by TrkA^{+} fibers nor in the intensity of them. This analysis is included in the new Figure 2, and support the idea that the reduced fluorescence intensity detected by CGRP in central fibers is not a consequence of a reduction in the number of peptidergic fibers innervating the spinal cord. However, we could not explain the decrease in the intensity of CGRP observed in central fibers. As the reviewer suggested we discuss this point in the Results section of the new version of the manuscript.

Regarding to the analysis of mechanoceptive and proprioceptive central innervation, although we consider that it is interesting to analyze them, in the current study we focus on the nociception based on the high co-localization of Etv4 and TrkA at embryonic stages previously described by our group (Fontanet et al 2013).

3) *The reviewer says: "The authors should better explain why these particular MMPs have been investigated and not others".*
We have analyzed the expression of some MMPs which has been previously described as potential targets of Pea3 transcription factors. In particular, the promotors of the MMPs analyzed have sites for Pea3 transcription factors. Moreover, there are several evidences, from our and other groups, that indicate that these MMPs are involved in the outgrowth of sensory neurons. This point has been clarified and extended by including references which support the selection of these particular set of MMPs.

4) *The reviewer mentions " The requirement of Etv4 for NGF induced expression of Upar is not convincing. The authors suggest that the downregulation of ECM remodeling proteins observed in DRG of Etv4 kO mice likely explains the observed axonal growth defects. This hypothesis could be tested in rescue experiments in PC12 cells"*

The induction of Upar in response to NGF has been previously described (Chen et al., 2008; Farias-Eisner et al., 2000). We have included these references in the new version of the manuscript. In our assay in sensory neurons we observed a substantial, but not significant increase, in the expression of *Upar* mRNA upon NGF stimulation in sensory neurons. However, we observed a significant difference when we compared the expression levels of *Upar* mRNA induced by NGF in control vs Etv4-KO mice, supporting that Etv4 mediates the expression of Upar in response to NGF.

Minor comments

We went along the text issues mentioned by the reviewer and made the changes following the reviewer´s suggestion and we have included the reference indicated by him/her.

-Regarding to the statistical analysis we have analyzed the normal distribution of all individual values (the variable) in each independent experiment. In example, for the variable: neurite length we have evaluated normal distribution (considering 30-100 neurons, depending on the experiment) with the Shapiro-Wilk test, and then we performed the Student t test with the mean value of each independent experiment.
Only when we analyzed the level of mRNA of the different ion channels we have directly assumed normal distribution of the variable, which come from a pool of DRG ganglia of 3 independent animals/genotype. This is because the *n*, in this case, is too small to test for normality. As the Mann Whitney test has little statistical power with small sample sizes (n=3), for RT-PCR experiments, the majority of the studies make the assumption of normality and test significance by using Student t test. For this reason, here we have used sample sizes (n= 3 mice/genotype), and performed Student t test to analyze significance. This has been clarified in the new version of the manuscript.

Second decision letter

MS ID#: DEVELOP/2022/200583

MS TITLE: Etv4 regulates nociception by controlling peptidergic sensory neuron development and peripheral tissue innervation

AUTHORS: Antonella S Rios, Ana Paula De Vincenti, Mailin Casadei, Jorge B Aquino, Pablo R Brumovsky, Gustavo Paratcha, and Fernanda Ledda

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.
The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Referee 2 asks for several clarifications that should be straightforward to deal with. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

Reviewer 1

Advance summary and potential significance to field

The study of the Etv4 mouse mutants has identified several interesting phenotypes related to nociception behaviors and peptidergic sensory axon growth/development. It provides a potential link between the role of transcriptional regulation, axon growth and channel expression, and sensory axon function.

Comments for the author

The revised manuscript by Rios et al has addressed most of the questions raised in the previous review. Although the mechanistic connections are yet to be fully established in vivo, the various phenotypes (in nociception, innervation, gene expression) observed in the Etv4 mutant mice are interesting to many people in the field and the proposed model will provide a new step forward in understanding the transcriptional control of sensory neuron development.
Reviewer 2

Advance summary and potential significance to field

The authors have done a good job in answering most of the reviewer's concerns. Regarding my specific comments on the statistics, the changes adequately answer my comments. I also appreciate the addition of experiments attempting to rescue the Etv4 phenotype by exogenously supplying MMP3 and MMP13 in the cultures of DRGs (via conditioned medium). Although I agree that it would have been nice to have a rescue of the Trpv1-associated capsaicin-sensitivity phenotype, achieving this is technically more challenging in vivo than the in vitro MMP rescue, and such an experiment should not be mandatory for accepting the paper.

Overall, I believe the manuscript has been improved and should be acceptable for publication, even though I still have a number of small comments. I provide a pdf with first my main comments, followed by the manuscript text with suggested changes highlighted in yellow.

Comments for the author

Summary

The authors have done a good job in answering most of the reviewer's concerns. Regarding my specific comments on the statistics, the changes adequately answer my comments. I also appreciate the addition of experiments attempting to rescue the Etv4 phenotype by exogenously supplying MMP3 and MMP13 in the cultures of DRGs (via conditioned medium). Although I agree that it would have been nice to have a rescue of the Trpv1-associated capsaicin-sensitivity phenotype, achieving this is technically more challenging in vivo than the in vitro MMP rescue, and such an experiment should not be mandatory for accepting the paper.

Overall, I believe the manuscript has been improved and should be acceptable for publication, even though I still have a number of small comments. The most important are written here, while I have included all minor text changes provided below my comments (highlighted in yellow).

Recommendations to Authors

Main comments:

1) Since the analysis of central projections revealed a lowering of CGRP level (figure 2E), possibly reflecting a lowered mRNA level (not investigated), I'm wondering if by using CGRP to detect peripheral axons, there isn't a bias in the detection threshold. It is not clear from the images shown in Figure 1 (at the magnification level chosen), if detectable signal represents one or several axons, and if detection is conditioned by a certain threshold in number of axons in a bundle. Using an independent marker to confirm the observations would have been more convincing. Since the authors have used TrkA to analyze central projections (Fig2.C and F), they could have used it as well to analyze peripheral terminals, and the manuscript would benefit from showing both results (as images). TrkA is adequate as an independent marker, as it is upstream of Etv4 and therefore unlikely to be affected by Etv4-deficiency.

2) Has the change in CGRP expression level also been detected by RT-PCR?

3) Given the change in central CGRP signal intensity, the conclusion (lines 176-177) that "Thus, together these findings suggest that Etv4 mainly affects peripheral peptidergic innervation." may be overstated. If CGRP lowering occurs both peripherally and centrally, and if other markers confirm the reduction in length and density of the peripheral but not central terminals, then the authors could conclude that aside from a lowering of CGRP expression levels, loss of Etv4 affected the morphology and density of peripheral but not central axons of peptidergic neurons.

4) The images chosen for Figure 1F (GFRa1/2) do not seem representative of the result in 1G (lack of change), as aside from the number of white arrows, the change in density seems visually relatively similar to the one seen in Figure 1B for CGRP.

5) As a conclusion to the first part (lines 197-199), I would add that the change in peripheral innervation observed in Etv4 mutants is therefore attributable neither to cell death nor to a cell fate conversion/change/phenotype.

6) For the part assaying axonal growth in cultured neurons, all the cultures have been done in the presence of NGF, and the authors did not compare conditions with and without NGF. In this context, it is not possible to conclude that Etv4 is required for the growth induced by NGF, but only that Etv4 is required for the neurite growth of neurons surviving in presence of NGF. The authors did compare conditions with and without NGF when evaluating gene expression levels in PC12 cells (fig. 5C), and primary neurons (Fig. 5E). While their results support the idea that Etv4 is required for induction by NGF of MMP3, MMP13 and MMP10, the same cannot be concluded from the Upar mRNA data for the following reasons:

Regarding Upar expression level (Figure 5E, last graph), having had n=4 (or more) in both groups would probably have helped improving statistics. But in the present situation (n=3), the increase in Upar expression induced by NGF in WT is not significant. Thus, even if the Upar mRNA level in presence of NGF is significantly lower in Etv4-ko than in wt, it is also lower in absence of NGF. In this case, the statistics do not allow you to conclude that Etv4 is required for the induction of Upar expression by NGF, it only allows you to conclude that Etv4 is required for promoting Upar expression in these neurons. If the authors want to maintain this conclusion, then they would need to increase the number of independent cultures. Otherwise, they should amend the conclusion to match available experimental evidence.

7) Regarding the rescue experiment in vitro, assaying axonal length in WT and Etv4-deficient neurons in absence or presence of exogenously supplied MMP3/13, what I see in Figure 5F is that addition of MMPs allows reaching the level of control neurons in absence of MMPs. It's not clear if MMPs induce an additional increase in neurite length in WT neurons. The images on suggest a 2 fold difference, whereas the graph suggests a more modest difference (visually around 1.3 fold change) with no indication whether this change is significant or not. I suggest amending the conclusion with the following sentence: Our results indicate that these MMPs were able of compensating the outgrowth deficiency exhibited by Etv4-deficient neurons, supporting the idea

their induction by Etv4 is necessary for the Etv4-mediated axonal growth of NGF-dependent sensory neurons.

It would have been good to assess in these same experiments whether the growth cone area also returned to WT size upon addition of exogenous MMPs or not. If yes, then the two proteins may be sufficient to account for this too, whereas if not, the authors could have concluded that other Etv4 targets may be required to sustain growth cone size.

Minor comment:

1) Make sure to correct all genes in italics, and proteins in non-italic, capital letters.

Reviewer 3

Advance summary and potential significance to field

We are satisfied that the authors have addressed most of our concerns and consider the manuscript as it is now acceptable for publication. We would like however to
stress again that parametric tests have been used to assess statistical significance of the data collected, while non-parametric ones would have been more appropriate given the small number of biological replicates per conditions.

Line 151 : please correct « pepetidergic » .

Comments for the author

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Second revision

Author response to reviewers' comments

Thanks to the reviewers for the positive comments on our work and for their suggestions that have improved the manuscript.

Regarding the comments of the reviewer 2.
1, 2, 3) The reviewer mentions:
- “I´m wondering if by using CGRP to detect peripheral axons, there isn´t bias in the detection threshold. It is not clear if detectable signal represents one or several axons”.
Regarding to this point, we have used identical settings to take and analyze the images corresponding to WT and KO mice. In order to quantify the number of fibers, we have measured the CGRP free nerve endings crossing the dermal-epidermal border. We didn't observe a differential formation of axons in bundle between Etv4 and KO mice.

-The reviewer says: “Since the authors have used TrkA to analyze central projections, they could have used it as well to analyze peripheral terminals”.

We tried to use another marker, such as TrkA to analyze the peripheral innervation, but unfortunately the skin keratinocytes present a strong reaction against this antibody that did not allow us to visualize the fibers innervating the epidermis.

- The reviewer suggests analyzing the levels of CGRP by RT-PCR in Etv4-KO and control animals.
We have analyzed the *CGRP* mRNA level in the ganglia of Etv4-KO and WT animals by RT- PCR. Our data indicate that there are no differences in *CGRP* mRNA levels in Etv4-KO compared to control mice (n=3 animals per genotype), indicating that CGRP is an adequate marker to study the innervation in our system. Here, we are including a figure showing this data (Rebuttal Fig 1). We have included this information in the manuscript as data not shown. If the reviewer or the editor consider it necessary, we can include this control experiment as a supplementary figure.

Thus, this finding reinforces those indicating that the differences we observed in the skin innervation are due to changes in the axonal growth of sensory fibers into the epidermis. Taken into consideration the reviewer suggestion, we modified the general conclusion of this section in the new version of the manuscript for "Thus together these findings suggest that Etv4 affects the morphology and density of peripheral but not central axons of peptidergic neurons", which we consider is more appropriate than the previous one.

[NOTE: We have removed a figure which was provided for the referees in confidence.]

1) The reviewer states that "the image chosen for Figure 1F (GFRa1/2) do not seem representative of the result in 1G (lack of change between Etv4-KO and control mice), as aside from the number of arrows, the change in density seems visually relatively similar to the one seen in Figure 1B for CGRP".
We thank the reviewer for this indication. We have replaced the images of GFRa1/GFRa2 staining in Etv4-KO mice (Fig.1F). In this new image the number of GFRa1/2^{+} fibers observed in the epidermis in Etv4-KO (indicated with arrowheads) is similar than the number of fibers observed in control mice. We consider that this new image better reflects the absence of difference in the non-peptidergic innervation between Etv4-deficient and control animals.

2) The reviewer suggests including a conclusion to the paragraph in which we described that Etv4 is dispensable for DRG neuronal viability. We thank the reviewer for this comment. Following his/her suggestion, we have included the suggested sentence in the new version of the manuscript.

3) Following the suggestion of the reviewer, in the description of the experiments in which we evaluate in vitro outgrowth of sensory neurons coming from Etv4-KO or control animals maintained in the presence of NGF, we have clarified that Etv4 is required for the neurite growth of neurons surviving in the presence of NGF (or NGF-responsive neurons or NGF- dependent neurons) but not induced by NGF, as in these assays we do not compare the conditions with vs. without NGF.

Regarding to the effect of NGF and Etv4 on *Upar* mRNA induction, the reviewer says "….it only allows you to conclude that Etv4 is required for promoting Upar expression in these neurons"

We have reformulated the conclusion about this molecule, and in the new version we only conclude that "Etv4 is required for promoting Upar expression in NGF-responsive sensory neurons".

4) Regarding to the rescue experiments we have included the sentence suggested by the reviewer in the conclusion "Our results indicate that these MMPS were able of compensating the outgrowth deficiency exhibited by Etv4-deficient neurons…..."

Regarding to the measurement of the growth cone area in the rescue experiments, we have done this new experiment on plastic wells and not on glass coverslips, and we couldn´t get good images under the confocal microscope to analyze the growth cones area appropriately. We consider this is an interesting point and we will continue analyzing the effect of Etv4 in the expression of molecules involved in growth cone remodeling.

Regarding to the minor comments we went through all the suggestions made by the reviewer that are indicated in the new version containing the track changes in red.

Third decision letter

MS ID#: DEVELOP/2022/200583

MS TITLE: Etv4 regulates nociception by controlling peptidergic sensory neuron development and peripheral tissue innervation

AUTHORS: Antonella S Rios, Ana Paula De Vincenti, Mailin Casadei, Jorge B Aquino, Pablo R Brumovsky, Gustavo Paratcha, and Fernanda Ledda
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.