

Targeting Prostaglandin E2 Receptor 2 in Schwann Cells Inhibits Inflammatory Pain but not Inflammation

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this manuscript, Nassini et al present an interesting finding that EP2 in Schwann cells (SCs) is critical to sustain PGE2-induced mechanical allodynia. They used both pharmacological blockade and genetic knock-down to target EP2 of SCs, and further delineated the cellular mechanism in SCs. Although the PGE2 associated mechanism has been well known with a body of literature published more than a decade ago, the contribution of SCs is novel. In light of a recent report that SC-released PGE2 maintain normal excitability of sensory neurons during development (Kantarci et al., Cell, 2024), the study may potentially expand the scope of the contribution of SCs to nociception. However, I have serious concerns regarding technical issues and the strength of the evidence in support of their key claims.

Scientific comments:

1. As the efficacy of pharmacological blockade and genetic knock down might be limited by some untoward side effects, the authors need to provide rigorous controls to support their claims. Notably, it was previously reported that intraplantar injection of EP3 or EP4 but not EP1 or EP2 antagonists, inhibited PGE2-induced paw-licking (Kassuya et al., BJP 2007). In contrast, in current study, blocking EP3 with L-798,106 did not affect non-evoked behavior (Fig. S1s). This discrepancy likely results from the specificity of the small molecules which form the foundation of current study. Moreover, Ptges3 is expressed actively in sciatic nerve of adult mice (Kantarci et al., Cell 2024), and the authors demonstrated that functional PGE2 downstream pathways exists in SCs. However, endogenous PGE2 is unlikely to induce mechanical allodynia unless exogenous PLAA or AA was used (Fig. 2). Therefore, the finding may not be physiologically relevant. Thus, specific deletion using conditional knockout mice is critical to address the discrepancies in the literature. Notably, both EP2 (Johansson et al., J Neurosci 2013; Liu et al., PNAS 2019) and EP4 (JAX) flox lines are already available.

2. With their best efforts, the authors used AAV-shRNA approach to selectively to knock-down Ptger2 and Ptger4 expression in Plp+ cells. Although Plp-scramble was tested in vivo, it was not used as a control in main figures and compared directly with the behavior of Plp-shPtger2 mice. Instead, WT mice without carrying Cre or CreER transgene were used. Moreover, the backbone AAV vector for scramble RNA ("pAAV[FLEXon]-CMV LL-rev(EGFP-5' miR-30E-BfuAI ORF_44bp BfuAI-3' miR-30E)- rev(LL)-WPRE") seems to be different from the shRNA vector ("pAAV[FlexOn]-CMV-EGFP-[shRNA]-mCherry"). This non-standard practice of shRNA knock-down strategy may impact the behavior outcomes after transduction.

3. Importantly, it was not clear if a good transduction was achieved. Presenting % of lineage specific marker among GFP+ cells (Fig. S3 and S4) will not address this. Instead, %GFP+/Sox10+, %GFP+/S100+; and %GFP+/NeuN+ should be presented. Moreover, it was noted that ~ 30% transduced cells (GFP+) in PlpCreER+ sciatic nerves were neither SOX10+ nor S100+ (Figs. S3e/g); and 40% of GFP+ cells in the AdvillinCre DRG were not NeuN+ (Figs. S4c-d). The results certainly raise the concerns of non-specific targeting and support the rationale to use conditional knockout mice.

Critiques on writing:

It is "painful" to go through this manuscript due to its substandard writing and weakness in interpreting the literature. The authors should have carefully combed through this manuscript before their submission. Otherwise, the readers would certainly doubt the teams' rigor in their efforts.

1. Introduction: the authors missed an opportunity to better introduce the relevant background/concepts and explain the study rationale. After highlighting the challenges in studying EP4, they abruptly drifted to their EP2 findings without further EP4 coverage. It is confusing to readers. A minor comment, abbreviation of Schwann cells was directly used without first mentioning.

2. Line 94-97 of Results: References #3-5 were cited to support that "we assessed the two temporally distinct responses reported in previous studies3-5...". However, Ref #4 is a review; both Ref #3 (rat nociceptive behavior induced by burn injury and formalin) and #5 (e-phys) have nothing to do with PGE2-induced nociceptive behavior. In addition, the authors

concluded that mechanical allodynia was “delayed” after spontaneous nociceptive behavior evoked by intraplantar PGE2 injection. However, this argues against the literature: the change in paw withdrawal threshold was documented within 2-10 mins after the intraplantar injection of PGE2 (Taiwo Y et al., Brain Res 1987; Neuroscience 1989).

3. AKAP:

a. Line 307/Fig. 4: It is confusing to the general audience with limited knowledge of AKAP as it was not mentioned that AKAP79 is a human orthologue and AKAP150 for rodents.

b. Line 332: after testing Plp-AKAP150-bPAC mice, the authors then started to introduce the experiment of selective silencing of SC AKAP150 in Plp-AKAP150 mice without describing how. I assume shRNA was used but could not find a definitive answer. After extensive searching, I was finally able to locate mAkap5 shRNA sequence listed in Methods. However, it was not explained that Akap5 is also known as Akap79.

c. In figure legends of Figs. 4m-n, which cDNA (human or murine) used in “AAV-AKAP79-LAPD (Plp-AKAP150-LAPD)” should be clarified although they share 93% homology.

It is possible that errors may occur during uploading or transferring from a sister journal which is beyond authors’ control. I then searched and found the identical copy of this manuscript from bioRxiv. Overall, this is a poorly written manuscript lack of well-controlled studies.

Minor comments

1. NSIADs remain to be life-saving and the side effects are serious but manageable. In addition, the claim that NSAID delays pain resolution was mainly derived from a CFA model which is not clinically relevant. Thus, authors may consider toning down the claim in Abstract.

2. As both AdvillinCre and AdvillinCreER lines are available, to minimize the confusion, please indicate that it was PlpCreER line used in this study.

3. Figs. 1h/l; 3g/k; 4d/e and S10e/h: please change “BCG” to “BGC”.

4. Fig. 1j vs Fig. 1i: qRT-PCR revealed higher-level Ptger2 expression in mouse sciatic nerves. However, antibody staining suggests otherwise.

5. Adding forskolin, a direct activator of adenylate cyclase, into primary Schwann cell culture, is common for promoting survival. As cells were already primed by forskolin, the results of cAMP sensor, cADDIs, to monitor cAMP production should be cautiously interpreted.

6. Although it is not discussed, PGE2-evoked thermal pain hypersensitivity can be used as an independent control to support SC-TRPA1 function.

7. Figs. 4b-c/4m-n and Figs. S11g-i: as red light may arguably have analgesic effect (Cheng et al., J Pain 2021), the appropriate control should be Control/light/(Cg/ CFA; PGE2/PLAA/AA).

8. It needs to be mentioned that Plp is also expressed in satellite cells (Avraham et al., Nat Comm 2020; Tiwari et al., Cell Report 2024).

Reviewer #2

(Remarks to the Author)

In this paper, Nassini et al. focus on early pain and delayed allodynia involving PGE2 and systematically investigate the PGE receptors involved, using pharmacological and genetic approaches. As a result, the authors found that Schwann cell (SC) EP2 receptor is involved in delayed allodynia, but not in the elicitation of inflammatory response itself, and that EP2 receptor stays at the plasma membrane without showing ligand-dependent internalization, contributing to cAMP production in the vicinity of the plasma membrane (nanodomain). They further proposed that this PKA activation within this nanodomain may cause allodynia, presumably by causing Trpa1 channel activation and H2O2 production, sensitizing nerve stimuli adjacent to the SC. These results suggest the possibility that PGE2-elicited two responses, delayed allodynia and increased inflammatory responses can be separately controlled, providing a new perspective on pain regulation. Furthermore, the role of cAMP nanodomains in SC, as demonstrated by the elaborate optogenetics in this study, contributes to our understanding of the pathogenesis of neuropathic pain. Therefore, this reviewer believes that this paper contains new findings that could potentially be acceptable, if several concerns shown below should be addressed.

Major concerns

1) The key to EP2, but not EP4, controlling the cAMP nanodomain is that EP2 stays at the plasma membrane without showing internalization. In this regard, for example, if EP2 and EP4 were exchanged only in the C-terminus, would the response of the cAMP nanodomain be opposite? If the authors can add structural biology results of EP2/EP4 receptors, it would provide a more profound insight.

2) In relation to the above, is it possible that AKAP79/150 tethers EP2 to the plasma membrane? It would be interesting to see if there is a difference in their affinities between EP2 and EP4 using NanoBit.

3) The EP2 receptor, cAMP nanodomain, PKA activation, and the scaffold function of AKAP have been well demonstrated by a series of analyses, but the molecular mechanism from Trpa1 activation to H2O2 production is unfortunately unclear. For example, it would be helpful if the authors could show the effects of TRPA1 phosphorylation by PKA on Ca response and H2O2 production.

4) What molecules produce H2O2? And how do sensory nerves recognize H2O2?

5) In Fig. S5, f and g, if it is good that knockdown of EP4 in DRG decreases acute pain elicited by an EP4 agonist, why does

allodynia is weakened by Adv-EP4 knockdown?

6) It is very important to examine endogenous PGE2 production within tissues treated with PGE2, PLAA, AA, Cg and CFA (in Fig. 2e and Fig. S7a). In the PGE2-EP2 or PGE2-EP4 signaling, endogenous PGE2 levels are often increased by positive loop of EP2/cAMP/PKA/COX pathway, and therefore, the authors should examine whether EP2 or EP4 agonist administration increases endogenous PGE2 levels.

Reviewer #3

(Remarks to the Author)

In their manuscript titled “Targeting the Schwann Cell EP2/cAMP Nanodomain to Block Pain but not Inflammation”, the authors investigate the mechanism of Prostaglandin E2 (PGE2)-evoked pain and protective inflammation. Overall, they find that Schwann cell EP2 receptor activation is responsible for mechanical allodynia observed after PGE2 injection or in clinical inflammatory models, but this mechanism is not associated with inflammation. The authors first show that local intraplantar injection of PGE2 into the mouse hind paw resulted in a non-evoked nociceptive and a delayed allodynia that were mediated by EP4 and EP2 receptors, respectively. They then showed that Schwann cell (SC) EP2 receptors are responsible for sustained allodynia, which was reduced in mice with SC-specific EP2 knockdown. The authors have several exciting discoveries that increase the mechanistic understanding of SC-EP2 driven allodynia with a series of very elegant experiments. First, the authors show that optogenetic stimulation of focal cAMP production in Schwann cells induces mechanical allodynia and that SC-EP2 driven allodynia is mediated by membrane-localized PKA activation. They further identified the downstream mechanism with their finding that the AKAP79/150 scaffold protein induces cAMP/PKA activation in the membrane. Blocking the AKAP79-PKA interaction via the peptide inhibitor st-Ht31 inhibited allodynia. Inhibiting AKAP/PKA interaction in SCs also reduced carrageenan- and CFA-induced allodynia. Lastly, the authors demonstrated that selective silencing of SC EP2 was able to produce an early and sustained attenuation of allodynia without reducing inflammation.

Overall, we are very excited with the discovery of Schwann cell driven mechanical allodynia in clinical inflammatory models or following PGE2 injection. Most studies in the literature solely focus on the role of EP receptors in neurons and studies that describe the role of EP receptors in Schwann cells are scarce. The finding that inhibiting Schwann cell-EP2 driven response alleviates mechanical allodynia but allows recovery through inflammation is very intriguing and novel. This marks the Schwann cell driven pathway as an important therapeutic target to remove persistent inflammatory pain while sustaining the protective and regenerative inflammatory mechanisms. The authors also use a breadth of experiments to test their findings with appropriate controls, and their experiments are conducted with rigor, proper animal numbers, and the difference in sexes is explained clearly. Overall, we are very excited about these findings and strongly support this study. For improved interpretation of results, we suggest the authors address the following:

Major:

1. There does not appear to be complete AAV-mediated silencing of EP2 or EP4 in DRGs (Supplementary Fig. 4a and b). Although there is a decrease by half in expression levels, the remaining receptors can still be a confounding variable in the interpretation of results. We recommend addressing this as a shortcoming of this study. We agree that obtaining a new mouse line with floxed alleles of *Ptger2* for specific knockout in Schwann cells may be beyond the scope of this manuscript, however, discussing this point in the manuscript is helpful.
2. To further verify that AAV-silencing has no off-target effects, it would be helpful to quantify whether AAV injections cause any changes in the total number of DRGs or SCs. We also would like authors to consider adding quantification of myelin, at least through showing Myelin basic protein staining, following injections into the sciatic nerve.
3. In supplementary figures 13 and 14, the authors describe different mechanisms of TRPA1 activation in neurons versus Schwann cells. The authors find that knocking down TRPA1 in DRG neurons attenuates allodynia, knocking down TRPA1 in Schwann cells also alleviates allodynia. If the authors could include a diagram of downstream signaling in DRG axons with different paradigms versus the one in Schwann cells, this would be helpful in describing the differences in TRPA1 channel activation.
4. Is it possible to stain for endogenous AKAP79/150 and PKA to detect the localization of the endogenous proteins in the Schwann cell membrane in vivo? This will help to establish an orthogonal method for the mechanisms suggested by the overexpression of full length Akap5 constructs.
5. We noticed that separate COX inhibitors were used throughout the manuscript, indomethacin and diclofenac. It would be helpful to know why each was used in their respective experiments. It would also be more rigorous that the results are the same using separate COX inhibitors, and that the results are not specific to only one drug.

Minor:

6. It would be helpful to include a table of the various activators/inhibitors/vectors used throughout the manuscript for easy reference.
7. In addition to the mRNA level quantification of the target EP receptors, it would be helpful to also include the quantification of protein levels from the immunostaining experiments. Having descriptive quantification will supplement the images used to

show decreases in the expression of the receptors.

8. The authors state that “several findings suggest that SC PKA/TRPA1 phosphoactivation by EP2/cAMP sustains calcium-dependent oxidative stress signal” elicited through H₂O₂ formation. It would be helpful to include citations that show H₂O₂ induces mechanical allodynia.

9. The authors mention that the non-specificity of antagonists in previous studies precludes the identification of EP receptor subtypes involved in inflammatory pain. How did they test the effectiveness and specificity of the inhibitors used in their study? A discussion of how these agonists and antagonists and their concentrations were determined and supporting data showing their specificity would be helpful.

10. The authors show that SC-EP2 knockdown doesn't attenuate non-evoked pain after PGE₂ injection, but it seems to do so in CFA or Carrageenan models. Is that due to a different behavioral test? Does PGE₂ injection cause grimace behavior?

11. Since authors are directly testing PGE₂ receptors, throughout the text I think it would be clearer to change “PG-mediated” to “PGE₂-mediated”.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this resubmission, the authors have addressed many of my concerns from the prior review, with new control experiments or justifiable reasons to power their conclusions. However, a few important issues remain unresolved.

1. The authors emphasized that “clarification of the ambiguous findings produced with older non-specific agonists/antagonists, is the primary contribution of our study”. They demonstrated that BGC20-1531, an EP4 antagonist which was first described in 2009, “produced marginal reduction of PGE₂-evoked allodynia” (Fig 1i; Fig S1k). It failed to reduce both mechanical allodynia and paw edema induced by Cg (Fig S8c) or CFA (Fig S8d). Notably, several newer EP4 antagonists were subsequently developed. EP4 antagonist 74/77 identified through computational docking significantly reduced mechanical allodynia induced by PGE₂, Cg or CFA (Gahbauer S et al., Nat Comm 2023). Furthermore, this compound was found to have anti-inflammatory property which significantly reduces CFA-induced paw edema. The conflicting result in the literature further heightens my concerns about “the specificity of the small molecules which form the foundation of current study” and empowers my prior comment that “specific deletion using conditional knockout mice is critical to address the discrepancies in the literature”.

2. Intriguingly, conditional deletion of TRPA1 in either SCs (Plp-Trpa1 mice) or sensory neurons (Adv-Trpa1 mice) reduced mechanical allodynia evoked by PGE₂ (Fig S13j; Fig S14 m-o), Cg or CFA (Fig 4o-p; Fig S14 m-o). The anti-allodynic effect was similar when compared to the treatment of indomethacin (Fig S7 h-m and Fig 2i-j). Zappia KJ et al (eNeuro 2017) suggested that non-neuronal TRPA1 is not sufficient to maintain mechanical sensory function. The increased injury-induced expression of TRPA1 (protein and mRNA) in the DRG within 24 hrs following CFA injection is also well documented (Obata K et al., JCI 2005; da Costa et al., PAIN 2010; Zhang Q et al., Cell Reports 2023). It is less likely that the CFA-evoked mechanical allodynia mainly results from “TRPA1-dependent ROS release (from SC) that targets neuronal TRPA1”. Other factors or alternative explanations merit considering for the data interpretation.

3. Line 441, “Although NSAIDs transiently inhibit inflammatory pain in patients, they markedly delay its resolution. This delayed resolution of pain, recapitulated in the CFA mouse model of inflammatory pain by treatment..” This statement is misleading. Parisien et al used statistical modelling to conclude that individuals with acute back pain were at increased risk of developing chronic back pain if they reported NSAID usage. However, to confirm this, they need a clinical trial with appropriate controls, a clinical gold standard. Instead, the supporting evidence came from a mouse model of inflammatory pain. Thus the statement should be revised to “Although NSAIDs transiently inhibit inflammatory pain in patients, they markedly delay its resolution in the CFA mouse model of inflammatory pain..”.

Minor comments:

--In Fig S3a: DRG staining with EP2 was mislabeled as “EP4”.

Reviewer #2

(Remarks to the Author)

The authors responded appropriately and courteously to this reviewer's concerns. Congratulations on an exciting paper that will make a significant contribution to our understanding of the long-unknown mechanism of prostaglandin-induced pain.

Reviewer #3

(Remarks to the Author)

We thank the authors for their revisions, additional experiments and clarifications that address our previous concerns. We are excited for the publication of this very interesting manuscript investigating the role of Schwann cell–EP2 signaling in inflammation and mechanical allodynia.

We suggest three minor edits for the manuscript:

1. In the introduction section, authors highlight the conflicting reports on EP4-signaling in mechanical hypersensitivity; but there is little mention of previously studied roles for EP2-signaling in inflammation, mechanical allodynia and pain. We suggest addition of such background information related to previous studies and inclusion of related citations.
2. For the audience who may be unfamiliar with the role of TRPA1 in pain, a citation for the statement provided in Line 365 may be beneficial.
3. The inclusion of the reagent tables in the Supplementary Info was very helpful. It would be more beneficial if the tables also included the catalog numbers for the reagents.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have addressed my prior concerns thoroughly. The addition of the new antagonist data and discussion on the study limitation has substantially improved the manuscript to support their conclusions. I recommend this article for publication.

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript, Nassini et al present an interesting finding that EP2 in Schwann cells (SCs) is critical to sustain PGE2-induced mechanical allodynia. They used both pharmacological blockade and genetic knock-down to target EP2 of SCs, and further delineated the cellular mechanism in SCs. Although the PGE2 associated mechanism has been well known with a body of literature published more than a decade ago, the contribution of SCs is novel. In light of a recent report that SC-released PGE2 maintain normal excitability of sensory neurons during development (Kantarci et al., Cell, 2024), the study may potentially expand the scope of the contribution of SCs to nociception. However, I have serious concerns regarding technical issues and the strength of the evidence in support of their key claims.

Scientific comments:

1. As the efficacy of pharmacological blockade and genetic knock down might be limited by some untoward side effects, the authors need to provide rigorous controls to support their claims. Notably, it was previously reported that intraplantar injection of EP3 or EP4 but not EP1 or EP2 antagonists, inhibited PGE2-induced paw-licking (Kassuya et al., BJP 2007). In contrast, in current study, blocking EP3 with L-798,106 did not affect non-evoked behavior (Fig. S1s).

This discrepancy likely results from the specificity of the small molecules which form the foundation of current study. Moreover, Ptges3 is expressed actively in sciatic nerve of adult mice (Kantarci et al., Cell 2024), and the authors demonstrated that functional PGE2 downstream pathways exists in SCs. However, endogenous PGE2 is unlikely to induce mechanical allodynia unless exogenous PLAA or AA was used (Fig. 2). Therefore, the finding may not be physiologically relevant. Thus, specific deletion using conditional knockout mice is critical to address the discrepancies in the literature. Notably, both EP2 (Johansson et al., J Neurosci 2013; Liu et al., PNAS 2019) and EP4 (JAX) flox lines are already available.

As highlighted by the Reviewer, clarification of the ambiguous findings produced with older non-specific agonists/antagonists, is the primary contribution of our study. As an example of previous uncertainties, we reference the paper by Birrell, M.A. & Nials, A.T. in the *Br J Pharmacol* (PMID: 21595650): ‘*At last, a truly selective EP₂ receptor antagonist*’. In our hands, and in contrast with Kassuya et al. (2007, PMID: 17310141), our EP3 selective antagonist (L-798,106) failed to reduce PGE2-evoked transient (licking/lifting) and sustained (mechanical allodynia) pain-like responses. This is not surprising, considering the extensive literature that consistently shows that PGE2-evoked pain is initiated by increased intracellular cAMP (Taiwo et al., 1989, PMID: 2557557; Taiwo and Levine, 1991 PMID: 1722888; Pitchford & Levine, 1991, PMID: 1724065; Khasar et al., 1995, PMID: 7753383). The solidly established observation that EP2 and EP4 receptor engagement is positively coupled with adenylyl cyclase activity strongly supports their role in PGE2-evoked pain-like responses. In contrast, as EP3 reduces adenylyl cyclase activity (Moreno JJ, 2017, PMID: 27940058), its implication in PGE2-evoked and cAMP-mediated pain signals seems very unlikely. To finally disprove that EP3 enhances inflammatory pain, Natura et al. reported in *PNAS* (2013, PMID: 23904482) that EP3 activation was able to reduce, rather than increase, pain-like responses evoked by PGE2. See the new sentences at page 6, line 3.

The very recent observation published by Cell (Kantarci et al., 2024, PMID: 39142281) that Schwann cells, expressing Ptes3, release PGE2 to promote Nav channel expression and DRG

neuron sensitization, pertains to a maturational function, prominent in embryonic development, and independent from inflammation. In fact, Kantarci et al. (2024, PMID: 39142281) reported: ‘..... suggesting the excitability-inducing effect did not result from an inflammatory response in Schwann cells’. In our models, we measured and reported mechanical hypersensitivity with the von Frey filament at 30 min (PGE2 and carrageenan, respectively) post-injection, but as correctly underlined by the Reviewer, the response to PGE2 assessed with the paw pressure test could be documented at earlier time points (2-10 min) (Taiwo et al., 1987, PMID: 2823989 ; Taiwo et al., 1989, PMID: 2557557). These quick changes are, however, incompatible with the PGE2-mediated transcriptional processes reported by Kantarci et al. (2024, PMID: 39142281). Finally, under inflammatory conditions (carrageenan or CFA), the PGE2 most likely derives from a large variety of cells of the inflamed milieu, while the contribution of SCs may be minimal, if any.

Inflammatory response requires a local, on-demand increase in prostaglandin tissue levels, that can be recapitulated by the injection of different agents: the prostaglandin precursors (AA); the enzyme that activates the prostaglandin cascade (PLA); inflammatory mediators (carrageenan, CFA). Although different prostaglandins may be released after AA or PLA injections, the observations that both agents increased PGE2 tissue level (Fig. 2) and selective EP2 antagonism or *Ptgr2* silencing in Schwann cells abated the indomethacin-dependent mechanical allodynia support the view that PGE2 is the main, if not the sole, mediator of this response. Further, the ability of selective EP2 antagonism or Schwann cell *Ptgr2* silencing to attenuate mechanical allodynia and grimace response to the proinflammatory agents, carrageenan and CFA, underlines the pathophysiological role of PGE2 in these pain responses. The extensive use of carrageenan and CFA models (Fehrenbacher et al., 2012, PMID: 22382999; Fischer et al. 2017, PMID: 28666464; Seibert et al., 1994, PMID: 7991575) to study prostaglandin-induced inflammation and NSAIDs emphasizes the translational and pathophysiological value of the two tests.

We propose that the present data, obtained with the use of the most recent and selective antagonists of PGE2 receptor subtypes, coupled with AAV-selective Schwann cell/DRG neuron silencing of receptors and related intracellular signaling pathways supports our hypothesis. However, we agree to expand the limitations of the study (page 17, line 14).

2. With their best efforts, the authors used AAV-shRNA approach to selectively to knock-down *Ptger2* and *Ptger4* expression in Plp+ cells. Although Plp-scramble was tested in vivo, it was not used as a control in main figures and compared directly with the behavior of Plp-sh*Ptger2* mice. Instead, WT mice without carrying Cre or CreER transgene were used.

We thank the Reviewer for this observation. We agree that Plp-scrambled mice are a better control. Accordingly, these data are now moved to the main figures (Fig. 1r).

Moreover, the backbone AAV vector for scramble RNA (“pAAV[FLEXon]-CMV LL-rev(EGFP-5' miR-30E-BfuAI ORF_44bp BfuAI-3' miR-30E)- rev(LL)-WPRE”) seems to be different from the shRNA vector (“pAAV[FlexOn]-CMV-EGFP-[shRNA]-mCherry”). This non-standard practice of shRNA knock-down strategy may impact the behavior outcomes after transduction.

Thank you for your comment. The vector backbone and the primers for the cloning strategy used for the shRNA were incorrectly indicated; in fact, both the shRNAs and the scramble RNA share the same backbone with BfuAI restriction sites for cloning. We have now corrected the Methods section accordingly (page 36, line 17).

3. Importantly, it was not clear if a good transduction was achieved. Presenting % of lineage specific marker among GFP+ cells (Fig. S3 and S4) will not address this. Instead, %GFP+/Sox10+, %GFP+/S100+, and %GFP+/NeuN+ should be presented.

We thank the Reviewer for this observation. The fraction label of the y-axis was inverted by mistake. The revised figures S3 e,f,g and S4 c,d now correctly report: %GFP+/Sox10+, %GFP+/S100+ and %GFP+/NeuN+.

Moreover, it was noted that ~ 30% transduced cells (GFP+) in PlpCreER+ sciatic nerves were neither SOX10+ nor S100+ (Figs. S3e/g); and 40% of GFP+ cells in the AdvillinCre DRG were not NeuN+ (Figs. S4c-d). The results certainly raise the concerns of non-specific targeting and support the rationale to use conditional knockout mice.

We agree that the GFP and SOX10, S100 and NeuN are not fully co-expressed in some cells. The discrepancy most likely arises from the different subcellular protein localizations (GFP cytoplasm; S100, transitory in cytoplasm before secretion; SOX10 and NeuN, nucleus) that, depending on the slice orientation may not be captured evenly within the 10 µm cryosection slices. The discrepancy in the detection of the biomarkers localized in two or more different intracellular compartments due to the slice orientation is now reported in the Methods section (page 41, line 1).

Critiques on writing:

It is “painful” to go through this manuscript due to its substandard writing and weakness in interpreting the literature. The authors should have carefully combed through this manuscript before their submission. Otherwise, the readers would certainly doubt the teams’ rigor in their efforts.

We did our best to ameliorate the text and its reading.

1. Introduction: the authors missed an opportunity to better introduce the relevant background/concepts and explain the study rationale. After highlighting the challenges in studying EP4, they abruptly drifted to their EP2 findings without further EP4 coverage. It is confusing to readers. A minor comment, abbreviation of Schwann cells was directly used without first mentioning.

We thank the Reviewer for this general comment. Within the limitation of the space constraints of the journal, some sentences have been added to better explain the study rationale and the move towards the EP2 study (page 5, line 18; page 6, line 3 and 12). We apologize for using the acronym, SCs, in the text without mentioning the full name. This has been corrected.

2. Line 94-97 of Results: References #3-5 were cited to support that “we assessed the two temporally distinct responses reported in previous studies3-5...”. However, Ref #4 is a review; both Ref #3 (rat nociceptive behavior induced by burn injury and formalin) and #5 (e-phys) have nothing to do with PGE2-induced nociceptive behavior. In addition, the authors concluded that mechanical allodynia was “delayed’ after spontaneous nociceptive behavior evoked by intraplantar PGE2 injection. However, this argues against the literature: the change in paw withdrawal threshold was documented within 2-10 mins after the intraplantar injection of PGE2 (Taiwo Y et al., Brain Res 1987; Neuroscience 1989).

We thank the Reviewer for this criticism. We used the terms ‘acute’ for licking/lifting behavior as it is an almost immediate response, whereas mechanical allodynia, which cannot be assessed immediately, was (inaccurately) defined as ‘delayed’. However, we agree that the terms ‘acute’ and ‘delayed’ are not entirely appropriate as they depend on the timing of the assessment. In view of the Reviewer’s critique, throughout the revised manuscript the term ‘acute’ for the licking/lifting behavior has been changed to ‘transient’, as it last <20 min, whereas the term ‘delayed’ for mechanical allodynia has been changed to ‘sustained’, as it lasts hours.

References #3-5 have been changed quoting more appropriate papers.

3. AKAP:

a. Line 307/Fig. 4: It is confusing to the general audience with limited knowledge of AKAP as it was not mentioned that AKAP79 is a human orthologue and AKAP150 for rodents.

The analogy and correlation between the human AKAP79 and rodent AKAP150 are now mentioned in the Results section (page 12 line 7).

b. Line 332: after testing Plp-AKAP150-bPAC mice, the authors then started to introduce the experiment of selective silencing of SC AKAP150 in Plp-AKAP150 mice without describing how. I assume shRNA was used but could not find a definitive answer. After extensive searching, I was finally able to locate mAkap5 shRNA sequence listed in Methods. However, it was not explained that Akap5 is also known as Akap79.

The sentence has been revised (page 12 line 32).

c. In figure legends of Figs. 4m-n, which cDNA (human or murine) used in “AAV-AKAP79-LAPD (Plp-AKAP150-LAPD)” should be clarified although they share 93% homology.

The sentence has been revised.

It is possible that errors may occur during uploading or transferring from a sister journal which is beyond authors' control. I then searched and found the identical copy of this manuscript from bioRxiv. Overall, this is a poorly written manuscript lack of well-controlled studies.

We hope that additional controls may satisfactorily support our hypothesis and that the text has been ameliorated.

Minor comments

1. NSIADs remain to be life-saving and the side effects are serious but manageable. In addition, the claim that NSAID delays pain resolution was mainly derived from a CFA model which is not clinically relevant. Thus, authors may consider toning down the claim in Abstract.

Although we agree that NSAID adverse events should be manageable, NSAIDs have been reported to be responsible for 30% of hospital admissions in the UK for ADRs, mainly due to bleeding, heart attack, stroke, and renal damage (Pirmohamed et al., 2004, PMID: 15231615). Deaths from NSAIDs remain very high, as they caused more deaths than from road traffic accidents and twice as many deaths as from asthma or cervical cancer (Courtney et al., 2002, PMID: 12176799). Thus, identification of cellular and molecular pathways selectively implicated in PG-induced pain may help to direct pharmacological research to more specific targets for safer analgesics.

We also acknowledge the critique about the clinical relevance of the carrageenan and CFA model. We agree that most, if not all, preclinical models have inherent limitations when extrapolating findings to clinical settings. However, as reported above, the widespread use of the carrageenan and CFA model underlines the translational and predictive value of these tests regarding prostaglandin-induced pain and inflammation (Fehrenbacher et al., 2012, PMID: 22382999; Burek et al., 2022, PMID: 34510137; Fischer et al. 2017. PMID: 2866644; Sotocinal et al. 2011, PMID: 21801409), and the consequences of these actions. Here, our aim was to use tests that produce prostaglandin-dependent inflammation and pain-like responses and to investigate the underlying mechanisms with the final goal to decouple pain from inflammation. Accordingly, our intention was not to mimic any specific human disease, but rather to identify targets for more effective and safer analgesics. Nevertheless, to mitigate our conclusion, we have revised the claim in the discussion (page 17, line 28). However, this was not possible in the abstract due to word limit (150 words).

2. As both AdvillinCre and AdvillinCreER lines are available, to minimize the confusion, please indicate that it was PlpCreER line used in this study.

Mouse strains have been better defined throughout the revised manuscript.

3. Figs. 1h/I; 3g/k; 4d/e and S10e/h: please change “BCG” to “BGC”.

Typos have now been corrected.

4. Fig. 1j vs Fig. 1i: qRT-PCR revealed higher-level Ptger2 expression in mouse sciatic nerves. However, antibody staining suggests otherwise.

Using the qRT-PCR analysis, we aimed to demonstrate the expression of the investigated gene, rather than quantify protein levels and the associated functional responses. Large-scale analyses of mRNA expression and protein production (Greenbaum et al., 2003, PMID: 12952525, Beyer et al., PMID: 15326222) have underlined the limited correlation between the two readouts, because of several factors, including the translational regulation and protein *in vivo* half-lives.

5. Adding forskolin, a direct activator of adenylate cyclase, into primary Schwann cell culture, is common for promoting survival. As cells were already primed by forskolin, the results of cAMP sensor, cADDIs, to monitor cAMP production should be cautiously interpreted.

We agree with this critique of the Reviewer. We underline that the forskolin concentration in the SC culture medium was 2 μ M, whereas the concentration used to increase cAMP levels was much higher (100 μ M). The possibility, although remote, that the very small dose used for culturing Schwann cells may affect the response to PGE2 and forskolin is now added on page 38 line 10.

6. Although it is not discussed, PGE2-evoked thermal pain hypersensitivity can be used as an independent control to support SC-TRPA1 function.

We thank the Reviewer for the suggestion. Indeed, we had collected data (not included in the original manuscript for space reasons) that confirm previous findings (Moriyama et al., 2005, PMID: 15813989) that heat hypersensitivity by PGE2 is markedly reduced in TRPV1 KO mice or by a first-generation EP1 antagonist (ONO-8713) and in global EP1 knockout mice. We found that the last-generation EP1 antagonist, SC-51322, and TRPV1 global deletion failed to reduce mechanical allodynia, but attenuated heat hypersensitivity elicited by PGE2, thus indicating, as correctly proposed by the Reviewer, that thermal (TRPV1/EP1) and mechanical (TRPA1/EP2) responses are mechanistically different. Unfortunately, Moriyama et al., (2005, PMID: 15813989) did not report any result on mechanical hypersensitivity, possibly because, consistent with our present data, they found no reduction in TRPV1 and EP1 knockout mice. The new data are now added to Suppl Fig 15, and page 15 line 1.

7. Figs. 4b-c/4m-n and Figs. S11g-i: as red light may arguably have analgesic effect (Cheng et al., J Pain 2021), the appropriate control should be Control/light/(Cg/ CFA; PGE2/PLAA/AA).

We acknowledge the importance of including appropriate controls to account for any confounding effects of light treatment on pain outcomes. Based on your suggestion, the revised manuscript includes experiments previously performed (and not reported in the original manuscript for space reasons), that evaluated the effect of red light in control mice injected with PGE2, AA, PLAA carrageenan, and CFA. Under these conditions, we did not observe any effect of red light (Fig. 4b,c,m,n Suppl Fig. 11 g,h,i) (page 12, line 3).

8. It needs to be mentioned that Plp is also expressed in satellite cells (Avraham et al., Nat Comm 2020; Tiwari et al., Cell Report 2024).

In line with the Reviewer's comment regarding the Plp presence in satellite glial cells, in the original manuscript we assessed whether AAV-shRNA affect Plp expression in these cells. Results reported in Supplementary Fig. 3d exclude this possibility and confirm the selectivity of AAV-shRNA for Schwann cells. However, we have added a sentence to better explain the significance of the results of the control experiments. (page 6, line 34).

Reviewer #2 (Remarks to the Author):

In this paper, Nassini et al. focus on early pain and delayed allodynia involving PGE2 and systematically investigate the PGE receptors involved, using pharmacological and genetic approaches. As a result, the authors found that Schwann cell (SC) EP2 receptor is involved in delayed allodynia, but not in the elicitation of inflammatory response itself, and that EP2 receptor stays at the plasma membrane without showing ligand-dependent internalization, contributing to cAMP production in the vicinity of the plasma membrane (nanodomain). They further proposed that this PKA activation within this nanodomain may cause allodynia, presumably by causing Trpa1 channel activation and H2O2 production, sensitizing nerve stimuli adjacent to the SC. These results suggest the possibility that PGE2-elicited two responses, delayed allodynia and increased inflammatory responses can be separately controlled, providing a new perspective on pain regulation. Furthermore, the role of cAMP nanodomains in SC, as demonstrated by the elaborate optogenetics in this study, contributes to our understanding of the pathogenesis of neuropathic pain. Therefore, this Reviewer believes that this paper contains new findings that could potentially be acceptable, if several concerns shown below should be addressed.

Major concerns

1) The key to EP2, but not EP4, controlling the cAMP nanodomain is that EP2 stays at the plasma membrane without showing internalization. In this regard, for example, if EP2 and EP4 were exchanged only in the C-terminus, would the response of the cAMP nanodomain be opposite? If the authors can add structural biology results of EP2/EP4 receptors, it would provide a more profound insight.

While we appreciate the significance of the Reviewer's suggestion, we would like to underline that selective receptor agonists/antagonists and cell-specific silencing of EP receptors and intracellular signaling molecules robustly support our hypothesis that inflammatory pain is mediated by EP2 in Schwann cells, and EP2 response depends on events confined to the membrane nanodomain. Therefore, the otherwise valuable experiments suggested by the Reviewer may not be part of the present already dense and extensive paper but may be the object of future research.

2) In relation to the above, is it possible that AKAP79/150 tethers EP2 to the plasma membrane? It would be interesting to see if there is a difference in their affinities between EP2 and EP4 using NanoBit.

Also in this case, although the point raised by the Reviewer may be of interest, it is beyond the aim of the study and may be the object of future research.

3) The EP2 receptor, cAMP nanodomain, PKA activation, and the scaffold function of AKAP have been well demonstrated by a series of analyses, but the molecular mechanism from Trpa1 activation to H2O2 production is unfortunately unclear. For example, it would be helpful if the authors could show the effects of TRPA1 phosphorylation by PKA on Ca response and H2O2 production.

We agree with the Reviewer's comment. Unfortunately, to the best of our knowledge there is no antibody that reliably detects phosphorylated TRPA1. Thus, to at least partially reply to the interesting point of the Reviewer, we have added PGE2-induced calcium responses in cultured

Schwann cells using the PKA inhibitor, H89, and the NOX1 inhibitor, ML171. Additionally, we also measured PGE2-induced H₂O₂ production both in the absence and presence of H89 and ML171 (Suppl Fig 14c,d and page 14, line 19).

4) What molecules produce H₂O₂? And how do sensory nerves recognize H₂O₂?

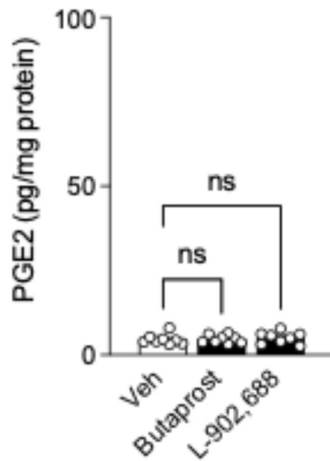
To answer to the Reviewer's question, we performed new experiments with the NOX1 inhibitor (ML171). The new data fill the gaps on the pathway that, from the association between PKA and TRPA1 in Schwann cells, signals pain: the PKA and TRPA1 association elicits a calcium raise that enables NOX1 to release H₂O₂, thus producing a feed-forward mechanism that, *via* TRPA1, amplifies and sustains the oxidative stress that eventually targets neuronal TRPA1 (Suppl Fig 14c-g and page 14, line 19).

5) In Fig. S5, f and g, if it is good that knockdown of EP4 in DRG decreases acute pain elicited by an EP4 agonist, why does allodynia is weakened by Adv-EP4 knockdown?

We thank the Reviewer for this question that provides the opportunity to underline the difference (complicated by conflicting results in the existing literature) between inflammation produced by exogenous vs. endogenous PGE2. Injection of the selective EP4 agonist, L-902,688, targeted EP4 in nerve fibers to elicit transient spontaneous behaviors (licking/lifting) and sustained mechanical allodynia, as further indicated using EP4 selective antagonist. This conclusion was supported by the ability of EP4 silencing in DRG neurons to attenuate both responses. However, this scenario was only minimally replicated with PGE2 injection, where the EP4 antagonist only marginally reduced PGE2-evoked mechanical allodynia, and was not replicated at all by endogenous PGE2. In fact, AA, PLAA, carrageenan, and CFA failed to evoke any licking/lifting behavior, and EP4 antagonism did not reduced mechanical allodynia and grimace behavior. The reasons for the difference between endogenous vs. exogenous PGE2 were discussed in the original manuscript; they may be due to the high PGE2 concentration achieved in the relatively small tissue volume at the injection site that enables the drug to target some nerve fibers. In contrast, the production and diffusion of endogenous PGE2 may not replicate the suddenly high unphysiological concentration (Fig. 2e) produced by the injection of exogenous PGE2. We would like to underline that the reason for the different response to exogenous vs. endogenous PGE2, which remains unknown, is beyond the aim of the present paper and may be a subject for future studies.

6) It is very important to examine endogenous PGE2 production within tissues treated with PGE2, PLAA, AA, Cg and CFA (in Fig. 2e and Fig. S7a). In the PGE2-EP2 or PGE2-EP4 signaling, endogenous PGE2 levels are often increased by positive loop of EP2/cAMP/PKA/COX pathway, and therefore, the authors should examine whether EP2 or EP4 agonist administration increases endogenous PGE2 levels.

In line with the Reviewer's comment, we have performed additional experiments by i.pl. injection of butaprost or L-902,688, EP2 and EP4 agonists, respectively, followed by the measurement PGE2 in tissue homogenates. The negative results (see below) are not included in the revised manuscript due to space constraints.



Reviewer #3 (Remarks to the Author):

In their manuscript titled “Targeting the Schwann Cell EP2/cAMP Nanodomain to Block Pain but not Inflammation”, the authors investigate the mechanism of Prostaglandin E2 (PGE2)-evoked pain and protective inflammation. Overall, they find that Schwann cell EP2 receptor activation is responsible for mechanical allodynia observed after PGE2 injection or in clinical inflammatory models, but this mechanism is not associated with inflammation. The authors first show that local intraplantar injection of PGE2 into the mouse hind paw resulted in a non-evoked nociceptive and a delayed allodynia that were mediated by EP4 and EP2 receptors, respectively. They then showed that Schwann cell (SC) EP2 receptors are responsible for sustained allodynia, which was reduced in mice with SC-specific EP2 knockdown. The authors have several exciting discoveries that increase the mechanistic understanding of SC-EP2 driven allodynia with a series of very elegant experiments. First, the authors show that optogenetic stimulation of focal cAMP production in Schwann cells induces mechanical allodynia and that SC-EP2 driven allodynia is mediated by membrane-localized PKA activation. They further identified the downstream mechanism with their finding that the AKAP79/150 scaffold protein induces cAMP/PKA activation in the membrane. Blocking the AKAP79-PKA interaction via the peptide inhibitor st-Ht31 inhibited allodynia. Inhibiting AKAP/PKA interaction in SCs also reduced carrageenan- and CFA-induced allodynia. Lastly, the authors demonstrated that selective silencing of SC EP2 was able to produce an early and sustained attenuation of allodynia without reducing inflammation.

Overall, we are very excited with the discovery of Schwann cell driven mechanical allodynia in clinical inflammatory models or following PGE2 injection. Most studies in the literature solely focus on the role of EP receptors in neurons and studies that describe the role of EP receptors in Schwann cells are scarce. The finding that inhibiting Schwann cell-EP2 driven response alleviates mechanical allodynia but allows recovery through inflammation is very intriguing and novel. This marks the Schwann cell driven pathway as an important therapeutic target to remove persistent inflammatory pain while sustaining the protective and regenerative inflammatory mechanisms. The authors also use a breadth of experiments to test their findings with appropriate controls, and their experiments are conducted with rigor, proper animal numbers, and the difference in sexes is explained clearly. Overall, we are very excited about these findings and strongly support this study.

For improved interpretation of results, we suggest the authors address the following:

Major:

1. There does not appear to be complete AAV-mediated silencing of EP2 or EP4 in DRGs (Supplementary Fig. 4a and b). Although there is a decrease by half in expression levels, the remaining receptors can still be a confounding variable in the interpretation of results. We recommend addressing this as a shortcoming of this study. We agree that obtaining a new mouse line with floxed alleles of *Ptger2* for specific knockout in Schwann cells may be beyond the scope of this manuscript, however, discussing this point in the manuscript is helpful.

We agree with the Reviewer's comment on the incomplete efficiency of the silencing procedure, and accordingly, a sentence has been added to more cautiously discuss the results (page 17, line 14).

2. To further verify that AAV-silencing has no off-target effects, it would be helpful to quantify whether AAV injections cause any changes in the total number of DRGs or SCs. We also would like authors to consider adding quantification of myelin, at least through showing Myelin basic protein staining, following injections into the sciatic nerve.

To accomplish the request of the Reviewer to further verify any unwanted non-specific and damaging effects of the silencing procedure, we have assessed the myelin basic protein (MBP) staining in sciatic nerve trunks in control and AAV EP2/EP4 Plp mice and neuron number in lumbar DRGs in control and AAV EP2/EP4 Adv mice. Results indicate that the AAV infections did not change MBP staining (expressed as mean value intensity) and the number of DRG neurons (Suppl Fig 5a,b and page 7, line 10).

3. In supplementary figures 13 and 14, the authors describe different mechanisms of TRPA1 activation in neurons versus Schwann cells. The authors find that knocking down TRPA1 in DRG neurons attenuates allodynia, knocking down TRPA1 in Schwann cells also alleviates allodynia. If the authors could include a diagram of downstream signaling in DRG axons with different paradigms versus the one in Schwann cells, this would be helpful in describing the differences in TRPA1 channel activation.

As suggested by the Reviewer, a diagram has been inserted in supplementary Fig 14p.

4. Is it possible to stain for endogenous AKAP79/150 and PKA to detect the localization of the endogenous proteins in the Schwann cell membrane *in vivo*? This will help to establish an orthogonal method for the mechanisms suggested by the overexpression of full length Akap5 constructs.

We appreciate the comment of the Reviewer. Although the localization of the endogenous proteins in the Schwann cell membrane *in vivo* would be of interest, to the best of our knowledge this type of evaluation *in vivo* does not seem to be possible.

5. We noticed that separate COX inhibitors were used throughout the manuscript, indomethacin and diclofenac. It would be helpful to know why each was used in their respective experiments. It would also be more rigorous that the results are the same using separate COX inhibitors, and that the results are not specific to only one drug.

We agree that the choice to use two different NSAIDs was not well explained in the original manuscript. Indomethacin was chosen because it is commonly used in preclinical studies to obtain complete prostaglandin inhibition. In the CFA experiments reported in Fig. 5, we used diclofenac to replicate previous results that this drug reduced pain but delayed its resolution in mice and humans (Parisien et al., 2022, PMID: 35544595), and because it is one of the most used NSAIDs to treat inflammatory pain (Van Walsem et al., 2015, PMID: 25879879; Atzeni et al., 2018, PMID: 29873010). The successful replication of the results in mice (Parisien et al., 2022, PMID: 35544595) allowed us to show the prominent role of Schwann cell EP2 in pain but not inflammation. Regarding the use of another anti-inflammatory drug, Parisien et al., 2022 (PMID:

35544595) already expanded their investigation to dexamethasone with results identical to those obtained with diclofenac, indicating that different anti-inflammatory drugs shared the same side effect to prolong the pain recovery. In line with the aim of the study, our confirmatory findings with diclofenac were intended to demonstrate the exclusive proalgesic role of EP2 and its Schwann cell intracellular pathway (AKAP150), rather than a proinflammatory role. Finally, to minimize the use of animals (ARRIVE guidelines, McGrath, 2015, PMID 25964986), we decided not to use other NSAIDs in this part of the study. One sentence to better explain the use of anti-inflammatory drugs (indomethacin, diclofenac and dexamethasone) is now provided (page 15, line 15).

Minor:

6. It would be helpful to include a table of the various activators/inhibitors/vectors used throughout the manuscript for easy reference.

Following the suggestion of the Reviewer, tables with all activators/inhibitors/vectors used throughout the manuscript have been added as Suppl Tables 1, 2 and 3.

7. In addition to the mRNA level quantification of the target EP receptors, it would be helpful to also include the quantification of protein levels from the immunostaining experiments. Having descriptive quantification will supplement the images used to show decreases in the expression of the receptors.

In the original manuscript we provided (Suppl Fig 3, 4, 5) semiquantitative analyses (Rcoloc) of the protein expression in tissues from control and silenced animals. The experiments show that silencing procedures produced significant reductions in protein expression.

8. The authors state that “several findings suggest that SC PKA/TRPA1 phosphoactivation by EP2/cAMP sustains calcium-dependent oxidative stress signal” elicited through H₂O₂ formation. It would be helpful to include citations that show H₂O₂ induces mechanical allodynia.

Findings and papers showing the role of H₂O₂ in mediating mechanical allodynia are now reported and cited, respectively (page 14, line 19).

9. The authors mention that the non-specificity of antagonists in previous studies precludes the identification of EP receptor subtypes involved in inflammatory pain. How did they test the effectiveness and specificity of the inhibitors used in their study? A discussion of how these agonists and antagonists and their concentrations were determined and supporting data showing their specificity would be helpful.

We thank the Reviewer for this question, which allows us to add information that, for reasons of space constraints, were not included in the original manuscript. The doses of the four antagonists for their respective EP receptors have been chosen by dose-response curves (not reported). However, more importantly, effectiveness and selectivity of the selected doses were proven by the following observations: EP2 and EP4 antagonists were effective in PGE₂-induced mechanical but not heat hypersensitivity (now reported in Suppl Fig. 15); the EP1 antagonist reduced heat but not mechanical hypersensitivity (now reported in Suppl Fig. 15); EP3 activation reduces rather than increases pain (Natura et al, 2013, PMID: 23904482), in line with the failure of our EP3 antagonist to affect the response to PGE₂. All the other drugs were used according to previous literature. A sentence has been added to page 15 line 1 (see also Suppl Fig 15).

10. The authors show that SC-EP2 knockdown doesn't attenuate non-evoked pain after PGE₂ injection, but it seems to do so in CFA or Carrageenan models. Is that due to a different behavioral test? Does PGE₂ injection cause grimace behavior?

The Reviewer raises an interesting issue regarding the different pain-like responses produced by exogenous vs. endogenous PGE₂. PGE₂ (i.pl.) evoked locally in the injected paw transient (<20

min) non-evoked pain-like behavior and sustained (~4 hours) mechanical allodynia, but failed to elicit any visible grimace behavior. Data showing the failure of PGE2 to produce grimace are reported in the original manuscript (Suppl Fig 8h). We do not have a final explanation for the difference between PGE2 and carrageenan or CFA which, however, as previously reported (Sotocinal et., Mol Pain 2011, PMID: 21801409), elicit grimace behavior. It is possible that a small, but highly concentrated PGE2 dose may elicit a sharp activation of a few nerve fibers, that, although transient, is sufficient to elicit non-evoked responses. However, its rapid tissue clearance may prevent grimace behavior development. Carrageenan inflammation lasts 24 hours and CFA >20 days. The persistent inflammatory processes elicited by carrageenan and CFA (24 h and 20 days, respectively) suggest a slowly mounting PGE2 production, sustained enough to elicit grimace behavior. While we agree that the unforeseen difference between the two non-evoked pain-like responses (transient pain-like behavior and prolonged grimace) deserves further research, we considered that it was beyond the scope of the present study.

11. Since authors are directly testing PGE2 receptors, throughout the text I think it would be clearer to change “PG-mediated” to “PGE2-mediated”.

We agree with the wise suggestion of the Reviewer and, accordingly, PG-mediated has been changed to PGE2-mediated when data and comments relate to pain-like responses, that are robustly reduced by targeting EP receptors for PGE2. However, regarding inflammation produced by carrageenan and CFA that are inhibited by indomethacin, but not by EP receptor antagonism/silencing, we maintained the general abbreviation PGs.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the Reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reply to Editor and Reviewer comments

General. We thank the Editor and Reviewers for their valuable suggestions and criticisms, which we believe have been satisfactorily addressed in the revised manuscript. In particular, we have provided additional support for the role of Schwann cell EP2 by using other antagonists: two for EP4 and one for EP2. Overall, the results obtained with five distinct PGE2 receptor antagonists, three against EP4 and two against EP2, align with findings from AAV-mediated silencing techniques targeting EP2 and EP4 in Schwann cells and DRG neurons. These results should resolve doubts regarding the specificity of the pharmacological and genetic interventions. Additionally, the change in the title from "blocks" to "inhibits" is intended to moderate our conclusion and message.

Reviewer #1 (Remarks to the Author):

In this resubmission, the authors have addressed many of my concerns from the prior review, with new control experiments or justifiable reasons to power their conclusions. However, a few important issues remain unresolved.

1. The authors emphasized that “clarification of the ambiguous findings produced with older non-specific agonists/antagonists, is the primary contribution of our study”. They demonstrated that BGC20-1531, an EP4 antagonist which was first described in 2009, “produced marginal reduction of PGE2-evoked allodynia” (Fig 1i; Fig S1k). It failed to reduce both mechanical allodynia and paw edema induced by Cg (Fig S8c) or CFA (Fig S8d). Notably, several newer EP4 antagonists were subsequently developed. EP4 antagonist 74/77 identified through computational docking significantly reduced mechanical allodynia induced by PGE2, Cg or CFA (Gahbauer S et al., Nat Comm 2023). Furthermore, this compound was found to have anti-inflammatory property which significantly reduces CFA-induced paw edema. The conflicting result in the literature further heightens my concerns about “the specificity of the small molecules which form the foundation of current study” and empowers my prior comment that “specific deletion using conditional knockout mice is critical to address the discrepancies in the literature”.

Response. Thank you for these comments. As noted in our responses to the initial review, we agree that experiments using conditional knockout mice with EP2 deletion specifically in Schwann cells would provide additional evidence supporting the role of EP2 in Schwann cell-mediated pain. In the current study, we used a viral-mediated approach to selectively knock down EP2 in Schwann cells and DRG neurons and verified cell-specific silencing. This method is widely recognized for its efficiency and specificity in gene silencing within targeted cell populations (e.g., PMID: 33558455; PMID: 30710128). It is important to note that the development of conditional knockout mice does not always guarantee complete or exclusive deletion within the targeted cell type (e.g., PMID: 40186303; PMID: 32027825; PMID: 23554062; PMID: 17579640). Compensatory changes in gene expression may also confound the findings in such animals. Additionally, the development of conditional knockout mice would require ethical approval and several breeding generations, which could take one to two years to complete. This timeline would substantially delay the publication of our work without necessarily altering the main conclusions of our study. In the discussion, we now acknowledge that conditional silencing of EP2 in Schwann cells would provide further confirmation of our findings and will be explored in future research (Line 531).

To address the reviewer’s concern and the editors’ recommendation regarding the specificity of EP antagonists, we conducted additional experiments using two further EP4 antagonists, CJ-42794 (used in the by Gahbauer et al. Nat Comm 2023; PMID: 38057319) and ONOAE3-208. We also tested another EP2 antagonist, TG6-10-1. To confirm the selectivity of these antagonists (all administered via intraplantar injection), we assessed their ability to block the effects of selective

agonists of EP2 (butaprost) and EP4 (L-902,688) on pain-like behaviors in mice. Subsequently, we evaluated whether these antagonists influenced pain-like behaviors and paw edema in the carrageenan and CFA models. Our results show that both ONOAE3-208 and CJ-42794 attenuated non-evoked nociception and allodynia to a selective EP4 agonist, L-902,688, and non-evoked nociception to PGE₂. They also reduced non-evoked nociception to PGE₂ and caused minimal decrease in allodynia to PGE₂. Neither compound affected allodynia to the EP2 agonist, butaprost, or allodynia, grimace and paw oedema to carrageenan and CFA (Supplementary Fig. 2 and 9). Conversely, the EP2 antagonist, TG6-10-1, did not affect responses to the EP4 agonist, L-902,688, or non-evoked nociception to PGE₂, but effectively blocked allodynia to PGE₂ and the EP2 agonist, butaprost, and allodynia and grimace, but not paw edema to carrageenan and CFA (Supplementary Fig. 2 and 9). To the best of our knowledge, these findings provide the most precise analysis to date of PGE₂ receptor involvement in pain and inflammatory responses in mice, specifically focusing on local mechanisms induced by carrageenan and CFA. Through meticulous examination of antagonist selectivity, our findings with two EP2 antagonists and three EP4 antagonists closely align with data obtained via AAV-mediated silencing techniques. These results collectively indicate that EP2 is the primary PGE₂ receptor mediating pain-like behaviors triggered by inflammatory stimuli, while EP4 appears to have a lesser, non-essential role in this context. See Line 130.

We have several reservations about the conclusions of the paper by Gahbauer et al. (Nat Comm 2023) cited by the reviewer. Their study describes the effects of a novel EP4 antagonist (74/77) on mechanical allodynia and paw oedema induced by carrageenan and CFA, as well as the actions of an earlier EP4 antagonist, CJ-42794, on carrageenan-evoked mechanical allodynia. While EP4 antagonists produced statistically significant reductions, the effect sizes were modest (around 15-20%) compared to the approximately 70% inhibition observed with cyclooxygenases inhibitors (Seibert et al, PNAS 1994, PMID: 7991575; and the present study). This suggests that EP4 antagonism results in only partial mitigation of the mechanical allodynia, and the receptors responsible for the remaining approximately 50% of the prostaglandin-mediated response remain unidentified. A key difference between our study of Gahbauer et al. lies in the route of antagonist administration - our study employed local (intraplantar) delivery, whereas Gahbauer et al. used systemic (oral or intraperitoneal) administration. This difference may account for the relatively small effects observed with EP4 antagonists in their study, effects that were minimal or absent with local administrations. The modest impact on mechanical thresholds and paw thickness reported by Gahbauer et al. might be mediated through EP4-mediated systemic mechanisms, resulting in a small reduction of the overall pro-inflammatory burden.

Furthermore, Gahbauer et al. administered an unusually small dose of carrageenan (30 µg/kg). This small dose caused a minimal increase in paw thickness (from ~1.7 mm to ~2.2 mm, a 24% increase). This contrasts with the greater increases (>200%) observed with commonly used doses (Iadarola et al, Pain 1988, PMID: 2906426; Morris, CJ Carrageenan-Induced Paw Edema in the Rat and Mouse, Methods in Molecular Biology (MIMB, volume 225); Seibert et al, PNAS 1994, PMID: 7991575). Therefore, the accuracy of assessing the inhibitory action of 77 on an overall increase of 24% is questionable when compared to that elicited by the usual higher dose of CFA that caused a 225% increase in paw thickness (1.8 mm to ~4.8 mm), similar to what we found in the current study and comparable to values commonly reported in the literature.

We were surprised that the paper by Gahbauer et al. failed to adequately describe the statistical tests used to evaluate the results (for example, it is unclear whether multiple comparisons were appropriately performed) or to include information about the determination of experimental replicates.

In the revised manuscript, we reference the work of Gahbauer et al. and compare our findings (Line 491).

2. Intriguingly, conditional deletion of TRPA1 in either SCs (Plp-Trpa1 mice) or sensory neurons (Adv-Trpa1 mice) reduced mechanical allodynia evoked by PGE2 (Fig S13j; Fig S14 m-o), Cg or CFA (Fig 4o-p; FigS14 m-o). The anti-allodynic effect was similar when compared to the treatment of indomethacin (Fig S7 h-m and Fig 2i-j). Zappia KJ et al (eNeuro 2017) suggested that non-neuronal TRPA1 is not sufficient to maintain mechanical sensory function. The increased injury-induced expression of TRPA1 (protein and mRNA) in the DRG within 24 hrs following CFA injection is also well documented (Obata K et al., JCI 2005; da Costa et al., PAIN 2010; Zhang Q et al., Cell Reports 2023). It is less likely that the CFA-evoked mechanical allodynia mainly results from "TRPA1-dependent ROS release (from SC) that targets neuronal TRPA1". Other factors or alternative explanations merit considering for the data interpretation.

Response. Thank you for raising these additional considerations. The study by Zappia et al., supports the role of neuronal TRPA1 in pain signaling, showing that tissue-specific deletion of TRPA1 from sensory neurons is sufficient to reduce mechanical sensitivity while having no effect on thermal sensitivity. These findings align with our results, which show attenuated mechanical but not thermal sensitivity in mice with conditional silencing of TRPA1 in DRG neurons. Zappia et al conclude, and we concur, that neuronal TRPA1 is 'sufficient' for maintaining mechanical sensory function. They also suggest that TRPA1 expressed in other cell types is not sufficient to sustain mechanical sensory function. However, in another section of the paper they state: *'That is, although the present experiments cannot exclusively preclude any non-neuronal expression of TRPA1, the results do in fact justify'*. Our data support the proposal that the TRPA1 responsible for signaling to the neuronal TRPA1, thus enhancing sensitivity to mechanical stimuli, is located in Schwann cells, which were not investigated in their study. The model proposed by Zappia et al. would be valid if TRPA1 in Schwann cells acted in parallel with neuronal TRPA1 to transmit the pain signal. In contrast, our hypothesis posits that TRPA1 in Schwann cells is arranged in series with neuronal TRPA1, meaning both channels are necessary and sufficient to signal pain.

We agree that TRPA1 upregulation may occur under inflammatory conditions or tissue injury, and we do not dismiss this possibility. However, responses dependent on TRPA1 or ROS were already evident 30 minutes to 1 hour after injections of PGE2, butaprost, and carrageenan, making the contribution of TRPA1 overexpression in DRG neurons at these time points unlikely. Additionally, the ability of oxidative stress byproducts to target TRPA1 has been extensively demonstrated (Andersson et al, J Neurosci 2008, PMID: 18322093; Trevisani et al PNAS 2008, PMID: 17684094; Eberhardt et al J Biol Chem 2012, PMID: 22740698; Taylor-Clark et al J Physiol 2008, PMID: 18499726). We also provide evidence that ROS scavengers reduce inflammatory allodynia. Nevertheless, in the revised version of the manuscript, we have moderated the conclusion that ROS may be the sole mediators implicated in TRPA1 activation (Line 543).

3. Line 441, "Although NSAIDs transiently inhibit inflammatory pain in patients, they markedly delay its resolution. This delayed resolution of pain, recapitulated in the CFA mouse model of inflammatory pain by treatment.." This statement is misleading. Parisien et al used statistical modelling to conclude that individuals with acute back pain were at increased risk of developing chronic back pain if they reported NSAID usage. However, to confirm this, they need a clinical trial with appropriate controls, a clinical gold standard. Instead, the supporting evidence came from a mouse model of inflammatory pain. Thus the statement should be revised to "Although NSAIDs transiently inhibit inflammatory pain in patients, they markedly delay its resolution in the CFA mouse model of inflammatory pain..".

We thank the reviewer for the comment. Following the suggestion we have changed the sentence accordingly (Line 458)

Minor comments:

--In Fig S3a: DRG staining with EP2 was mislabeled as “EP4”.

We apologize for the oversight, the label in Supplementary Fig 3a has been corrected accordingly.

Reviewer #2 (Remarks to the Author):

The authors responded appropriately and courteously to this reviewer's concerns. Congratulations on an exciting paper that will make a significant contribution to our understanding of the long-unknown mechanism of prostaglandin-induced pain.

We thank the reviewer for the positive feedback.

Reviewer #3 (Remarks to the Author):

We thank the authors for their revisions, additional experiments and clarifications that address our previous concerns. We are excited for the publication of this very interesting manuscript investigating the role of Schwann cell–EP2 signaling in inflammation and mechanical allodynia.

We thank the Reviewer for the appreciation of our work.

We suggest three minor edits for the manuscript:

1. In the introduction section, authors highlight the conflicting reports on EP4-signaling in mechanical hypersensitivity; but there is little mention of previously studied roles for EP2-signaling in inflammation, mechanical allodynia and pain. We suggest addition of such background information related to previous studies and inclusion of related citations.

We thank the reviewer for the suggestion. To help readers understand the scientific background of the paper, we have moved the findings from the literature on EP2 in peripheral pain and EP receptors in Schwann cells from the discussion section to the introduction (Line 69 and Line 82).

2. For the audience who may be unfamiliar with the role of TRPA1 in pain, a citation for the statement provided in Line 365 may be beneficial.

We thank the reviewer for the suggestion. We have added relevant citations. (Line 381)

3. The inclusion of the reagent tables in the Supplementary Info was very helpful. It would be more beneficial if the tables also included the catalog numbers for the reagents.

In response to the suggestion, we have updated supplementary tables to include the catalogue numbers for all listed reagents

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.