Peer Review File

Editorial note: The figure numbering has changed during the course of the review process

Manuscript Title: Neural circuit basis of placebo pain relief

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referee #1 (Remarks to the Author):

Chen et al. have delineated a neural pathway from the rACC to the Pn that encodes and regulates what they term "placebo analgesia conditioning" (PAC).

However, although the circuit influences several phenotypes associated with the conditioning paradigm, it does not necessarily modulate an endogenous pain pathway in a manner identical to placebo analgesia in patients, instead, it may only signal anticipatory pain-reducing behaviors in mice, which is certainly interesting and novel but needs to be fully clarified. The manuscript requires a major revision to deal with this, and several additional control experiments.

Major comments:
1. I suggest they drop the term placebo for their findings since this will just create confusion. Frankly they are not modeling human placebo analgesia but "pain relief expectation". The title also needs to be changed to something like "A cortico-cerebellar circuit modulating anticipatory pain"
2. A set of published data suggests that placebo analgesia serves as a learning experience where patients get clinical benefit via contextual conditioning without prior exposure to the treatment itself (Colloca et al., 2013 Pain). However, in this study, the PAC paradigm subjects the animals to the "treatment" box (300C) during the 3 days of the conditioning phase and therefore it is directly only implicating an anticipatory behavior and not acute placebo analgesia. The authors need to carefully explain the differences between their approach and the standard appearance of placebo analgesia and can certainly conjecture in the Discussion that while it may contribute, it is not the same as a human placebo.
3. Does the conditioning change other pain-related behaviors in response to mechanical or chemical stimuli?
4. The post-conditioning test of PAC used noxious heat (48 deg) for both chambers, if this assay captures pain relief anticipation, animals shouldn't prefer chamber 2 when both chambers are set to a warm temperature (30 deg) during the test phase. Please confirm if this is the case.
5. Naloxone should be given during the conditioning phase to show that the PAC paradigm promotes endogenous opioid signaling in mice.
6. Heterogeneous cell type recordings across different phases during PAC (Fig. 2) fail to clarify an association with endogenous analgesia versus anticipatory signaling on crossing the chambers. Please consider re-examining this experiment using the Oprd1Cre line, as for the RNASeq in Fig.5.
7. Aligning neural firing to the time of crossing could represent a motor-related response. Please add a supplement control group (M1/2 to Pn) that relates functionally to locomotion and not anticipation of pain relief.

8. Fig.4 evaluated sensory responses by optogenetically targeting a mixture of cell populations along ACC to Pn pathway, but not PAC-associated cell types. Please consider using FosCreER to label cells, using a similar strategy to that in Fig.1e for the behavioral experiments.
9. Sequencing data were not derived from rACC-projected Pn neurons but a heterogeneous local population (Fig.5). What is the % of ACC projection neurons / local population neurons in the Pn?

Minor comments:
1. In Fig.1 Placebo analgesia was tested only 1 day after conditioning. Please provide a longer temporal profile of the placebo effect (3-7 days) to validate PAC efficiency. In addition, the frequency of behavior bouts is critical besides latency, so incorporate this metric as well.
2. Fig.1f and g need quantification of the histology.
3. Please record and compare activities during 1st licking, rearing, and jumping on post-PAC to validate the encoding of sensory reflex.
4. The feedforward inhibition mechanism (fig.3i-3l) can explain why rACC neurons' firing pattern changes from tonic to phasic, but cannot explain why they fire more action potentials in the beginning (line 218, fig.3b), do the authors have another interpretation for this?
5. The second and fourth data points in NpHR group in Fig.4f seem to have significance compared to eYFP and ChR2. Please reanalyze the statistics.
6. NpHR inhibition of Oprd1+ neurons (fig.4j) should also be done during conditioning.
7. Please map rACC-Pn projection-specific population in Oprd1Cre mice and repeat the optogenetic experiments in Fig.5 i and j.
8. Is it correct that cortical tissue was aspirated above to implant the GRIN lens? Does that mean that the upper layers of the cortical column of the ACC are gone? If so, does this change the output of the ACC-PN neurons?
9. The relationship between calcium activity and crossing behavior in Figure 2d, is hard to interpret. You might expect high activity at -2s and then lower or sustained activity moving to a crossing event if it signaled pain-relief expectation. Rather, it seems like the number of neurons that have a high level of activity increased after training. What is the activity of these neurons like at longer time windows, say up to 10 seconds? Are there subtypes of neurons that cluster differently across time?
10. The data in Ext Fig 6, where non-PN projecting neurons display reduced activity compared to PN projecting neurons, suggests that PAC induces a change in the local network connectivity similar to that which occurs in S1 (Okada et al., 2021 Sci. Adv). Do you see this in rACC?
10. Figure 4f: please show raw frequency value curves rather than delta.
11. The authors claim the Ca2+ activity of rACC→Pn neurons increased progressively during PAC which is consistent with the learning-based nature of placebo analgesia'(lines 208-209). But they only showed a single time point during conditioning (day 6) for the imaging data (fig.2e,f). Have the authors done imaging on d4 and d5 as well? If yes, please show the data.
12. In Extended Data Figure 5, the argument is made that rACC-PN neurons do not display significant increases in activity but the heatmaps presented in 5b and d look as though there are populations that increase in activity and decrease in activity and are time-locked to the stimulus.

Referee #2 (Remarks to the Author):

This study describes a novel means of investigating expectation-related pain in a placebo paradigm to show that 'placebo' analgesia is mediated by a rostral ACC-pontine nucleus circuit that is more active when expectations of pain relief are experimentally induced. Proposed to be an opioidergic mechanisms owing to the high abundance of opioid receptors in the Pn and disruption of placebo analgesia when inhibited, the authors findings reveal a novel target for pain relief (the rACC-Pn circuit). The broad implications of the study, as a framework for investigating neural circuits involved in descending modulatory control, are important.

This is a highly original study with novel significance.

The combined use of a novel model to induce expectation-mediated analgesia, neural Ca imaging in behaving animals, single RNA seq, ephys and optogenetics leads to a highly sophisticated experimental approach.

The conclusions are in keeping with the high quality of the data and I believe them to be robust.

Apologies if missed, but it would be prudent to share detail of labelling efficiencies for the viral targeted strategies.

This is a well written, clear and lucid paper.

Referee #3 (Remarks to the Author):

In this manuscript by Chen and colleagues, an array of modern techniques are used to provide evidence for the involvement of an ACC-->Pn neural circuit subserving placebo analgesia in the mouse. On the plus side, this is to my knowledge the first proposed neural circuit for placebo, and the experiments here are expertly performed and convincing. On the other hand, aspects of this work are not as novel as the authors claim, and I was left thinking that the most interesting finding here was only hinted at, not directly shown. Although the behavioral placebo paradigm here is "novel" in the sense that no one has ever done it quite this way before, this is nowhere near the first rodent model of placebo analgesia. Similarly, although it is claimed that the ACC-->Pn labeling was "unexpected" and that the pontine nuclei have "no known pain-related function", a cursory PubMed search turned up two papers showing ACC-->Pn connections using anterograde tracing (Shi et al., Mol. Pain, 2022; Fillinger et al., Brain Struct. Func., 2017). In addition, two fMRI studies have found pain-related activation of the pontine nuclei (Hubbard et al., 2016; Sclocco et al., 2016). While it is certainly interesting to nail down a circuit involving the pontine nuclei related to placebo analgesia, what I was really interested to see was direct evidence for involvement of the cerebellum in placebo analgesia. That was never shown here in any way. I realize I'm asking a lot here, of course, but in 2023 aren't neural circuits from limbic regions to brain stem regions a dime a dozen?

Another concern is related to the authors conceptualization of placebo as a "contextual, cue-based learning phenomenon". To my understanding that is only part of what placebo is, and the less-interesting part at that. Placebo can involve conditioning, yes. If I'm given a pill and told it's an analgesic, my history of successful analgesia against headaches of ibuprofen will have conditioned me to respond with analgesia to the pill. But placebo is also expectation and desire, and this requires no new learning. If I believe the doctor that this is an analgesic, and I want it to be an analgesic, it will be. This is the part that is difficult to model in animals. The authors have indeed set up an expectation using conditioning here, of course, which is fine if not novel. They refer to this expectation as one of "pain relief", and that the mice develop a "preference" for chamber 2. I think this is the wrong way to think about it. Isn't it more accurate to think of them as having developed an aversion to chamber 1? And the expectation isn't of pain relief, is of the absence of a pain stimulus. That's not the same as pain relief. A close perusal of Figure 1 reveals some details that are hard to understand. How is it possible that a mouse would voluntarily spend even 20% of its time on a 48degC platform? The evidence of successful conditioning here is a single data point—Post—in Fig. 1b and 1c. The error bars conceal individual data. What percentage of the mice had more time in chamber 2 than chamber 1, or a longer latency to cross back compared to the control condition? What I'd really like to see, actually, are some experiments where after conditioning mice are confined to either chamber 1 or chamber 2.

The finding that the relevant Pn neurons are Oprd1-positive is interesting, but confusing. There is absolutely nothing in the placebo analgesia literature suggesting that delta-opioid receptors are in any way involved, and in fact there have been some studies specifically implicating mu-opioid receptors only. What happens if you repeated the naloxone experiment, but with naltrindole or another delta-specific antagonist?

Finally, it's 2023. What possible justification is left for doing experiments only in male mice? At the very least, the experiments in Figure 1a–d need to be repeated in female mice. I wouldn't be at all surprised if there are sex differences here.

Other issues include:
1. The first sentence of the paper, "Pain thresholds aren't fixed values", is odd. The paragraph would better begin with the next sentence.
2. In Fig. 1c, is what's being plotted the total latency over 4 days? It's not at all clear.
3. There's a typo ("cossing") in Fig. 2c's legend.
4. If the behavior in the Cond group is more different from Pre than that in the Post group, why are the neurons more active in the Post group?
5. I'm not sure the scatterplot combining different conditions is statistically valid.
6. In Fig. 4d I don't think "Withdrawal latency" is an appropriate y-axis label. No one is "withdrawing" from the hot plate.
7. In Fig. 4g I think the asterisks are supposed to be referring to On vs. Off within-group, no?
8. In Extended Data Fig. 1, wouldn't one expect a significant difference between saline and naloxone groups in graph b? Also, graph c is uninterpretable without Pre group data.

Author Rebuttals to Initial Comments:

Overview

We extend our sincere thanks to all 3 reviewers for their strong interest in our work, their constructive comments, and their helpful suggestions to improve the manuscript.

Reviewer #1 wrote: "...which is certainly **interesting and novel** but needs to be fully clarified**.**"

Reviewer #2 stated: "The **broad implications** of the study, as a framework for investigating neural circuits involved in descending modulatory control, are **important**." "This is a **highly original** study with **novel significance**." "The combined use of a **novel model** to induce expectation-mediated analgesia, neural Ca imaging in behaving animals, single RNA seq, ephys and optogenetics leads to a **highly sophisticated** experimental approach." "The conclusions are in keeping with the **high quality** of the data and I believe them to be **robust**." "This is a **well written, clear and lucid** paper."

Reviewer #3 said: "...this is to my knowledge the **first proposed** neural circuit for placebo, and the experiments here are **expertly performed and convincing.**"

We carefully considered and addressed each comment, including by performing numerous novel experiments (involving 240 mice) and analyses suggested by the reviewers. We provide below a list of the changes made in the revised manuscript.

In our point-by-point response, we cite the reviewers' comments in full, using *blue italic typeface*, and describe our responses and the resulting improvements to the revised manuscript in regular black typeface.

Summary of major additions to the paper in revision

- **Substantial edits to the text** to clarify points about which the reviewers sought more information or discussion. (Please see our point-by-point replies below; these edits are too numerous to list here.)
- **New subsection in the Main section** describing novel data from cerebellar Purkinje cells during placebo pain relief.
- **New subsection in the Discussion**, most notably providing clarity regarding the novelty of PAC as compared to previous animal models of placebo analgesia.
- **New subsection in the Discussion**, particularly discussing the involvement of the endogenous opioid peptides and receptors in placebo analgesia.
- **New experiments added in revision** (new **Fig.6**; **Extended Data Figs.1a-c, 1g-i, 2, 12d-f, 12g-j, 13a-b,** and **14**).

- We used a miniscope to directly record the activity of cerebellar Purkinje cells during PAC-induced pain relief expectations to investigate the role of the cerebellum in pain and its modulation (**Fig.6** and **Extended Data Fig.14**).
- We set both chambers to a neutral temperature of 30°C during the post-test of PAC to test whether the preference to chamber 2 of conditioned mice is indeed due to pain relief expectation (**Extended Data Fig.1a,b**).
- To investigate the role of the endogenous opioid system in the formation of pain relief expectation, we injected naloxone during the conditioning phase of PAC (**Extended Data Fig.1g,i**).
- We tested whether PAC can also relieve chemical pain by injecting formalin during a modified PAC post-test in which both chambers were set to innocuous 30°C (**Extended Data Fig.2a-c**).
- We examined whether PAC-induced pain relief expectation reduces thermal, mechanical, or chemical pain outside the PAC apparatus using the Hargreaves, von Frey, or formalin tests, respectively (**Extended Data Fig.2d,e**).
- We investigated the nocifensive behaviors of mice after confining them to either chamber 1 or chamber 2 during the post-test of PAC by blocking the opening between the two chambers of the PAC apparatus (**Extended Data Fig.2f,g**).
- We performed PAC with female mice to examine sex differences in PAC-induced analgesia (**Extended Data Fig.2h-j**).
- We photoinhibited *Oprd1*$^{+}$ Pn neurons during the conditioning phase of PAC to determine the extent to which they contribute to the formation of pain relief expectation (**Extended Data Fig.12d-f**).
- We utilized an AAV1 virus to specifically target the *Oprd1*$^{+}$ Pn neurons receiving rACC inputs and examined their role in PAC-induced analgesia using photoinhibition (**Extended Data Fig.12g-j**).
- We examined the role of both μ- and δ-opioid receptors (MOR and DOR, respectively) in PAC-induced analgesia by systemically injecting the selective MOR antagonist β-funaltrexamine or the selective DOR antagonist naltrindole, respectively (**Extended Data Fig.13a,b**).

- **<u>Revised figure panels showing new aspects of the data requested by the reviewers:</u>**
  - **Fig.1e,f** now show the frequency of nocifensive behavior bouts during the post-test of PAC.
  - **Fig.1h,i** display labeled ACC neurons and the density of these neurons in both the Ctrl and Cond groups.
  - **Fig.1k** shows the density of ACC neuron terminals found in each brain area in both the Ctrl and Cond groups.
  - **Extended Data Fig.4e,f** display the activity of rACC→Pn neurons during Pre, Day 5, and Day 6.
  - **Extended Data Fig.4g,h** show the proportion of cross-day-aligned rACC→Pn neurons exhibiting increased activity, compared across different phases of PAC.
  - **Extended Data Fig.5g,i** show the results of correlation analysis between the Ca^{2+} activity of rACC→Pn neurons and the locomotion of mice during the pre-test.
  - **Extended Data Fig.5j** shows the activity of rACC→Pn neurons for up to 10 seconds after the first crossing from chamber 1 to chamber 2 on the post-test day.
  - **Extended Data Fig.7a-j** show the Ca^{2+} activity of rACC→Pn neurons during innocuous mechanical, noxious mechanical, or noxious heat stimulation, by classifying these cells into two clusters based on their activity.
  - **Extended Data Fig.7k,i** show the Ca^{2+} activity of rACC→Pn neurons during licking and rearing behaviors.
  - **Extended Data Fig.10d** shows the change in paw withdrawal frequency in response to 6 different von Frey filaments during optogenetic manipulation of the rACC→Pn pathway.

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We appreciate the reviewer's positive and constructive comments.

Major comments:

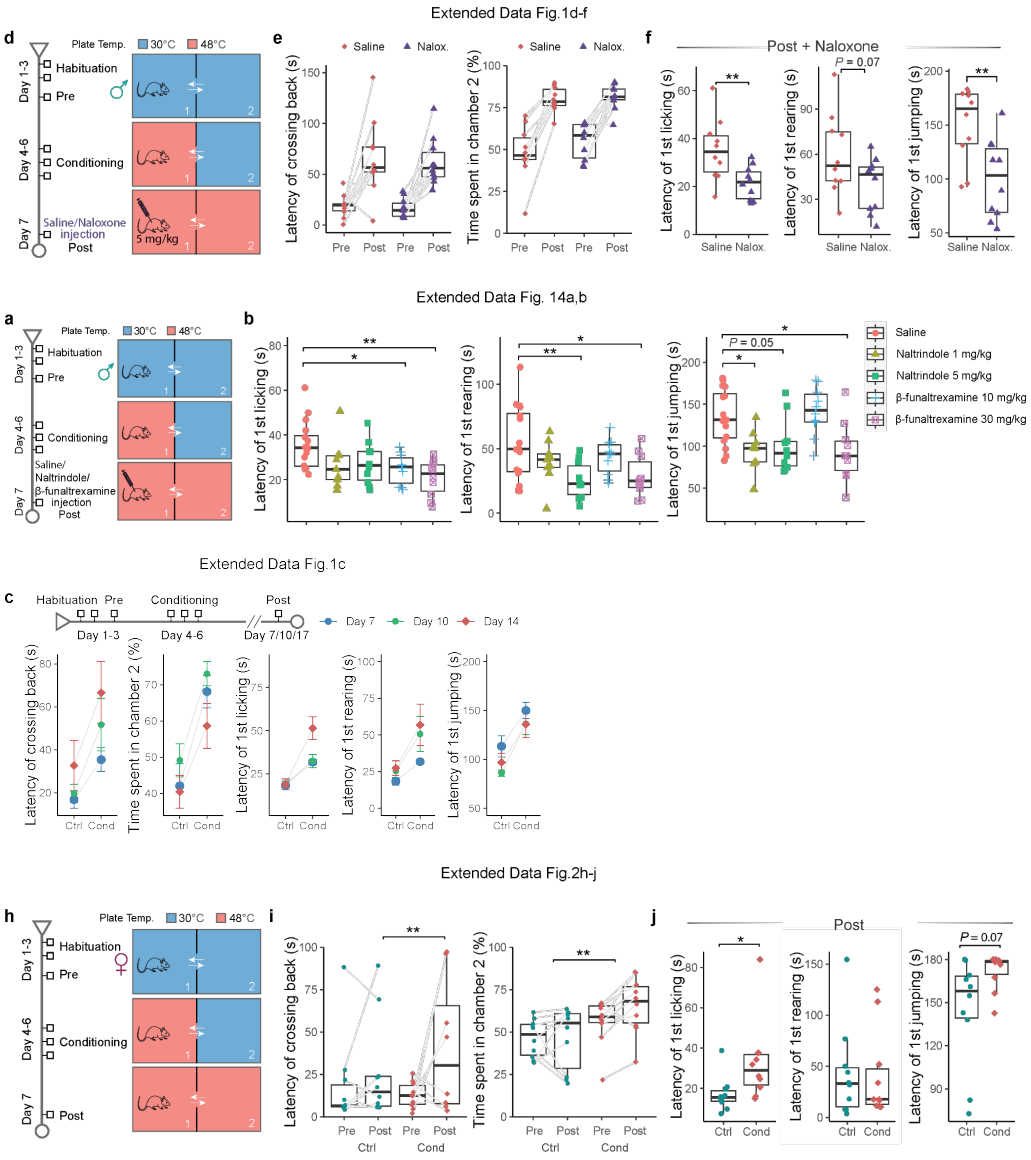
1. I suggest they drop the term placebo for their findings since this will just create confusion. Frankly they are not modeling human placebo analgesia but "pain relief expectation". The title also needs to be changed to something like "A cortico-cerebellar circuit modulating anticipatory pain"

We thank the reviewer for this suggestion and for noting that our behavioral assay models pain relief expectation. Interestingly, the terms "pain relief expectation" and "anticipatory pain relief" are also used in the literature on human placebo analgesia to describe and characterize this phenomenon [1–4]. Thus, in human placebo research, expectations have long been emphasized as crucial to the generation of placebo effects [5–10]. Similarly, it is widely accepted that expectation of pain relief drives placebo analgesia [1,9,10]. Crucially, our mouse behavioral paradigm faithfully recapitulates essential features of human placebo analgesia, including: its dependency on endogenous opioids (Extended Data Fig.1d-f and new data in Extended Data Fig.13a,b), the long-lasting analgesic effect (new data in Extended Data Fig.1c), and sex differences (new data in Extended Data Fig.2h-j). Please see the corresponding Figure panels below.

Nevertheless, we appreciate the complexity of human placebo analgesia and have modified the introduction accordingly. We have also included a new paragraph in the Discussion section explicitly acknowledging the distinctions and limitations of our model compared to human placebo analgesia.

Overall, although we concur with the reviewer that the PAC-induced analgesic effect in our mouse model may not be as complex as human placebo analgesia, PAC recapitulates fundamental features

of human placebo analgesia, and thus serves as a useful rodent model of the human phenomenon. Therefore, to clearly communicate the subject of the study for a broad audience, we believe that in this manuscript it is appropriate to use the broader term “placebo” analgesia. However, we will respectfully defer to the Editor's final judgment in this regard.



2. A set of published data suggests that placebo analgesia serves as a learning experience where patients get clinical benefit via contextual conditioning without prior exposure to the treatment itself (Colloca et al., 2013 Pain). However, in this study, the PAC paradigm subjects the animals to the “treatment” box (30C) during the 3 days of the conditioning phase and therefore it is directly only implicating an anticipatory behavior and not acute placebo analgesia. The authors need to carefully explain the differences between their approach and the standard appearance of placebo analgesia and can certainly conjecture in the Discussion that while it may contribute, it is not the same as a human placebo.

We appreciate the reviewer's comment and agree with the observation that “patients gain clinical benefit via contextual conditioning without prior exposure to the treatment itself.” In humans, factors such as social learning and verbal suggestion can indeed create an expectation for pain relief, which is crucial for initiating placebo analgesia. However, it is important to note that these expectations are generally rooted in previous experiences in which pain was successfully alleviated by some form of analgesic treatment, independent of any specific study. As a result, the efficacy of placebo analgesia can vary significantly across individual subjects due to their diverse past experiences with pain relief.

To control for such variability, many human placebo analgesia studies (as also discussed in Colloca et al., 2013) include a conditioning phase [11–13]. For example, an inert cream might be applied while the temperature of a thermal probe is reduced, and subjects are informed that the cream is analgesic. This approach is analogous to our PAC model, in which, as the reviewer noted, a “treatment” chamber serves to condition pain relief expectations.

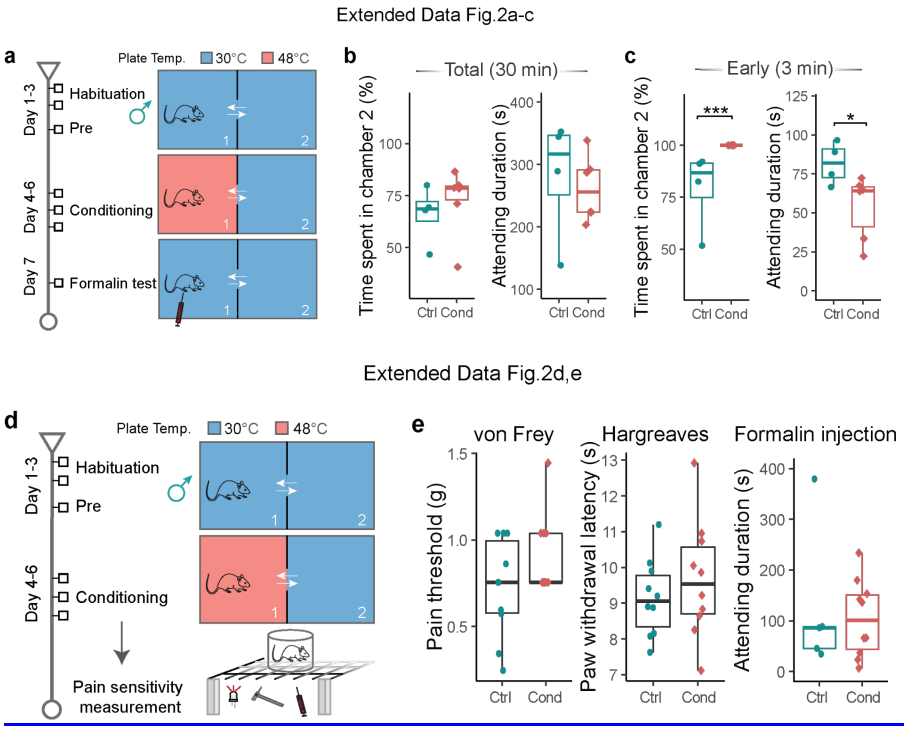
Nonetheless, we acknowledge the importance of distinguishing between our animal model and human placebo analgesia. To address this and the previous comment, we have modified the introduction and incorporated a thorough delineation of the differences and constraints of our model in comparison to human placebo analgesia in the Discussion.

3. Does the conditioning change other pain-related behaviors in response to mechanical or chemical stimuli?

This is an insightful and important question. We agree that it would be intriguing to explore whether pain relief expectation conditioned by thermal pain during PAC can produce generalized analgesia against other pain modalities. While our current PAC apparatus does not permit the examination of mechanical pain, it does allow for the assessment of chemical pain. Thus, we investigated how mice responded to formalin paw injections on the post-test day of PAC, with both plates set to innocuous 30°C (Extended Data Fig.2a). When quantified over the course of the entire 30-minute trial, we found that the total time spent attending to the affected paw was similar between the Ctrl and Cond groups, and mice in both groups spent a comparable amount of time in chamber 2 (Extended Data Fig.2b). Notably, however, in the first 3 minutes of the trial, mice in the Cond group demonstrated a stronger preference for chamber 2 after formalin injection and exhibited reduced attending behavior compared to those in the Ctrl group (Extended Data Fig.2c). These observations indicate that pain relief expectations conditioned by thermal pain during PAC can indeed reduce sensitivity to other pain modalities within the context of the PAC apparatus.

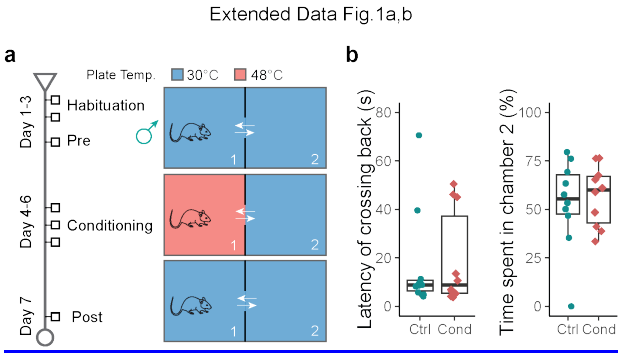
Further, we conducted experiments to determine whether mice show alterations in their sensitivity to thermal, mechanical, and/or chemical pain following the PAC conditioning phase, but outside of the PAC apparatus. These data show no noticeable change in sensitivities to thermal, mechanical, or chemical pain when the tests were conducted outside of the PAC environment (Extended Data Fig.2g-h). These findings suggest that the analgesia induced by PAC is specifically due to an expectation of pain relief associated with chamber 2 of the PAC apparatus.

We thank the reviewer for this constructive question and have incorporated these data into the revised manuscript.



4. The post-conditioning test of PAC used noxious heat (48 deg) for both chambers, if this assay captures pain relief anticipation, animals shouldn't prefer chamber 2 when both chambers are set to a warm temperature (30 deg) during the test phase. Please confirm if this is the case.

We agree with the reviewer and have conducted this experiment. The results, as shown below, are in line with the expectation that the preference for chamber 2 is absent when both chambers are set at 30°C during the post-test of PAC. The results have been incorporated into the revised manuscript (Extended Data Fig.1a,b).



5. Naloxone should be given during the conditioning phase to show that the PAC paradigm promotes endogenous opioid signaling in mice.

We appreciate the reviewer's recommendation. We considered performing this experiment before our first submission but initially had reservations about it. First, we were concerned that, regardless of the experimental outcome, the stress caused by multiple days of injections could confound interpretation of the results. Second, investigations into the role of the endogenous opioid system in human placebo analgesia typically involve administration of naloxone during the testing phase. Thus, to enable more direct comparisons between our study and these human studies, we chose to administer naloxone during the testing phase of PAC.

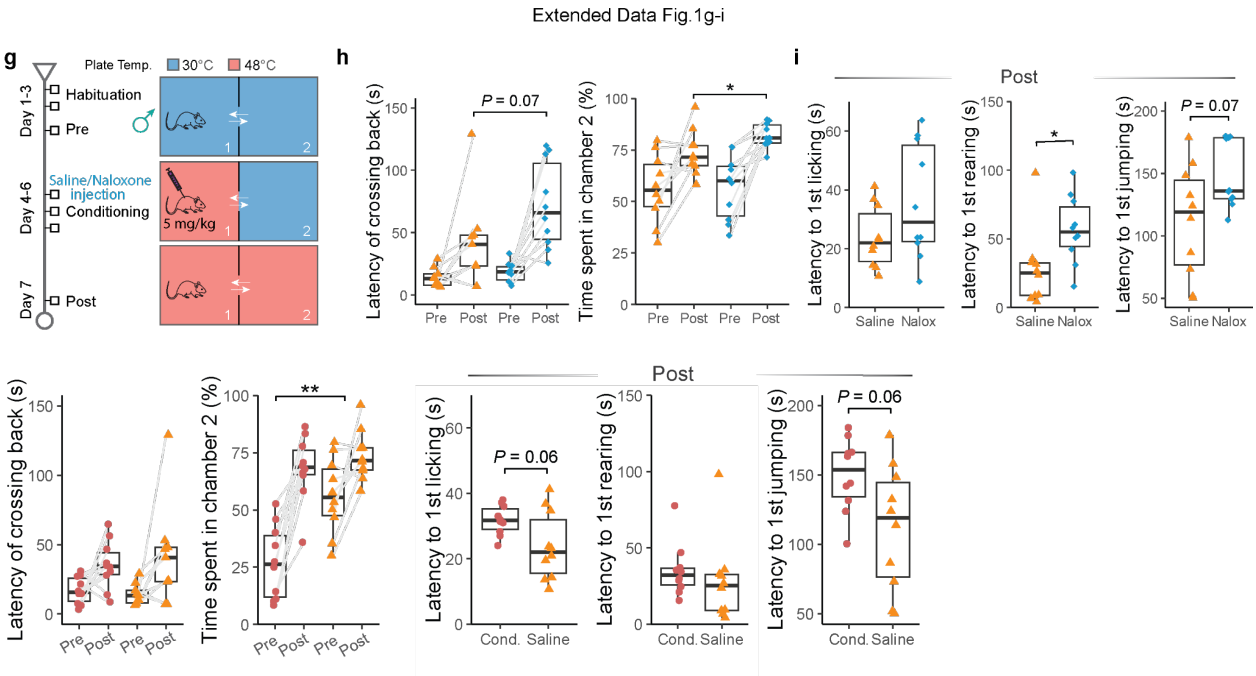
However, we concur with the reviewer regarding the importance of investigating the role of the endogenous opioid system in shaping pain relief expectation, particularly given that such studies are challenging in humans, and have performed the suggested experiment during the revision period. We injected naloxone (5 mg/kg) or saline (control) each day during the conditioning phase, 30 min prior to each PAC trial, to probe the involvement of the endogenous opioid system during the conditioning phase of PAC.

We found that, compared to mice injected with saline prior to each conditioning trial of PAC, mice injected with naloxone spent significantly more time in chamber 2 (Wilcoxon rank-sum test, $P < 0.05$) during the post-test, and also showed a trend of increased latency to revisit chamber 1 (Wilcoxon rank-sum test, $P = 0.07$), as shown below and in Extended Data Fig.1g-i. Naloxone injection had no significant effect on the latency of licking (Wilcoxon rank-sum test, $P = 0.17$), but significantly increased the latency of rearing (Wilcoxon rank-sum test, $P < 0.05$) and produced a trend toward prolonging the latency of jumping (Wilcoxon rank-sum test, $P = 0.07$) behaviors.

However, these differences between naloxone and saline injected groups are challenging to interpret, because multiple factors may affect endogenous opioid signaling during this experiment. Specifically, these altered behaviors could result from the stress associated with three consecutive

days of injections and the well-known phenomenon of stress-induced analgesia, which also involves the endogenous opioid system [14,15]. Thus, in saline-injected mice, stress-induced analgesia could reduce pain perception on the hot plate during the conditioning phase, weakening associative learning and PAC-induced analgesia (please see direct comparison between the saline-injected group and non-injected group provided below). In contrast, in naloxone-injected mice, naloxone would block endogenous opioid signaling, thereby limiting stress-induced analgesia during conditioning, resulting in similar PAC-induced analgesia compared to mice with no injection during conditioning (Fig.1b-d). Indeed, this similarity between the analgesic responses of mice with naloxone injection during conditioning and mice with no injection suggests that the endogenous opioid signaling plays no obvious role in the conditioning phase of PAC. Consistent with this interpretation, human studies suggest that neither associative learning nor conditional responses to pain cues depend on the endogenous opioid signaling [16].

We have incorporated these results into the revised manuscript (Extended Data Fig.1g-i).



6. Heterogeneous cell type recordings across different phases during PAC (Fig. 2) fail to clarify an association with endogenous analgesia versus anticipatory signaling on crossing the chambers. Please consider re-examining this experiment using the Oprd1Cre line, as for the RNASeq in Fig.5.

The reviewer raises important points.

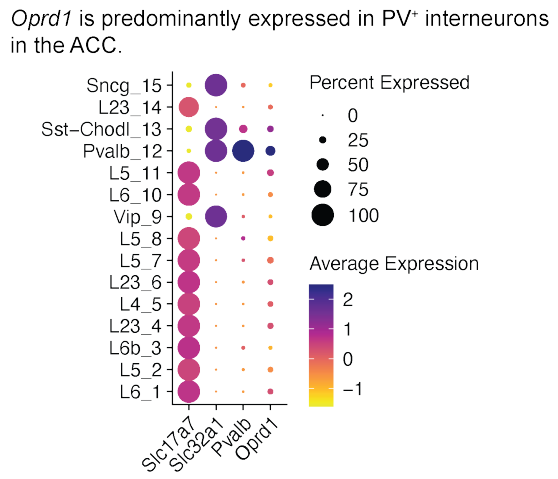
Regarding potential heterogeneity of the rACC→Pn neurons, we appreciate this concern. However, we note that previous studies have indicated that pyramidal tract neurons targeting specific subcortical areas are generally homogeneous, both genetically [17] and in terms of morphology and function [18–20]. Therefore, we believe our approach targets a predominantly homogenous population of rACC neurons defined by their projection to the Pn.

Conceptually, if rACC→Pn neurons were associated with endogenous analgesia rather than anticipatory signaling, we would expect the activity level of these neurons to correspond to the intensity of the noxious stimulation, namely, the chamber temperature. However, we found that rACC→Pn neuron activity progressively increased over the course of the conditioning phase (Extended Data Fig.4d,e), despite that the noxious chamber temperature remains consistent throughout this phase. Similarly, on the post-test day, both chambers are set at 48°C, yet we observed different rACC→Pn neuron activity during the first border crossing from chamber 1 to chamber 2 versus the first crossing back (Fig. 2i-k). Together, the progressive increase during the conditioning phase and varying responses to identical noxious stimuli in both the conditioning and post-test phases suggest that the activity of these neurons is associated not with endogenous analgesia, but indeed with anticipatory signaling. Furthermore, we found that neither noxious thermal nor noxious mechanical stimuli alter the activity of rACC→Pn neurons compared to innocuous mechanical touch (Extended Data Fig.7), further suggesting that these neurons are not involved in endogenous analgesia.

While our findings suggest that rACC→Pn neurons are predominantly associated with the expectation of pain relief rather than with endogenous analgesia, we acknowledge that this was not made clear in our initial submission. Therefore, we have added a few sentences to both the Main and Discussion section of the manuscript to clarify this crucial point.

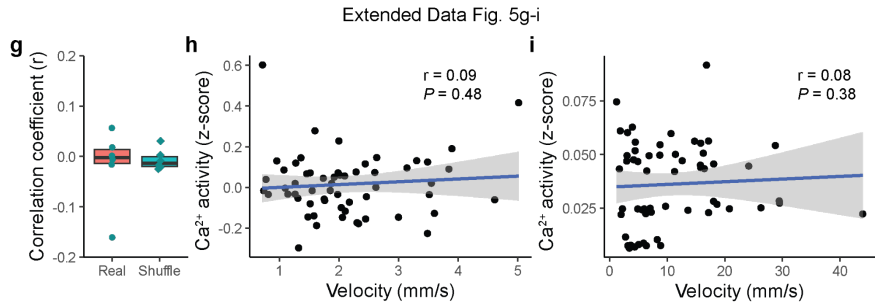
Additionally, based on our transcriptomic data (provided below) and our previous study [21], the majority of *Oprd1*$^{+}$ neurons in the rACC are parvalbumin-positive interneurons. Given this, using *Oprd1*^{Cre} mice would not effectively serve our goal of identifying the projection neurons in the rACC responsible for placebo analgesia.

Lastly, to clarify, the RNA-seq experiment was conducted using WT mice, not the *Oprd1*^{Cre} line. Our goal was to characterize the gene expression patterns across all neurons in the Pn, not only the *Oprd1*$^{+}$ neurons. We chose this approach to provide a comprehensive and publicly accessible dataset for the Pn, which remains a minimally explored brain region. Given that this dataset includes *Oprd1*$^{+}$ neurons and that we can filter these neurons based on *Oprd1* expression during our data analysis, we believe this broader approach offers substantial benefits for our research objectives.



7. Aligning neural firing to the time of crossing could represent a motor-related response. Please add a supplement control group (M1/2 to Pn) that relates functionally to locomotion and not anticipation of pain relief.

We thank the reviewer for this suggestion and agree that we should exclude this possibility. While imaging neurons in the motor cortex is one approach, we believe that our current data can effectively address this concern. Specifically, we conducted a correlation analysis between the Ca^{2+} activity of rACC→Pn neurons and the velocity of mice on the pre-test day across three different conditions: throughout the entire pre-test day, during intervals with pronounced Ca^{2+} activity of rACC→Pn neurons, and during periods when the mice were moving at high speeds. Our analysis revealed no significant correlation between these variables, which strongly suggests that the activity of rACC→Pn neurons is not principally associated with motor functions. We believe that this additional analysis should adequately address the reviewer's concerns, and we have incorporated these findings into the revised manuscript to clarify this important point (Extended Data Fig.5g-i).



8. Fig.4 evaluated sensory responses by optogenetically targeting a mixture of cell populations along ACC to Pn pathway, but not PAC-associated cell types. Please consider using FosCreER to label cells, using a similar strategy to that in Fig.1e for the behavioral experiments.

We appreciate the reviewer's insightful comment. As noted above, our approach to target rACC neurons projecting to Pn is unlikely to involve diverse cell types, given that previous studies have suggested these neurons are generally homogeneous, both genetically [17] and in terms of morphology and function [18–20].

Regarding the use of TRAP2 mutant mice, there are known challenges associated with this method, such as low labeling efficiency and biases in labeling efficiency between molecularly and functionally heterogeneous cell types [22]. Thus, we hope the reviewer will appreciate our rationale for choosing modulating axon terminals to directly and specifically test the function of the rACC→Pn pathway in pain (Fig.4 and Extended Data Fig.12d-f).

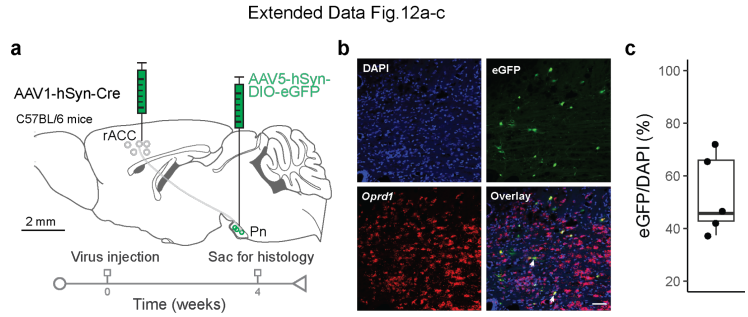
Nevertheless, we acknowledge the reviewer's point that not all rACC→Pn neurons may be involved in mediating placebo analgesia. To address this, we have incorporated further elaboration on this aspect in the revised Discussion.

9. Sequencing data were not derived from rACC-projected Pn neurons but a heterogeneous local population (Fig.5). What is the % of ACC projection neurons / local population neurons in the Pn?

We thank the reviewer for this comment. Yes, our single-cell RNA sequencing included all Pn neurons, not only the ones receiving rACC inputs. To clarify, as mentioned in another response above, our goal was to characterize the gene expression patterns across all neurons in the Pn to provide a comprehensive and publicly accessible dataset for the Pn, which remains a minimally explored brain region. We believe that this broad approach not only enriches the current understanding of Pn neurons but also lays the groundwork for studies that may wish to delve deeper into specific subsets of Pn neurons. Indeed, these gene expression data prompted us to further investigate the direct connection between rACC neurons and *Oprd1*$^{+}$ Pn neurons (Fig.5g,h and Extended Data Fig.12a-c) and examine the role of *Oprd1*$^{+}$ Pn neurons in PAC-induced analgesia (Fig.5i,j and Extended Data Fig.12g-j).

In addition, it is important to note the technical challenges inherent in selectively sequencing the Pn neurons that receive rACC inputs. To specifically target and dissect these neurons would require the anterograde transneuronal transport capabilities of the AAV1 virus [23]. However, the AAV1 virus exhibits limited labeling efficiency and specificity for this application, which could introduce complications in the experimental procedure and potentially confound the results.

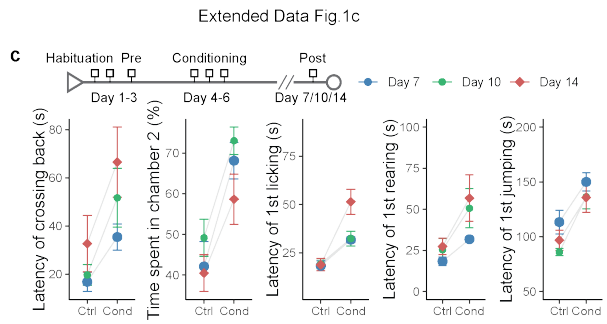
We can, however, use the AAV1 virus to answer the reviewer's question regarding the proportion of Pn cells receiving projections from the rACC. Specifically, we introduced an AAV1 virus encoding Cre into the rACC and an AAV5 virus expressing eGFP in a Cre-dependent manner into the Pn. This approach labeled approximately 53% of cells in rACC-projecting Pn regions with eGFP, suggesting that this proportion of cells receives inputs from the rACC. We have included these results in the revised manuscript (Extended Data Fig.12b,c), with the caveat that the AAV1 virus exhibits limited labeling efficiency and specificity for this application.



Minor comments:

1. In Fig.1 Placebo analgesia was tested only 1 day after conditioning. Please provide a longer temporal profile of the placebo effect (3-7 days) to validate PAC efficiency.

We thank the reviewer for these suggestions. In response, we conducted additional experiments to assess the post-test at additional time points: 3 days and 7 days after the conditioning phase of PAC. Our results revealed that the analgesic effect induced by PAC persists for at least 7 days following the conditioning phase, notably analogous to the persistence of human placebo analgesia [24–26]. These findings have been incorporated into the revised manuscript (Extended Data Fig.1c).



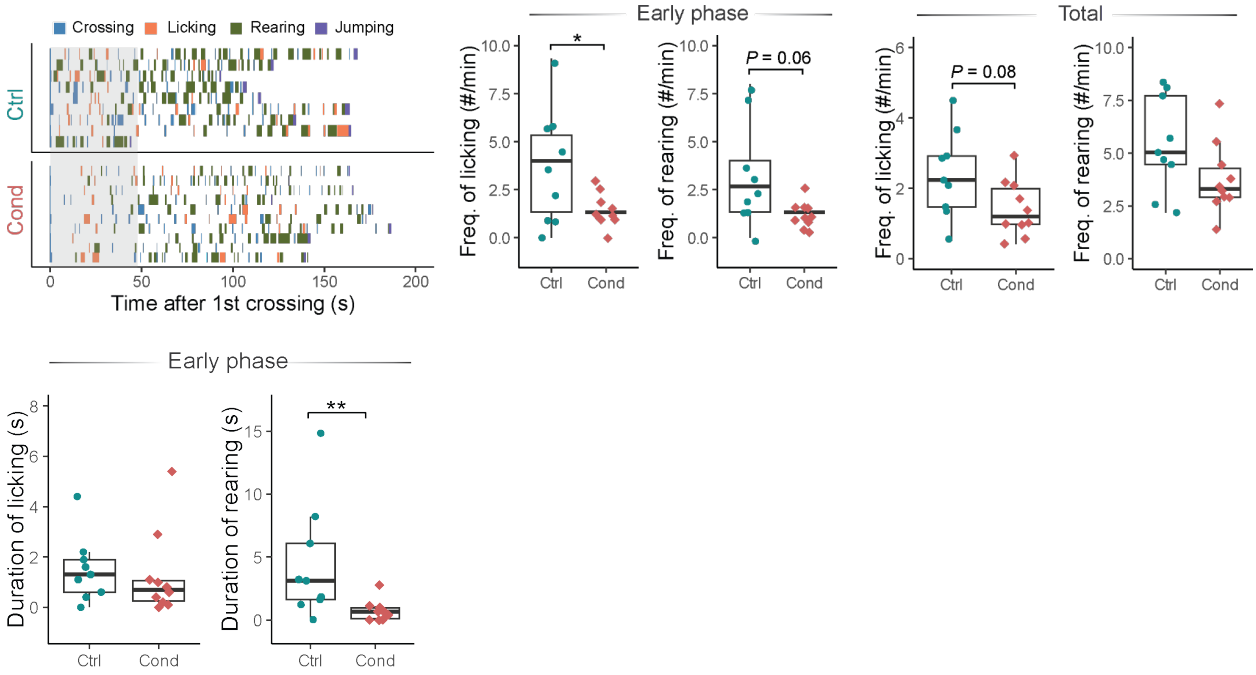
In addition, the frequency of behavior bouts is critical besides latency, so incorporate this metric as well.

We also concur with the reviewer regarding the importance of including the frequency of behavior bouts as a data point. To address this, we have generated a raster plot that displays the nocifensive behaviors of Ctrl versus Cond mice throughout their respective recording periods (Fig.1e). This raster plot captures the reduction in both frequency and duration of these behaviors. Additionally, it highlights the period before the violation of pain relief expectation on the post-test day, when mice perceive that chamber 2 is unexpectedly hot.

Based on these data, we estimated the duration of PAC-induced analgesia to be roughly 45 s after arriving in chamber 2. Accordingly, we calculated the frequency of nocifensive behavior bouts over two time periods: the early phase (*i.e.*, the first 45 s after crossing) and the total duration (*i.e.*, the entire time spent in the PAC apparatus after the first border crossing).

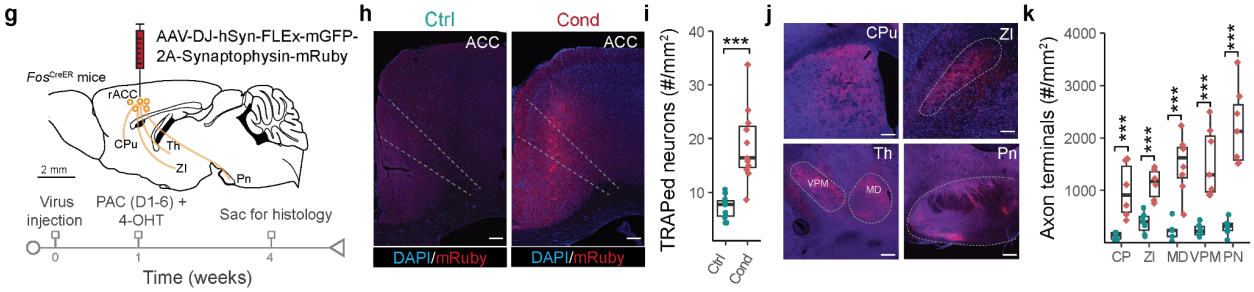
Over the entire recording period, mice in the Cond group tended to show less licking (Wilcoxon rank-sum test, $P = 0.08$) and rearing (Wilcoxon rank-sum test, $P = 0.11$) behavior compared to the Ctrl group, although this difference was not statistically significant. In the early phase, however, the Cond group exhibited significantly less licking (Wilcoxon rank-sum test, $P < 0.05$), and significantly shorter duration of rearing behaviors (Wilcoxon rank-sum test, $P < 0.01$).

In the revised manuscript, we have included three additional boxplots summarizing these findings (Fig. 1f). Note that, in order to prevent tissue damage, we terminated the experiment if mice began to jump. For this reason, we could not compare the frequency of jumping behavior between the groups.



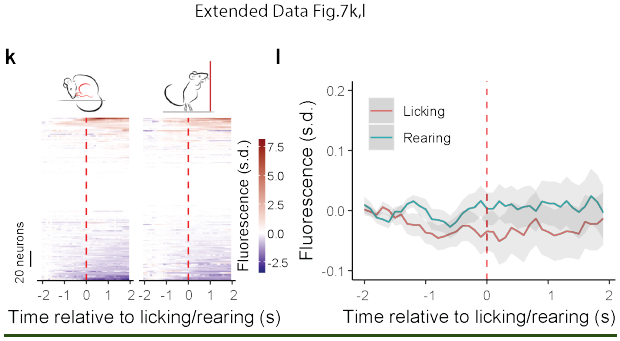
2. Fig.1f and g need quantification of the histology.

We appreciate the reviewer's suggestion. In response, we conducted a comparative analysis of the density of labeled neurons in the ACC between the Ctrl and Cond groups. We also examined the density of mRuby signals, indicative of putative axonal terminals, in each targeted brain region for both groups (Figure 1g-k).



3. Please record and compare activities during 1st licking, rearing, and jumping on post-PAC to validate the encoding of sensory reflex.

We thank the reviewer for this recommendation. We performed this analysis and found no significant change in the activity of rACC→Pn neurons during licking or rearing behaviors during post-test. These data align with our finding that neither noxious thermal nor noxious mechanical stimulation alters the activity of rACC→Pn neurons compared to innocuous mechanical stimulation (Extended Data Fig.7), suggesting that these neurons do not encode nocifensive behaviors. Mice with miniscope implants rarely displayed jumping behavior during the 3-minute experiment, so we could not evaluate rACC→Pn neuron activity during jumping. These new data are presented in Extended Data Fig.7k,l of the revised manuscript.



4. The feedforward inhibition mechanism (fig.3i-3l) can explain why rACC neurons' firing pattern changes from tonic to phasic, but cannot explain why they fire more action potentials in the beginning (line 218, fig.3b), do the authors have another interpretation for this?

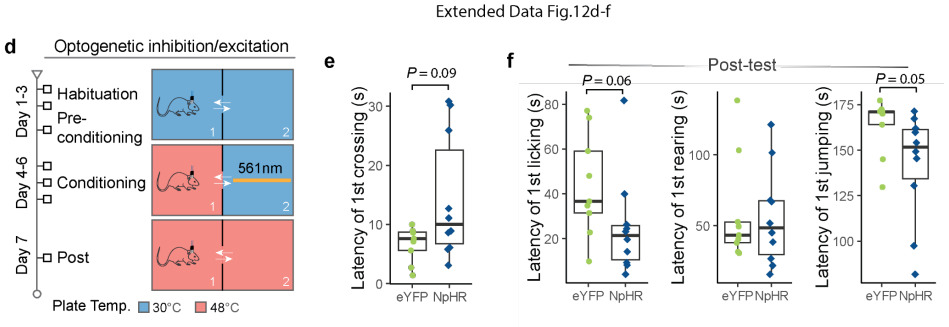
The reviewer raises an important point. An increased number of action potentials is indicative of burst firing, a dynamic state in which a neuron repeatedly fires discrete groups ("bursts") of spikes. Burst firing can facilitate physiological functions such as synaptic transmission, synaptic plasticity, and signal encoding. The observed increase in burst firing is consistent with our calcium imaging and electrophysiological data suggesting enhanced activity of rACC→Pn neurons following PAC-conditioning. Although our study did not specifically investigate how reduced fast feedforward inhibition (FFI) affects burst firing, previous research [27–29] has demonstrated the regulatory role of FFI in burst firing. We have added text to the Discussion section to clarify this interpretation.

5. The second and fourth data points in NpHR group in Fig.4f seem to have significance compared to eYFP and ChR2. Please reanalyze the statistics.

We thank the reviewer for bringing this to our attention. After conducting a thorough reanalysis of these data, we confirm that there is no significant difference for the second data point between groups (two-way ANOVA, $F_{2, 156} = 72.846$, $P < 0.001$; Tukey post-hoc test, $P = 0.71$ for eYFP and NpHR comparison, $P = 0.99$ for eYFP and ChR2 comparison, $P = 0.24$ for ChR2 and NpHR comparison). The fourth data point also shows no significant difference between NpHR and eYFP groups (two-way ANOVA, $F_{2, 156} = 72.846$, $P < 0.001$; Tukey post-hoc test, $P = 0.43$ for eYFP and NpHR comparison, $P < 0.001$ for eYFP and ChR2 comparison, $P < 0.001$ for ChR2 and NpHR comparison).

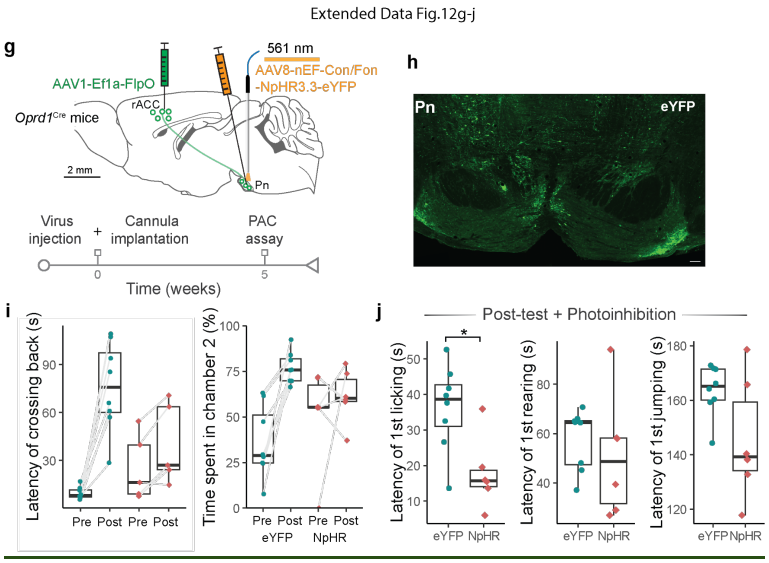
6. NpHR inhibition of Oprd1+ neurons (fig.4j) should also be done during conditioning.

We agree with the reviewer that it would be interesting to examine whether *Oprd1*$^{+}$ Pn neurons contribute to learning during the conditioning phase of PAC. Thus, we targeted *Oprd1*$^{+}$ Pn neurons by injecting a virus expressing the inhibitory opsin eNpHR into the Pn of *Oprd1*^{Cre} mice, followed by the bilateral implantation of optical fibers into the Pn. We found that photoinhibition of *Oprd1*$^{+}$ Pn neurons during the conditioning phase of PAC prolonged the latency of mice to cross the border and diminished the analgesic effect observed during the post-test, suggesting that *Oprd1*$^{+}$ Pn neurons also contribute to the conditioning phase of PAC. These findings have been incorporated into the revised manuscript (Extended Data Fig.12d-f).



7. Please map rACC-Pn projection-specific population in Oprd1Cre mice and repeat the optogenetic experiments in Fig.5 i and j.

We concur with the reviewer that investigating the involvement of *Oprd1*$^{+}$ Pn neurons receiving inputs from the rACC would provide greater specificity in understanding their contribution to placebo analgesia. To address this, we can utilize the anterograde transneuronal transport capabilities of the AAV1 virus to specifically target these *Oprd1*$^{+}$ Pn neurons (Extended Data Fig.12g). However, noting that the AAV1 virus has limited labeling efficiency and specificity for this purpose, we conducted this experiment with a small group of mice. We found that optogenetic inhibition of *Oprd1*$^{+}$ Pn neurons receiving ACC inputs diminished PAC-induced placebo analgesia, albeit only significantly for licking nocifensive behaviors, potentially due to the technical limitations of the approach and the small number of mice used in this study. We have incorporated these findings into the revised manuscript (Extended Data Fig.12g-j).

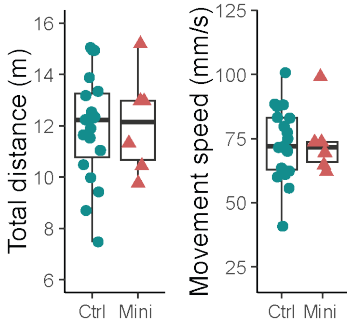


8. Is it correct that cortical tissue was aspirated above to implant the GRIN lens? Does that mean that the upper layers of the cortical column of the ACC are gone? If so, does this change the output of the ACC-PN neurons?

We thank the reviewer for highlighting this point. Indeed, when implanting a GRIN lens, a small amount of cortical tissue must be aspirated to accommodate the lens. This is standard procedure for this technique [30], which has been similarly employed in hundreds of studies.

While there is some disruption to the upper layers of the cortical column during implantation, the procedure has been optimized to minimize damage. Importantly, in our study, we observed no noticeable alterations in the general behavior of mice post-surgery. Specifically, we observed no differences in walking distance or moving speed between mice without miniscopes (Ctrl) and those with miniscopes (Mini) during the first day of PAC. We have included these data into the revised manuscript for clarification (Extended Data Fig.4d).

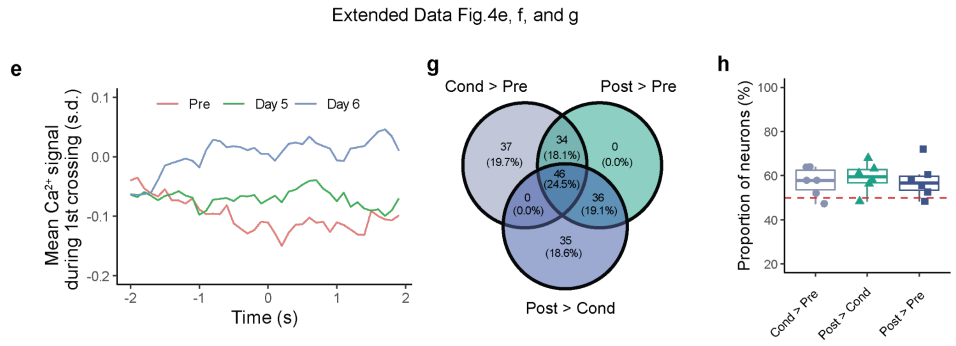
Extended Data Fig.4d



9. The relationship between calcium activity and crossing behavior in Figure 2d, is hard to interpret. You might expect high activity at -2s and then lower or sustained activity moving to a crossing event if it signaled pain-relief expectation. Rather, it seems like the number of neurons that have a high level of activity increased after training.

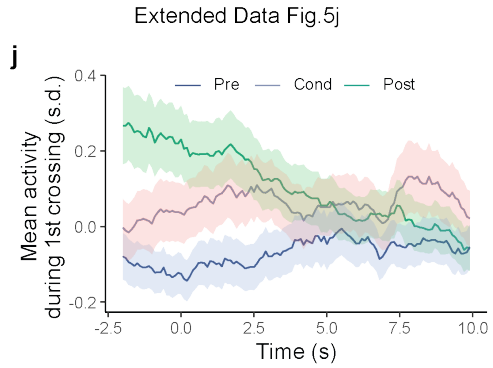
We concur with the reviewer's expectation that rACC→Pn neurons should exhibit sustained activity during anticipation of pain relief. Both our heatmap (Fig. 2d) and an updated figure (Extended Data Fig.4e), which displays the activity of rACC→Pn neurons against time during the Pre, Cond, and Post phases, support this hypothesis. On the topic of comparing the number of cells with heightened activity, the challenge arises from the inherent variability between individual neurons and the subjective nature of defining a high level of activity. However, our ability to track individual neuron activity across days enabled us to determine that 57.8% of the cross-day-aligned rACC→Pn neurons displayed increased activity post PAC-conditioning (57.8% ± 3.3%, one-sample *t*-test, $P < 0.05$;

Extended Data Fig.4g,h). This suggests that during pain relief expectation, both the number of highly activated neurons and the activity of several individual rACC→Pn neurons are increased. We have incorporated these findings into the revised manuscript.



What is the activity of these neurons like at longer time windows, say up to 10 seconds?

To address the reviewer's query regarding longer time windows, we have included a new plot (Extended Data Fig.5j) describing the activity of these neurons for up to 10 seconds after the first crossing from chamber 1 to chamber 2 on the post-test day. We note, however, that mice exhibited different behaviors post border crossing, which might influence the interpretation of these data. Nonetheless, we observed a decline in the activity of rACC→Pn neurons post border crossing during the Post phase, but not during the Pre or Cond phases, aligning with a diminished pain relief expectation due to the unexpectedly hot chamber 2 during the post-test.

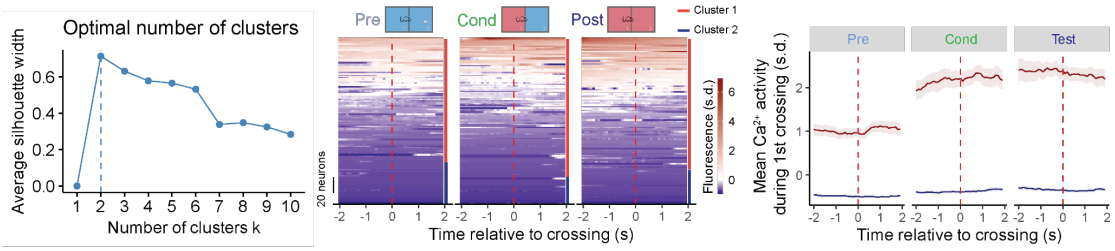


Are there subtypes of neurons that cluster differently across time?

We appreciate the reviewer's question. Based on our heatmap plots, rACC→Pn neurons do display diverse activity during crossing (Fig.2d,i). One approach to answer this question would be to perform

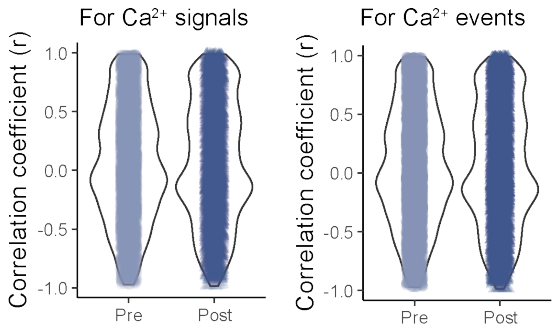
a clustering analysis to categorize rACC→Pn neurons into distinct subtypes based on the activity pattern of each neuron during pain relief expectation. To do this, we employed K-means clustering, an unsupervised machine-learning algorithm. First, we used silhouette analysis to determine the optimal number of clusters for our dataset, which we found to be 2 (provided below). K-means clustering successfully classified the rACC→Pn neurons into 2 clusters based on their activity pattern during the first crossing from chamber 1 to chamber 2 during Pre, Cond, and Post. This analysis revealed that while one cluster of neurons exhibited progressively increased activity during PAC, the other cluster showed stable activity across different phases of PAC, aligning with our previous findings at the population level (Fig.2e,f).

Nevertheless, given that we imaged a likely homogenous population[17–20] and that the primary aim of our study is to investigate the collective behavior of the rACC→Pn neuron population in pain perception, we have decided not to include this clustering analysis in the revised manuscript in order to prevent any potential misinterpretation of the data.



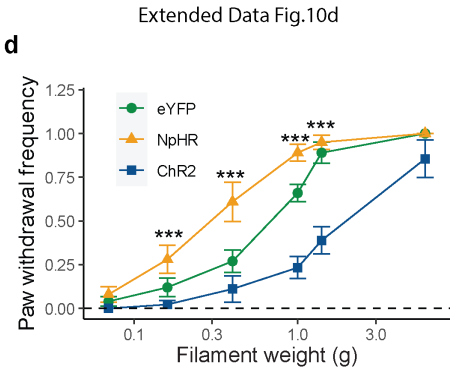
10. The data in Ext Fig 6, where non-PN projecting neurons display reduced activity compared to PN projecting neurons, suggests that PAC induces a change in the local network connectivity similar to that which occurs in S1 (Okada et al., 2021 Sci. Adv). Do you see this in rACC?

This paper (Okada et al., 2021) suggests that pain enhances the synchronization of neurons in the primary somatosensory cortex (S1), which is very interesting. We have conducted correlation analysis of both the Ca^{2+} fluctuations data and events data of the rACC→Pn neurons to examine their synchronization after PAC. However, we observed no significant increase in the correlation coefficient of rACC→Pn neural activity after PAC (data provided below).



10. Figure 4f: please show raw frequency value curves rather than delta.

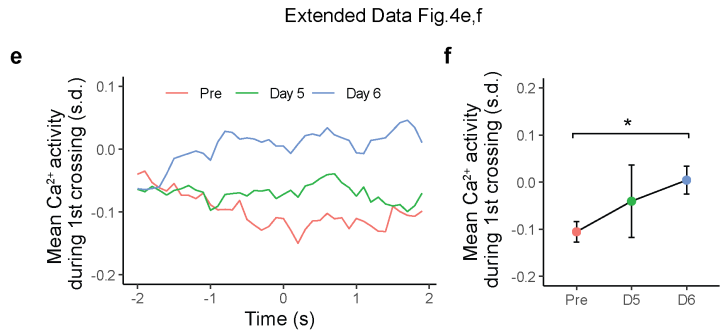
We thank the reviewer for this suggestion. In our original submission, after careful consideration, we decided to compare the change in light-induced withdrawal frequency between the eYFP, ChR2, and NpHR groups. Since each group encompasses two distinct conditions (light on and light off), we considered that using raw frequency would only allow us to present data from the light on condition for each group, whereas delta values allow us to provide a more comprehensive analysis by incorporating both conditions. However, we appreciate the merit of also showing raw frequency values as suggested by the reviewer, and have included a new figure in the revised manuscript to provide further clarity (Extended Data Fig.10d).



11. The authors claim the Ca2+ activity of rACC→Pn neurons increased progressively during PAC which is consistent with the learning-based nature of placebo analgesia'(lines 208-209). But they only showed a single time point during conditioning (day 6) for the imaging data (fig.2e,f). Have the authors done imaging on d4 and d5 as well? If yes, please show the data.

We thank the reviewer for bringing this issue to our attention. We have conducted this analysis and observed a progressive increase in mean activity of rACC→Pn neurons when comparing data from Day 3 (-0.11 ± 0.02 z-score), Day 5 (-0.04 ± 0.08 z-score), and Day 6 (0.004 ± 0.03 z-score). Additionally, we observed a higher variation in activity on Day 5 compared to Day 6, possibly

reflecting initial instability in learning and memory processes. We did not include Day 4 in this analysis because, during the first border crossing on Day 4, mice have not yet had an opportunity to form an expectation of pain relief. We have included these data in the revised manuscript (Extended Data Fig.4e,f).



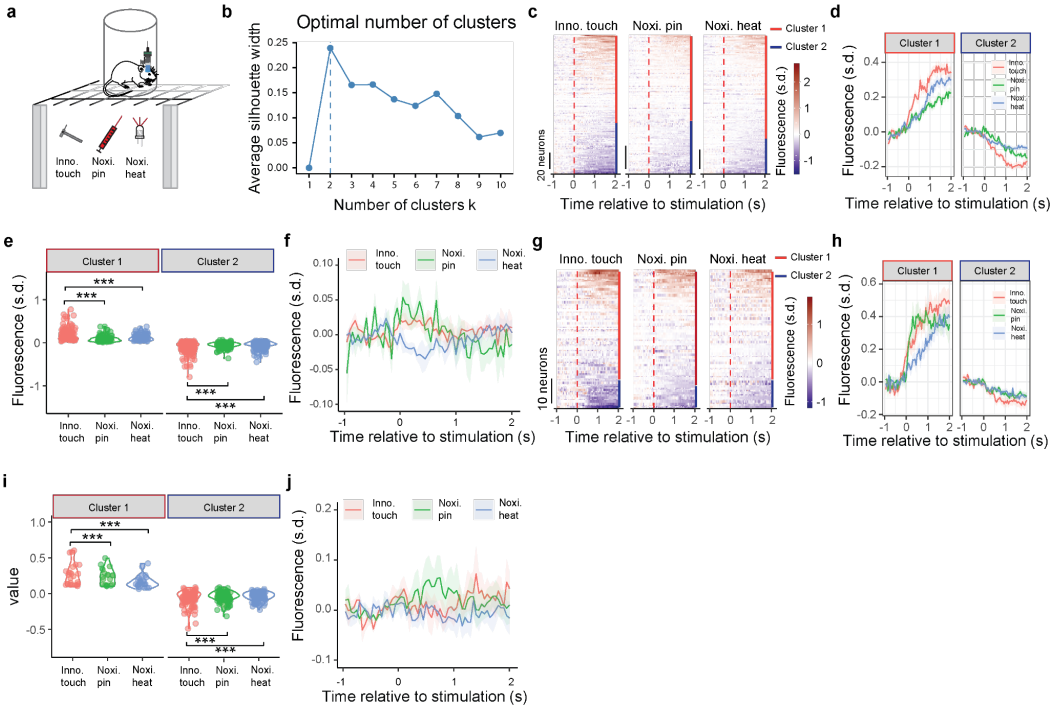
12. In Extended Data Figure 5, the argument is made that rACC-PN neurons do not display significant increases in activity but the heatmaps presented in 5b and d look as though there are populations that increase in activity and decrease in activity and are time-locked to the stimulus.

We agree with the reviewer that, based on the heatmaps, some rACC→Pn neurons showed increased or decreased activity time-locked to the stimulation. Accordingly, to further investigate the activities of these neurons during innocuous and noxious stimulation, we performed K-means clustering analysis. We found that, for each stimulus type (innocuous mechanical, noxious mechanical, or noxious heat stimuli), rACC→Pn neurons can be classified into two distinct subgroups, with one showing increased activity and the other showing decreased activity in response to the stimulation, as shown below.

Upon separate comparison of these two rACC→Pn neuron groups, we found that they exhibited similar patterns of activation and inhibition in response to either noxious mechanical or noxious thermal stimuli in the pinprick test or Hargreaves test, respectively. Interestingly, innocuous mechanical stimulation with a blunted needle triggered more pronounced activation and inhibition in these neurons, resulting in a comparable level of activity at the population level when compared to responses from noxious mechanical or thermal noxious stimuli.

Although these data are intriguing, interpreting the differential activation of individual neurons with the same projection in response to a stimulus is challenging and requires further studies. Thus, given that the primary aim of our study is to investigate the collective behavior of the rACC→Pn neuron population in pain perception, we believe it is still appropriate to conclude, based on their population-level activity, that these neurons are not specifically involved in pain. We have included

these analyses in the revised manuscript to provide additional clarity and depth to our findings (Extended Data Fig.7a-j).



Referee #2 (Remarks to the Author):

This study describes a novel means of investigating expectation-related pain in a placebo paradigm to show that 'placebo' analgesia is mediated by a rostral ACC-pontine nucleus circuit that is more active when expectations of pain relief are experimentally induced. Proposed to be an opioidergic mechanisms owing to the high abundance of opioid receptors in the Pn and disruption of placebo analgesia when inhibited, the authors findings reveal a novel target for pain relief (the rACC-Pn circuit). The broad implications of the study, as a framework for investigating neural circuits involved in descending modulatory control, are important.

This is a highly original study with novel significance.

The combined use of a novel model to induce expectation-mediated analgesia, neural Ca imaging in behaving animals, single RNA seq, ephys and optogenetics leads to a highly sophisticated experimental approach.

The conclusions are in keeping with the high quality of the data and I believe them to be robust. Apologies if missed, but it would be prudent to share detail of labelling efficiencies for the viral targeted strategies.

This is a well written, clear and lucid paper.

We thank the reviewer for these positive remarks.

Concerning the labeling efficiency of the virus: our study utilized multiple viruses for purposes ranging from Ca^{2+} imaging, to tracing, to optogenetic manipulation. As is widely recognized, the labeling efficiency of viruses is governed by a multifaceted interplay of factors, including the virus's serotype and titer, the selected promoter, the targeted cell types, and others.Thus, guided by existing literature, we have carefully chosen each of the viruses used in our study, rigorously confirmed their expression prior to data collection, and invested significant effort into calibrating and optimizing these parameters. However, due to the inherent variability of these factors, providing a precise value for labeling efficiency is challenging. To address this, we have included a statement in our revised manuscript acknowledging the limitations in this context.

Referee #3 (Remarks to the Author):

In this manuscript by Chen and colleagues, an array of modern techniques are used to provide evidence for the involvement of an ACC-->Pn neural circuit subserving placebo analgesia in the mouse. On the plus side, this is to my knowledge the first proposed neural circuit for placebo, and the experiments here are expertly performed and convincing.

We appreciate the reviewer's complimentary remarks.

On the other hand, aspects of this work are not as novel as the authors claim, and I was left thinking that the most interesting finding here was only hinted at, not directly shown. Although the behavioral placebo paradigm here is "novel" in the sense that no one has ever done it quite this way before, this is nowhere near the first rodent model of placebo analgesia. Similarly, although it is claimed that the ACC-->Pn labeling was "unexpected" and that the pontine nuclei have "no known pain-related function", a cursory PubMed search turned up two papers showing ACC-->Pn connections using anterograde tracing (Shi et al., Mol. Pain, 2022; Fillinger et al., Brain Struct. Func., 2017). In addition, two fMRI studies have found pain-related activation of the pontine nuclei (Hubbard et al., 2016; Sclocco et al., 2016).

We thank the reviewer for highlighting these pertinent references. While we were aware of previous publications describing the ACC to Pn projection, we initially overlooked the fMRI studies demonstrating the activation of the Pn during pain. This being noted, however, we consider that in such fMRI studies, the Pn is one of a very large number of brain structures that are labeled as activated during pain perception, without establishing the specific contribution of these activation patterns to pain experience. Further, although the Pn has been reported as active during pain experience, the role of the Pn in pain relief expectation had not been interrogated. Similarly, ACC connectivity has been explored extensively in rodents, resulting in the reporting of numerous ACC outputs, including the Pn, but without establishing a causal relationship with pain or with pain relief expectation. Our intention was to convey that, compared to other ACC subcortical output regions that have been extensively studied in the pain field, such as the PAG or amygdala, the contributions of the Pn to pain modulation generally and pain relief expectation specifically have never been reported. To provide more background and clarity on this point, we have updated the text to include these references, and expanded our discussion on this topic in the revised manuscript.

Regarding our behavioral paradigm, we acknowledge that PAC is not the first rodent model of placebo analgesia. Nonetheless, the specific experimental design of PAC is novel. Compared to earlier models [31–38], PAC confers simplicity and repeatability while eliminating the need to introduce a pharmacological treatment. Crucially, PAC-induced placebo analgesia replicates several key features of the human phenomenon, including reliance on the endogenous opioid system (Extended Data Figs.1d and 13a), persistence of the placebo effect over time (Extended Data Fig.1c), and sex differences (Extended Data Fig.2h-j). These features, to our knowledge, have not been reported with previous models. These advantages position PAC as a uniquely effective model for in-depth mechanistic investigations of placebo analgesia. However, we acknowledge the need for greater clarity on this point and have thoroughly explained which aspects of PAC are novel in the revised manuscript.

While it is certainly interesting to nail down a circuit involving the pontine nuclei related to placebo analgesia, what I was really interested to see was direct evidence for involvement of the cerebellum in placebo analgesia. That was never shown here in any way. I realize I'm asking a lot here, of course, but in 2023 aren't neural circuits from limbic regions to brain stem regions a dime a dozen?

We appreciate the reviewer's insightful comment and agree that providing the first direct evidence for the involvement of the cerebellum in placebo analgesia would further increase the impact of our study. In response, we have dedicated considerable effort during this revision period to imaging the Ca^{2+} activity of various cerebellar cell types in freely moving mice during PAC using a miniscope.

Toward this goal, we attempted to develop several preparations to image cerebellar calcium dynamics, including in the axon terminals of *Oprd1*$^{+}$ Pn neurons (diameter about 2–3 µm [39]) using *Oprd1*^{Cre} mice, in the somata of granule cells (diameter about 5–6 µm [40]) using *Math1*^{Cre} mice, or in the dendrites of Purkinje cells using WT mice. We have thus far encountered significant technical challenges associated with using a miniscope to image the terminals of *Oprd1*$^{+}$ Pn neurons or granule cell somata, primarily due to their small size. Similarly, one prior study [41] has reported difficulties associated with using a miniscope to image granule cell somata in *Math1*^{Cre} mice. However, we are pleased to report that we have successfully used a miniscope to image the dendritic Ca^{2+} activity of Purkinje cells during PAC.

Cerebellar Purkinje cells possess extremely elaborate dendritic trees, receiving excitatory synaptic input from a single climbing fiber (CF) originating from the inferior olivary, and from ~200,000 parallel fibers (PF) that relay information sent disynaptically from the Pn [42]. Spontaneous CF activity (1–2 Hz) triggers dendritic Ca^{2+} spikes that can pervade the entire dendritic tree, whereas PF inputs, depending on their activity level, can evoke more localized smaller to moderate dendritic spikes [43–46]. Moreover, when near coincident in time with CF input, PF activity can modulate the amplitude of the Purkinje cell dendritic Ca^{2+} spikes and lead to supralinear Ca^{2+} excitation [43,44,46,47]. Therefore, if the Pn indeed relays pain relief expectation from the rACC to the cerebellum via PFs, then PAC should increase the amplitudes of Purkinje cell dendritic Ca^{2+} spikes and the occurrence frequency of dendritic Ca^{2+} spikes that are large enough to be detected by Ca^{2+} imaging.

To test these predictions, we obtained data from 276 cross-day-aligned Purkinje cells, with an average daily total cell count of approximately 700 across 6 mice. To identify Purkinje cells that might encode pain relief expectation, we performed a classification analysis using the Ca^{2+} imaging data from these cross-day-aligned Purkinje cells (Extended Data Fig.14b-d). Based on Ca^{2+} activity during the first border crossing (with conditioned pain relief expectation), first crossing back (without conditioned pain relief expectation), and last border crossing (reduced or no conditioned pain relief expectation due to the expectation violation), the classification algorithm resolved two main classes of Purkinje cells (termed here clusters 1 and 2). Notably, we found that Purkinje cells of cluster 1, but not cluster 2, exhibited responses similar to those of rACC→Pn neurons during PAC, such as the progressively increased Ca^{2+} activity across days of PAC (Fig.6h,i) and elevated Ca^{2+} activity during first border crossing after conditioning, but not during the first crossing back or the last border crossing (Fig.6l-n and Extended Data Fig.14b-f) during the post-test. Importantly, Purkinje cells in cluster 1 displayed increased Ca^{2+} firing and more Ca^{2+} spikes with large amplitudes following PAC (Fig.6k and Extended Data Fig.14i). These findings support the idea that PF inputs active during the PAC assay drive the increased Ca^{2+} activity levels observed in Purkinje cells of cluster 1.

These data provide what we believe to be the first direct evidence, at the cellular level, for a role of the cerebellum in placebo analgesia, and in cognitive pain modulation more generally.

Numerous human functional brain imaging and animal studies have shown activation of the cerebellum during pain processing [48–50]. Generally, however, these cerebellar activity patterns during pain were considered to reflect the intent to move or subtle movements in the scanner, rather than pain experience itself. Thus, it has long remained unclear how and to what extent the cerebellum may participate in pain perception [51].

Our results show increased dendritic activity of Purkinje cells during pain relief expectation (Fig.6), revealing that the cerebellum plays a critical role in the cognitive-evaluative component of pain, consistent with recent studies suggesting the cerebellum's role in cognitive functions [52–55].By providing the first cellular-level evidence of the cerebellum's role in cognitive pain modulation and placebo analgesia, our work suggests that the cerebellum, through its extensive connections with the thalamus and critical nodes of the brainstem pain modulatory circuitry [56,57] such as the periaqueductal gray (PAG) and the rostral ventromedial medulla (RVM), may be an underexplored target for innovative pain management therapies. Thus, our study opens an entirely novel research direction for investigations of the circuit, cellular, neurophysiological, and molecular mechanisms by which the cerebellum modulates pain.

We thank the reviewer for prompting us to embark on this set of experiments which, although technically challenging, yielded worthwhile and highly impactful results.

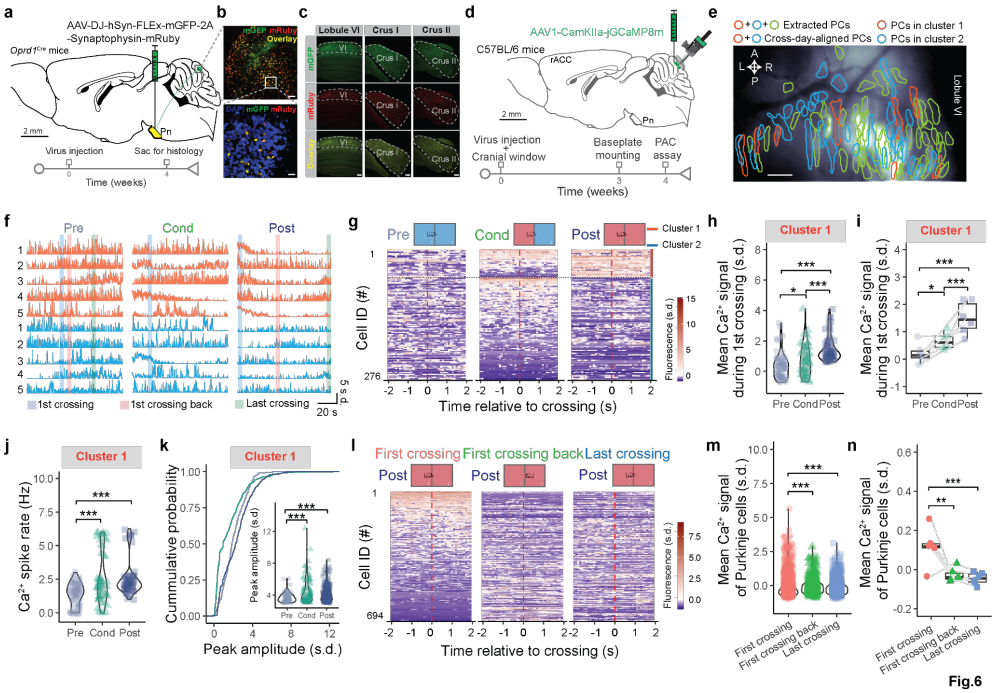
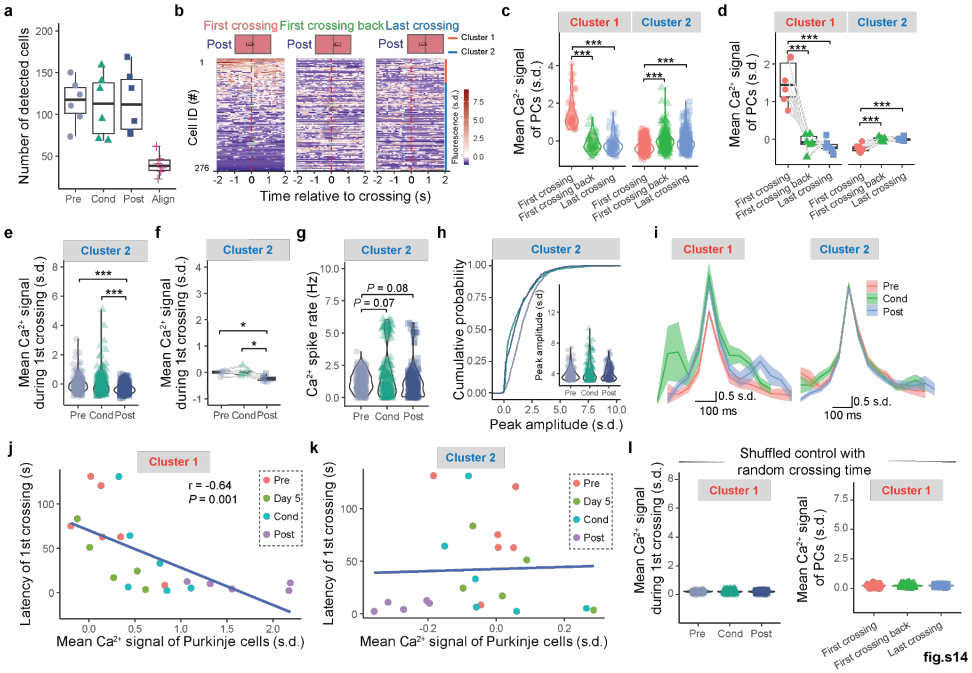


Fig.6



Another concern is related to the authors conceptualization of placebo as a "contextual, cue-based learning phenomenon". To my understanding that is only part of what placebo is, and the less-interesting part at that. Placebo can involve conditioning, yes. If I'm given a pill and told it's an analgesic, my history of successful analgesia against headaches of ibuprofen will have conditioned me to respond with analgesia to the pill. But placebo is also expectation and desire, and this requires no new learning. If I believe the doctor that this is an analgesic, and I want it to be an analgesic, it will be. This is the part that is difficult to model in animals. The authors have indeed set up an expectation using conditioning here, of course, which is fine if not novel. They refer to this expectation as one of "pain relief", and that the mice develop a "preference" for chamber 2. I think this is the wrong way to think about it. Isn't it more accurate to think of them as having developed an aversion to chamber 1? And the expectation isn't of pain relief, is of the absence of a pain stimulus. That's not the same as pain relief.

We thank the reviewer for this comment. We concur with the reviewer that "placebo is also expectation and desire, and this requires no new learning." In humans, factors such as social learning and verbal suggestions from doctors can indeed create a pain relief expectation, and a desire for pain relief can contribute to pain reduction. However, it is important to note that these expectations or desires are generally rooted in previous experiences in which pain was successfully alleviated by some form of analgesic treatment.

We also agree that "expectation" is the most interesting aspect of placebo analgesia. Indeed, this expectation aspect is the focal point of our study. In addition, we acknowledge the possibility that aversion to chamber 1 in mice could lead to a preference for chamber 2. However, we note that pain

aversion is required for the generation of a pain relief expectation, and that the two phenomena are thus inextricably related.

Conceptually, the mouse's aversion to chamber 1 and preference for chamber 2 during PAC is in our opinion similar to how humans behave when they perceive pain and generate expectations for pain relief. In human placebo analgesia, the expectation that pain perception will cease or be reduced after some intervention also stems from the aversion associated with a painful context/condition that may be escapable. This aversion provides the essential motivation to engage in a behavior that we expect will reduce pain. As we repeat this behavior to successfully reduce our pain experience, we learn to associate that behavior with pain relief, and eventually, engaging in this behavior can become sufficient to reduce pain. In this way, the aversion to the painful stimulus is fundamentally linked to the formation of pain relief expectation and therefore to placebo analgesia.

Concretely, our calcium imaging data support our claim that rACC→Pn neurons encode the expectation of pain relief, rather than a conditioned aversion to chamber 1. First, if these neurons encoded aversion to a painful chamber, we would expect them to show high activity when the mice return to chamber 1 from chamber 2 during the post-test after recognizing that chamber 2 is painful. Instead, however, we found that the activity of rACC→Pn neurons decreases after the expectation of pain relief is violated, despite that pain perception and aversion persist (Fig.2i-k). Second, continuously receiving nociceptive inputs from chamber 1 during the post-test should strengthen the aversion to chamber 1. However, the activity of rACC→Pn neurons decreased from 0.26 ± 0.03 z-score during the first crossing from chamber 1 to 2 to 0.05 ± 0.01 z-score during the last crossing (Fig.2i-k). Overall, our analysis indicates that the activity of rACC→Pn neurons closely aligns with the period of time during which we would predict that the mice are expecting pain relief, before manifesting a behavioral preference for chamber 2. Thus, we believe it is accurate to interpret the activity of rACC→Pn neurons as indicative of pain relief expectation. However, we agree with the reviewer that our initial explanation was unclear. To address this, we have clarified our logic in the Results section of the revised manuscript.

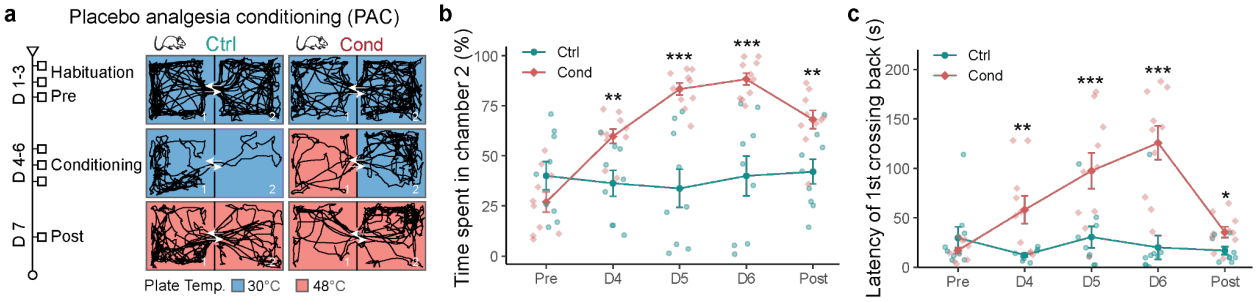
A close perusal of Figure 1 reveals some details that are hard to understand. How is it possible that a mouse would voluntarily spend even 20% of its time on a 48degC platform? The evidence of successful conditioning here is a single data point—Post—in Fig. 1b and 1c. The error bars conceal individual data. What percentage of the mice had more time in chamber 2 than chamber 1, or a longer latency to cross back compared to the control condition?

We thank the reviewer for this comment. Two primary factors contribute to the mice spending approximately 20% of their time on the 48°C plate. First, at the beginning of each PAC trial, the mouse is placed in chamber 1, entailing some period of time spent in chamber 1 before navigating to chamber 2. In addition, there is an occasional tendency for mice to return to chamber 1. Indeed, to ensure that we have enough time to observe PAC-induced analgesia during the post-test, we

chose to use 48°C instead of hotter temperatures often used for the hotplate (50–55°C) that could lead to tissue damage and more extreme pain. Data from our hot plate tests indicates that, at 48°C, mice typically take around 40 seconds to display nocifensive paw withdrawal, compared to 13 seconds at 52°C (Fig.4d)

To answer the reviewer's second question, during the post-test, 90% (9/10) of mice in the Cond group spent more time in chamber 2, while 80% (8/10) displayed a longer latency to cross back compared to mice in the Ctrl group. We concur that these data are important and apologize for their omission from the figure. We have updated the figure to include data from individual mice as requested (Fig.1b,c) and incorporated this information into the revised manuscript.

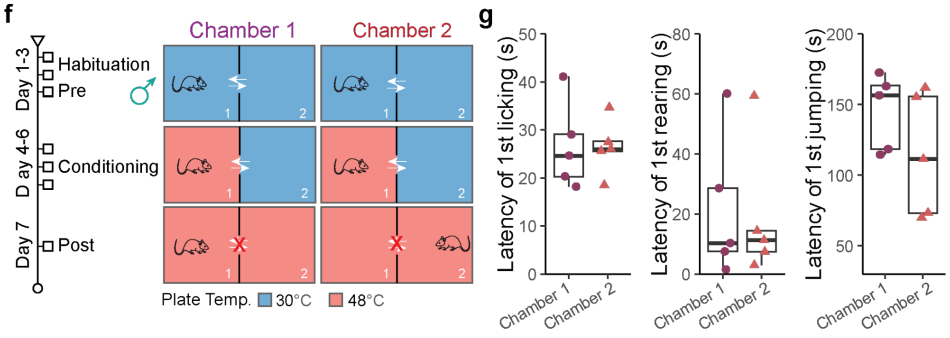
Fig.1a-c



What I'd really like to see, actually, are some experiments where after conditioning mice are confined to either chamber 1 or chamber 2.

We appreciate the reviewer's suggestion and have conducted the recommended experiment, confining mice to either chamber 1 or chamber 2 during the post-test of PAC by blocking the opening between the two chambers of the PAC apparatus. We found that mice displayed similar latencies to initiate nocifensive behaviors regardless of the chamber in which they were confined (Extended Data Fig.2f,g). This suggests that not only the context of chamber 2, but the entire context of the PAC trial (*e.g.*, beginning in chamber 1 and observing the opening that previously provided an opportunity for pain relief), is necessary to generate pain relief expectations and PAC-induced analgesia.

Extended Data Fig.2f,g



The finding that the relevant Pn neurons are Oprd1-positive is interesting, but confusing. There is absolutely nothing in the placebo analgesia literature suggesting that delta-opioid receptors are in any way involved, and in fact there have been some studies specifically implicating mu-opioid receptors only. What happens if you repeated the naloxone experiment, but with naltrindole or another delta-specific antagonist?

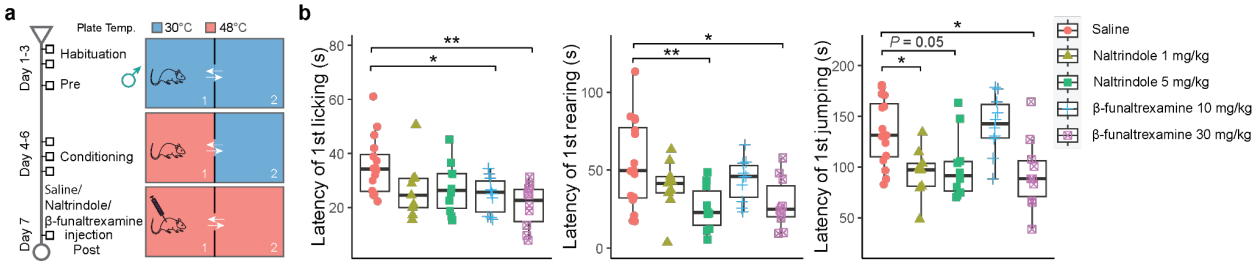
We agree with the reviewer. We have conducted the suggested experiments to determine if administration of a selective δ-opioid receptor (DOR) antagonist, naltrindole, could alter PAC-induced placebo analgesia. Additionally, we assessed the contribution of the μ-opioid receptor (MOR) using the selective antagonist β-funaltrexamine. Although the impact of these selective antagonists on the measured nociceptive behaviors (licking, rearing, and jumping) varied slightly, we found that both naltrindole and β-funaltrexamine can diminish the analgesia induced by PAC, at low or high doses (1 or 5 mg/kg for naltrindole, 10 or 30 mg/kg for β-funaltrexamine), with no clear dose-dependency. These reduced nocifensive behaviors following administration of selective DOR or MOR agonists indicate that both opioid receptors contribute to PAC-induced analgesia (Extended Data Fig.13a,b).

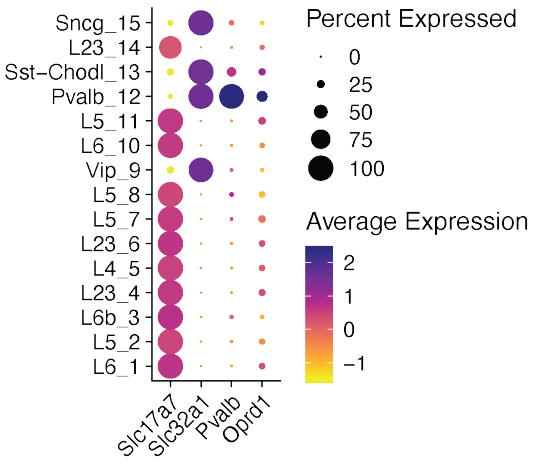
These data are consistent with previous rodent [58] and human [59,60] studies suggesting the involvement of MOR in placebo analgesia. Although the role of DOR in placebo analgesia has not been extensively investigated, one rodent study specifically examined this by microinjecting naltrindole into the rACC, and claimed that "opioid placebo analgesia is mediated exclusively through MOR" but not DOR [58]. While the findings from our naltrindole injection experiment diverge from this study, we believe this discrepancy is reasonable based on the expression of DOR in different neuron types in the cortex and caudal brain regions. Our previous study [21] and the ACC single-cell RNA-seq data for another ongoing project in the lab (provided below) show that DOR is predominantly expressed in parvalbumin (PV)-positive GABAergic interneurons within the ACC. However, the single-cell RNA-seq data from our current study (Fig.5) indicate that the *Oprd1*$^{+}$ neurons in the Pn are mainly glutamatergic excitatory neurons. Thus, microinjection of naltrindole into the rACC may disinhibit the already activated rACC→Pn pathway through inhibitory PV^{+} interneurons during placebo

analgesia, producing a limited effect, similar to our photoexcitation experiment (Extended Data Fig.5b,c). In contrast, systemic injections could directly inhibit the rACC-ponto-cerebellar pathway by reducing the activity of the excitatory DOR^{+} neurons in the Pn, thereby generating a more profound effect on placebo analgesia.

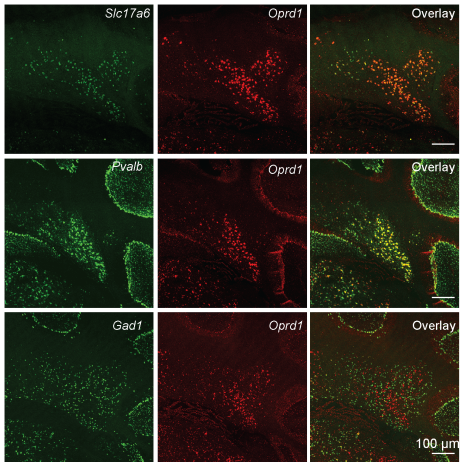
However, we acknowledge that it remains unclear how the endogenous opioid system participates in placebo analgesia. Data from our previous [21], current (Fig.5), and ongoing (provided below) studies, together with the single-cell RNA-seq data from our lab (provided below) and others [56,61] have suggested that both opioid peptides, particularly enkephalins, and the μ-, δ-, and κ-opioid receptors, are broadly distributed in each node of the cingulate cortico-ponto-cerebellar pathway. Thus, rigorously establishing the contribution to placebo analgesia of each opioid peptide and receptor, and where in this circuit and beyond, and whether via additive and/or synergistic effects, is a complex task that requires considerable time and effort and, notably, novel genetic models (*i.e.*, mice enabling conditional deletion of each opioid peptide or receptor in the rACC and/or Pn and/or cerebellum) that we are presently generating for these future studies. We have expanded our discussion on this topic in the revised manuscript accordingly.

Extended Data Fig.13a-b





Oprd1 expression in the ACC (ongoing project).

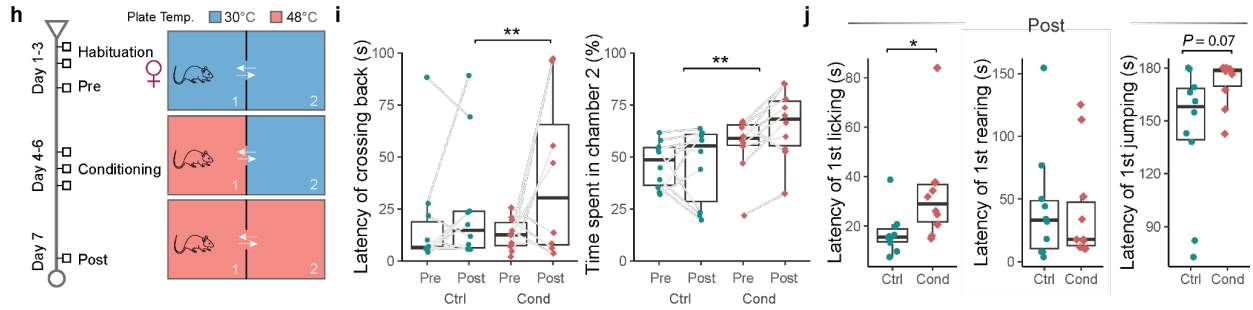


Oprd1 expression in the deep cerebellar nuclei (ongoing project).

Finally, it's 2023. What possible justification is left for doing experiments only in male mice? At the very least, the experiments in Figure 1a–d need to be repeated in female mice. I wouldn't be at all surprised if there are sex differences here.

We appreciate the reviewer's comment. Indeed, according to clinical studies, there appears to be significant variation in the placebo analgesia between male and female groups [62]. Consequently, we opted to use male mice to minimize intra-group variability. However, we agree with the reviewer that repeating PAC in females would provide useful information regarding sex differences. Thus, we have conducted the suggested experiments, and found that female mice displayed behavioral phenotypes similar to those of males during PAC, but with greater variability (Extended Data Fig.2h-j). These sexually dimorphic variability patterns align notably well with sex differences observed in human placebo analgesia [62], further underscoring the translatability of our mouse model to human placebo analgesia. We have included this information in the revised manuscript.

Extended Data Fig.2h-j



Other issues include:

1. The first sentence of the paper, "Pain thresholds aren't fixed values", is odd. The paragraph would better begin with the next sentence.

We have deleted the first sentence, as suggested.

2. In Fig. 1c, is what's being plotted the total latency over 4 days? It's not at all clear.

This figure shows the latency of mice to cross back from chamber 2 to chamber 1 for the first time during each trial. We apologize for this confusion. We have updated the y-axis label to "Latency of 1st crossing back (s)" to improve clarity.

3. There's a typo ("cossing") in Fig. 2c's legend.

We thank the reviewer for noticing this typo; we have corrected it.

4. If the behavior in the Cond group is more different from Pre than that in the Post group, why are the neurons more active in the Post group?

We appreciate the reviewer's question. Although mouse behavior indeed differed across the entire duration of the Pre, Cond, and Post trials, we examined the Ca^{2+} activity of rACC→Pn neurons within a specific 4-second window beginning 2 seconds before and ending 2 seconds after the first border crossing. When considering only this 4-second period, mouse behavior varied considerably less, particularly given that all mice in each condition were principally engaged in crossing the border (i.e, similar motor behaviors). Thus, these differences in rACC→Pn neural activity likely correspond not to differences in motor behaviors, but to other neural processes, such as pain relief expectation. This notion is supported by our data showing that locomotion does not correlate with rACC→Pn neural activity (Extended Data Fig.5g-i), reflexes, or pain behaviors (Extended Data Fig.7).

As shown in the calcium imaging data presented in Fig.2 and Extended Data Fig.4e,f, we observed that the mean activity of rACC→Pn neurons during the first border crossing increased on each successive day of PAC, from Day 3 to Day 7. This finding aligns with the idea that these neurons encode a learning-based phenomenon like expectation-based pain relief. Thus, on the post-test day, because the mice have been exposed to the highest number of previous conditioning trials, they should have the strongest expectation for pain relief, and therefore the highest rACC→Pn neural activity during first border crossing.

5. I'm not sure the scatterplot combining different conditions is statistically valid.

We acknowledge the reviewer's concern. Our Ca^{2+} imaging data indicate that the activity level of rACC→Pn neurons corresponds well with time points during which the mouse should be experiencing pain relief (Fig.2 and Extended Data Fig.4). In addition, the activity of rACC→Pn neurons progressively increases on each successive day of the conditioning phase (Extended Data Fig.4e,f), consistent with the learning-based nature of placebo analgesia. These findings motivated us to examine whether a correlation exists between the activity of rACC→Pn neurons and the crossing behavior during conditioning. We reasoned that mice with stronger pain relief expectation, as indicated by higher Ca^{2+} activity of rACC→Pn neurons, would cross the border sooner, regardless of

the phase of PAC. This analysis, which includes Day 3 (no expectation), Day 5 (first day of conditioned expectation), Day 6 (second day of conditioned expectation), and Day 7 (third day of conditioned expectation), revealed that rACC→Pn neural activity is indeed negatively correlated with the time of mice spent before border crossing, further indicating that rACC→Pn neurons encode pain relief expectation.

6. In Fig. 4d I don't think "Withdrawal latency" is an appropriate y-axis label. No one is "withdrawing" from the hot plate.

We agree. In order to avoid any potential confusion, we have changed this y-axis label to "Latency of 1st paw withdrawal (s)".

7. In Fig. 4g I think the asterisks are supposed to be referring to On vs. Off within-group, no?

We thank the reviewer for pointing this out. We agree, and have added asterisks to show both within-group and between-group comparisons.

8. In Extended Data Fig. 1, wouldn't one expect a significant difference between saline and naloxone groups in graph b? Also, graph c is uninterpretable without Pre group data.

Indeed, that is also what we originally expected. However, our results are actually consistent with one previous rodent study showing that naloxone prevented placebo analgesia without affecting the preference for the conditioning cue [37]. It is known that placebo analgesia involves both the cognitive expectation of pain relief and the physiological modulation of pain processing. Consistent with our results, previous studies have shown that naloxone can block placebo-induced analgesia [12,63,64]. Nevertheless, the influence of naloxone on expectations in general remains unclear. Our study, at least, suggests that naloxone may have a limited impact on encoding expectation.

Regarding Extended Data Fig.1c (Extended Data Fig.1f in the revised manuscript), we examine whether PAC-induced analgesic effects depend on the endogenous opioid system by comparing the differences in pain behaviors (licking, rearing, and jumping) between mice with and without conditioning on the post-test day. We did not compare data from Pre (Day 3) because both chambers are set to an innocuous 30°C at which mice do not display pain behaviors.

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Reviewer Reports on the First Revision:

Referee #1 (Remarks to the Author):

The authors have addressed most concerns with a lot of additional experiments and a re-analysis of in vivo calcium recording, behavior and AAV-based circuit mapping.
Their new data and explanations regarding the experiments look good, although whether placebo analgesia and anticipatory pain relief are identical is still an issue that needs to be mentioned.
1. The title of the paper ideally requires modification to something more specific (e.g., 'A cortico-cerebellar circuit modulating anticipatory pain').
2. The new results indicate that naloxone treatment during the conditioning phase has no profound effect (Extended Data Fig.1g-i), but there seems to be a trend towards reduced analgesia when the Oprd1+ Pn neurons were inhibited by NpHR (Extended Data Fig.12d-f). This suggests that another molecular cascade, rather than opioid signaling in the Oprd1+ Pn neurons may be responsible for the conditioning/associative learning process. A few sentences to clarify this in the discussion section would be beneficial. Since the NpHR data do not reach statistical significance, the authors cannot say 'Photoinhibition of Oprd1+ Pn neurons during the conditioning phase of PAC diminished PAC-induced analgesia observed on the post-test day (Extended Data Fig.12d-f).' They should either add more animals to confirm this or temper the language in the text.
3. Based on the references (18-20) provided by the authors, different long range projecting pyramidal tract neurons display distinct projection patterns. It will be helpful if the authors can elaborate on what cell types are involved in the rACC to Pn pathway.

Referee #2 (Remarks to the Author):

The authors have provided a huge amount of additional data/analyses and interpretation that these strengthens the paper considerably. I support publication and commend the authors commitment to responding in detail to each of the reviewer queries.

Referee #3 (Remarks to the Author):

I have rarely if ever seen such a responsive rebuttal, including a set of cerebellum experiments that I am gobsmacked the authors actually attempted. I am reasonably satisfied with their changes on all points except one. In defense of my criticism over their use of the word “novel” to describe their behavioral paradigm, the authors point out that their PAC model “replicates several key features of the human phenomenon”, citing opioid-dependence, persistence over time, and sex differences. I only agree with one of those: opioid-dependence. Persistence over time is not really a feature, and it's not clear that in humans placebo persists over time. In fact, there is much evidence that placebo responding in humans is a state, not a trait. I would drop this contention. Furthermore, although I am happy the authors redid their experiments in female animals, they didn’t really find any sex differences (variability doesn’t really count, and I guarantee a formal test of male vs. female variability would not yield a statistically significant difference because their sample sizes were far too low for such a comparison). Neither does Enck & Klosterhalfen. This is not a reason to be impressed with their model, and this contention should be dropped as well.

Author Rebuttals to First Revision:

We thank all three reviewers once again for their time and effort to improve our manuscript. We are grateful that they agree our revisions have addressed most of their concerns.

In our point-by-point response below, we cite the reviewers' remaining comments in full, using *blue italic typeface*, and describe our responses and the resulting improvements to the revised manuscript in regular black typeface.

Referee #1

The authors have addressed most concerns with a lot of additional experiments and a re-analysis of in vivo calcium recording, behavior and AAV-based circuit mapping. Their new data and explanations regarding the experiments look good, although whether placebo analgesia and anticipatory pain relief are identical is still an issue that needs to be mentioned.

We appreciate the reviewer's positive comments.

1. The title of the paper ideally requires modification to something more specific (e.g., 'A cortico-cerebellar circuit modulating anticipatory pain').

We thank the reviewer for this suggestion. We understand the viewpoint that, as for any animal model, the mouse model of placebo analgesia we developed may not be identical to placebo analgesia in humans. To address this concern, we noted this limitation in the Discussion of our previously revised manuscript. In addition, we agree that "placebo-like anticipatory pain relief" appropriately conveys this limitation, and we have now edited both the Abstract and the beginning of Main to include this exact phrasing. For the title, however, to clearly and concisely communicate the main finding to Nature's broad readership, we would like to suggest keeping the current title "Neural circuit basis of placebo pain relief".

2. The new results indicate that naloxone treatment during the conditioning phase has no profound effect (Extended Data Fig.1g-i), but there seems to be a trend towards reduced analgesia when the Oprd1+ Pn neurons were inhibited by NpHR (Extended Data Fig.12d-f). This suggests that another molecular cascade, rather than opioid signaling in the Oprd1+ Pn neurons may be responsible for the conditioning/associative learning process. A few sentences to clarify this in the discussion section would be beneficial. Since the NpHR data do not reach statistical significance, the authors cannot say 'Photoinhibition of Oprd1+ Pn neurons during the conditioning phase of PAC diminished PAC-induced analgesia observed on the post-test day (Extended Data Fig.12d-f).' They should either add more animals to confirm this or temper the language in the text.

We thank the reviewer for highlighting these results. We agree that these findings are interesting. We chose not to discuss them in the previous version of our manuscript due to the lack of conclusive evidence. We also considered the fact that our Discussion was already too long and needed to be shortened rather than expanded. Nevertheless, we agree with the reviewer that including a brief discussion of these findings in our manuscript is beneficial and have added a couple sentences in the Discussion.

Regarding our claim that 'Photoinhibition of Oprd1+ Pn neurons during the conditioning phase of PAC diminished PAC-induced analgesia…', we agree with the reviewer that softer language is appropriate given the current data. Therefore, we have changed the sentence to 'Photoinhibition of Oprd1+ Pn neurons during the conditioning phase of PAC showed a trend toward attenuating PAC-induced analgesia on the post-test day.' in our revised manuscript to temper the claim.

3. Based on the references (18-20) provided by the authors, different long range projecting pyramidal tract neurons display distinct projection patterns. It will be helpful if the authors can elaborate on what cell types are involved in the rACC to Pn pathway.

We appreciate the reviewer's suggestion to identify the specific cell types involved in the rACC→Pn pathway. While we recognize the importance of this inquiry for advancing our understanding and potential therapeutic targeting of this pain relief circuitry, we believe that a comprehensive investigation of which molecularly defined cell types are involved in the rACC→Pn pathway is beyond the scope of the current study. However, we agree this is a promising direction for subsequent research.

Referee #2:

The authors have provided a huge amount of additional data/analyses and interpretation that these strengthens the paper considerably. I support publication and commend the authors commitment to responding in detail to each of the reviewer queries.

We thank the reviewer for these positive remarks.

Referee #3:

I have rarely if ever seen such a responsive rebuttal, including a set of cerebellum experiments that I am gobsmacked the authors actually attempted. I am reasonably satisfied with their changes on all points except one.

We appreciate the reviewer's complimentary remarks and are grateful for the suggestion to undertake this set of experiments, which have yielded highly impactful results.

In defense of my criticism over their use of the word "novel" to describe their behavioral paradigm, the authors point out that their PAC model "replicates several key features of the human phenomenon", citing opioid-dependence, persistence over time, and sex differences. I only agree with one of those: opioid-dependence. Persistence over time is not really a feature, and it's not clear that in humans placebo persists over time. In fact, there is much evidence that placebo responding in humans is a state, not a trait. I would drop this contention. Furthermore, although I am happy the authors redid their experiments in female animals, they didn't really find any sex differences (variability doesn't really count, and I guarantee a formal test of male vs. female variability would not yield a statistically significant difference because their sample sizes were far too low for such a comparison). Neither does Enck & Klosterhalfen. This is not a reason to be impressed with their model, and this contention should be dropped as well.

We thank the reviewer for this feedback regarding our use of the term 'novel' for our PAC behavioral paradigm.

Regarding the persistence of placebo effects over time, we acknowledge that the term 'persistence over time' may be misleading. We modified it to 'recall of pain relief expectations over time' to more accurately reflect our findings and emphasize that placebo analgesia is a learning and memory-based phenomenon (Colloca and Miller 2011).

Regarding the variability between male and female mice in PAC-induced analgesia, we conducted a non-parametric Fligner-Killeen Test to compare their variability. We found a significant difference in licking ($P = 0.017$) and jumping ($P < 0.001$). However, we agree with the reviewer that the overall sex difference in PAC-induced analgesia is not obvious. Therefore, we now highlight the variability in PAC-induced pain relief, noting that such variability is also observed in human placebo analgesia (Colloca et al. 2013).

Overall, we appreciate the reviewer's concern regarding the use of the term 'novel', which we have now replaced by 'advanced' in the manuscript.