nature portfolio

Peer Review File

Mimicking opioid analgesia in cortical pain circuits

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

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
Summary: The authors aimed to study mu-opioid receptor related sub circuits in the anterior cingulate cortex and their role in nociceptive and motivational (affective) pain behavior, physiology and activity dependent genetics in mice. They claim 4 main findings: 1) They designed a dark chamber for observation of naturalistic pain behaviors combined with deep learning algorithms that they term 'LUPE' 2) using calcium imaging, immunohistochemistry, FISH, and single nucleus RNA sequencing they identify cohorts of ACC neurons that express mu opioid receptors, and characterize their role in morphine analgesia (focusing on painTRAP network) 3) they characterize this subcircuit in acute to chronic pain transition, and evaluate the transcriptomic changes in chronic pain over 3 months 4) they used a novel synthetic opioid promoter approach with optogenetics and DREADD techniques to stimulate opioid-relevant sub circuits in the ACC. This opioid mimicry approach reproduced the apparent activity dependent physiology, gene expression profiles and analgesic behavioral effects seen with morphine.

Overall, the authors should be commended and congratulated for undertaking such a massive project that uses multiple levels of analysis with RNA genetics, behavior (with multiple pain models), calcium imaging, whole brain clearing, optogenetics, synthesizing new viral promoters for MOR activation and DREADD techniques. This paper could have been split into 3 different papers and each would still have contained significant new findings or important extensions of prior findings.

This study is significant in that it adds rigorous characterization of structure and function of mu opioid-related subcircuits of ACC in mice, and its potential role in mediating pain acute vs chronic pain processing over previously unreported timescales using newer methods. However, conclusions regarding nociception versus affective dimensions of pain were less convincing. Further, while their results on mimicking opioid activation using a synthetic virus and DREADD methods is very novel and rigorous, the authors appear to have replicated both the LUPE method and anatomical/ physiological results of MOR subcircuits in acute pain from prior studies/ related methods that were not appropriately credited. As a whole this impressive study ties together a replication of many prior findings with new rigorous loss and gain of function experiments to further understanding of how opioid sub circuits in ACC mediate acute and chronic pain and the transition between them.

1. While the LUPE chamber is novel for observing naturalistic pain behavior, the power of this method stems from the deep learning method to characterize behavioral sequences and extract important component behaviors related to pain. This neuroethological approach to characterize behavioral sequences in pain has previously been published, and elaborated in detail in the context of acute pain in mice by the Abraira lab (including one of the corresponding authors Yttri).
The authors cite this important paper in passing but fail to acknowledge that the first result of this paper basically involves modifications on this prior method. (Bohic M et al., Neuron. 2023 Sep 20;111(18):2811-2830.e8. doi: 10.1016/j.neuron.2023.06.008.; PMCID: PMC10697150) See figures S27,28 and31 for comparison to the original paper.

2. The authors omit mention of significant prior work that has been done characterizing Mu opioid receptor sub circuits in the interestingly cortex using immunohistochemistry, patch clamp recording, and other tools. Their topographic results of mu opioid receptor mapping Appears to replicate these prior findings (here using RNA-seq methods), and extend them by comparing them to nociceptive activated neurons across multiple new time points.

a. See a brief list below of prior studies:
1.Qiu, X.-T. et al. Transcriptomic and proteomic profiling of the anterior cingulate cortex in neuropathic pain model rats. Front Mol Neurosci 16, 1164426 (2023).
2. Zamfir, M. et al. Distinct and sex-specific expression of mu opioid receptors in anterior cingulate and somatosensory S1 cortical areas. PAIN 164, 703 (2023).
3. Yao, Z. et al. A taxonomy of transcriptomic cell types across the isocortex and hippocampal formation. Cell 184, 3222-3241.e26 (2021). *This was cited by the authors in methods but the paper contains data that was replicated by this paper - no mention was made in the results or discussion.
4. Ochandarena, N. E., Niehaus, J. K., Tassou, A. & Scherrer, G. Cell-type specific molecular architecture for mu opioid receptor function in pain and addiction circuits. Neuropharmacology 238, 109597 (2023).

3. There is an overly simplistic notion in much literature, including in the introduction to this paper, that the ACC is specific or selective for mediating the affective component of pain, while significant evidence exists that nociceptive signals are processed among ACC neurons (including in this paper). The authors "(line 498) conclude that opioids as well as our opioid mimicry therapy inhibit cortical nociceptive functions to reduce the integration of negative valence information within the ACC resulting in reduced aversive arousal and attentional processes that bias the selective of nocifensive behaviors ." This sentence / claim is confusing and highlights the confusion around this point in the field. For example 1) what cortical nociceptive functions are the authors referring to ? 2) Attentional processes were not rigorously studied in this paper 3) 'Nocifensive behaviors' are not necessarily related to affective processing of pain. The following conclusion is another confusing example: Line 507) "We find that ACC nociception preferentially engages animals in affective-motivational pain attending and escape behaviors. "
a. The authors make the assumption that paw licking behavior reflects affective processes, while the gold standard in prior literature studying affective pain processing has been to use conditioned place preference protocols. It is possible that mice perform less paw licking responses because they experience less pain - and this reviewer is not aware of a clear way to dissociate this confound to support author's assumption above. A more convincing demonstration of affective behaviors would have tested some metrics related to depression or task that required significant motivation beyond licking a paw (such as walking to another chamber).
b. The discussion of 'specificity' of ACC circuits to pain unpleasantness, but also their key role in nociceptive should be made more conceptually clear in the discussion.

4. The classification of pain behaviors using Fisher decoder from neural data are very impressive and a notable finding. A small point: calculation of p-value does not require t-tests, but should be calculated directly from the shuffled label models (by calculating the probability that true values from models occur in shuffled models). Also, authors should report AUC-ROC instead of accuracy, which is less robust.

5. The software code provided by the authors appeared to be well commented but could not successfully be run because a data file that should have contained raw data was empty.

6. Some, points in the discussion should be adjusted to account for uncertainty in the literature. For example Line 468 "While opioids can be administered to the spinal cord…" This statement is not necessarily true, as to what is the 'primary' site of action that provides relief for opioids., most likely depends on what the pain generator is (spinal infusions can help completely when pain is primarily peripheral or spinally driven).

(Remarks on code availability)
The code appears well commented and in theory should run. However, the raw data (pkl) file is empty, so the code does not successfully run and so results are not able to be reproduced.

Referee #2

(Remarks to the Author)
Title Mimicking opioid analgesia in cortical pain circuits
This is an exciting, impactful, well execute set of studies, where James, Oswell, McCall, Hsu et al. propose that distinct mu opioid receptor expressing nociceptive neurons in the anterior cingulate cortex mediate the affectively aversive components of pain. To assess pain behaviors without bias of experimenter presence and manual behavior scoring, the authors develop a new experimental arena and computational behavioral analysis pipeline, collectively called LUPE. Using this technique, the authors determine the effective dose of morphine to produce “affective” analgesia in contrast to previous doses used to induce reflexive sensory analgesia in mice. Then they further uncover the neural basis of this effect through activity based “trapping” of pain responsive ACC neurons. Using whole brain imaging and single nucleus RNA sequencing, they identify the anatomical and genetic characteristics of these neurons as well as their projections. Lastly, using a custom intersectional genetic strategy, the authors demonstrate that manipulation of these nociceptive, mu opioid receptor expressing neurons in the ACC specifically attenuates affective pain behaviors in both acute and chronic pain models.

The authors have both developed major experimental and technological innovations which they use make unique observations about the complex pain encoding properties of the ACC. The development of an unbiased behavioral assay

and analysis pipeline addresses core issues of experimenter bias that have plagued pain research for decades. Importantly, they thoroughly characterize nociceptive ACC neurons, providing critical insights into the anatomical, genetic and circuit characteristics of these neurons and demonstrate that precision targeting of these neurons could be used to alleviate affective components of pain.

Overall, this is a tour de force, with an incredible and impressive amount of work included. Unfortunately, the amount of data is at times overwhelming and sparingly/inadequately described and the display of the data is confusing in several instances. This combination leaves the reader confused and wondering whether inclusion of less data would have given additional room for a more detailed and thorough description and synthesis of the major findings and impact. Further, in some instances the methods and logic for the interpretation of the data (or choice of analysis and display) is unclear or lacking. Finally, the authors should reconsider the order in which the figures are displayed and described- e.g. starting with LUPE felt slightly misleading since LUPE was then rarely used in the rest of the paper and/or if it was, the results were not clearly meaningful and other readouts were provided (e.g. attending). Additionally, ending on the tracing experiments felt anticlimactic/open ended. Overall, this paper presents important, impactful technical and scholarly contributions to the field of pain neuroscience; however, several recommendations are necessary for publication. These recommendations are detailed below to further clarify the behavioral readouts, operational definitions, and other methodological details throughout the paper. The authors base their primary conclusions on these novel readouts and analyses, but it is unclear how reliable, replicable and meaningful they are currently. In addition, reorganization and simplification of the paper should be strongly considered for maximum and widespread impact.

Key Points:
• Methodological detail in the Methods and in the Figures and legends – Insufficient methodological detail throughout the main text, methods and figure legends make it very difficult to discern necessary experimental details throughout the entire paper. Without describing ever instance, here are some examples: 1) In figures 2,4,5 there is a little black box/mouse symbol to display the use of LUPE (I gather, though this is not explicitly stated anywhere), but this symbol is difference from the symbol used for LUPE in fig 1- making it confusing to follow 2) The behavioral readouts and pain testing are consistent in Fig 1-2, then are different in the following Figures, but the justification is unclear, and the data was difficult to follow for this reason 3) The exact viruses used in Fig 5i-m (including serotype etc) is unclear. It is also unclear why the representative photomicrographs are included for k and m but not l. Further, the text describing the purpose for each of these tracing experiments is not clear and took a long time to figure out. 4) the AP coordinates and scale bars are not included for photomicrographs, making interpretation difficult. 5) It is unclear when males or females are being used (e.g. Fig 3a – how many of each sex?) – considering the known differences in pain thresholds and responses in males and females, this is critical information. There are additional examples throughout the manuscript. I suggest that the authors consider more consistency, simplicity and explicitly communicated methods throughout the manuscript.

• Affective behavioral definition and readouts
o In the main text, the authors reference " formalin-induced affective-motivational licking behavior" (line 124) and "reduces the selection of attentional recuperative behaviors (e.g., directed licking the effected somatic tissue)" (line 136). Multiple figures plot attending and licking behavior separately (e.g. Figure 4d, 4e). Clarifying how exactly these “licking” behaviors differ from each other and/or the licking behavior that is standardly used in the field. It is unclear how this relates to affect or motivation.
o The methods for quantifying “attending” behavior is not specified, nor are any references given for this scoring. It is briefly mentioned in the main text that “attending, guarding and escape” behaviors are quantified- but there are no specific details on what exactly this refers to. It is impossible to assess the validity of the data in the paper without explicit description of “attending” behavior and how this and licking equates to affective components of pain.

• Figure 1-5 -The authors quantify pain responses using the “% maximum possible effect” however this metric is not described anywhere in the main text. What is the % MPE of VF Mechanical Threshold? Where is the raw data for these graphs, can it be included in the supplement? It is difficult to compare these data to previously published work when it is displayed in this way.
• Fig 3 – Displaying this data using the N as the number of cells rather than N as the number of mice (with a single data point for each animal representing the average number of cells) may be displaying pseudoreplication rather than a meaningful outcome. It is unclear how many animals were used for this analysis. Have the authors analyzed the data and display as the average per animal? If there is not a high enough N, this should be clearly stated as a more descriptive finding rather than quantitative.
• Mapping the nociceptive hotspot- Here the authors state that Cg1 layers 2/3 followed by 5/6 are the “hotspot” with variable tagging in Cg2. However, the calcium imaging basically ablates Cg1 and is examining Cg2 due to grin lens placement (understandable, but needs to be addressed in the text somewhere and considered when drawing conclusions about the imaging and the other TRAP quantification).
• Figure 4 -The authors conclude that because chemogenetic targeting of the nociceptive MOR+ ACC population prolongs latency to “attending” (?) behaviors, these neurons specifically encode the affective component of pain.
o Figure 4o Results show that chemogenetic inhibition of these neurons induces analgesia to both acetone and 55-degree water droplet but does not affect von Frey thresholds. An alternative hypothesis to the author’s proposition that nociceptive MOR+ ACC neurons are necessary for the affective dimension of pain (in contrast to sensory-discriminative aspect of pain) is that these neurons are modality specific (i.e. are responsible for analgesia to thermal pain stimuli and not mechanical). Because the Von Frey assay does not allow for an equivalent measure of pain “affect” to the thermal assay, the encoding of pain modality versus pain dimension is not addressed by the current set of experiments. Can you quantify and show the “attending” behavior following mechanical stimulation so that the behavioral readouts are the same?
o Figure 4q the authors use unconventional methods to quantify affective pain such as “Delta time withdrawal to attend” and

“% Attending post 1st attending bout.” Further rationale should be provided to justify the use of this metric, as it does not appear to be validated with LUPE, previous literature or standard practice. In the absence of other readouts – it is unclear what these readouts really mean? Were there other parameters that were explored that were not significant (but maybe provide justification or context to the parameters that were chosen for display)?

• Figure 5 The authors conclude that because chemogenetic targeting of the nociceptive MOR+ ACC population diminishes attending behavior after acetone and 55-degree hot water droplet assays in chronic pain mice but not mechanical withdrawal threshold, manipulation of these neurons specifically modulates the affective dimension of pain.
o Figure 5E Lack of effect on withdrawal threshold compared to attending behavior following acetone and 55-degree heat stimuli may represent modality specificity rather than pain dimension specificity.
o Figure 5F Though the authors demonstrate a clear effect on lick instances (supporting the claim that these neurons are necessary for affective pain analgesia), there are no results displaying effects on withdrawal – which is needed to support their claim about the pain dimension specificity of this analgesic effect.
o Figure 5F-H The design of these plots makes it incredibly difficult for the reader to follow and interpret results. “Lick left/lick right” and “groom/rear” imply this is a ratio (?) and it is unclear why these data are shown on the same graph and split into separate graphs for controls and DREADD groups. This would be much easier to understand if separate plots for each behavior with control and treatment groups were displayed on the same plot to allow for clear comparison between groups.
o In the main text, references to this figure do not follow along with the alphabetical order of the panels- making it difficult to follow.
o Figure 5I-K are very difficult to follow.

• Fig. S10. Authors identify a ‘hot spot’ of nociceptive ACC neurons using TRAP2 activity dependent genetic capture and permanent fluorescent capture following exposure to a noxious thermal stimuli.

• C-D- While the additional experiment in f and g showing activity in this population is valence specific subsequent calcium imaging and single cell RNA sequencing experiments use coordinates from this “identified” hotspot, however these experiments use 2 different forms of pain, capsaicin induced pain and sciatic nerve transection induced pain. Perhaps authors can provide additional justification for why we would expect these populations to overlap, or alternatively authors may perform an additional TRAP/cFos pain experiment to demonstrate the overlap in activity across these different pain modalities.

• FigS12 The authors conclude that independent populations in the ACC encode valence
o Using activity dependent trapping, the authors compare populations activated by mating opportunity vs. those activated after pain stimulus administration. It is unclear if duration of stimulus, social quality of stimulus (i.e. mating opportunity requires presence of a conspecific while pain stimulus does not) and other properties of these stimuli are equivalent between the negative valence (pain) and positive valence (mating) stimuli. More equivalent positive valence stimuli should be used to properly test this hypothesis.

(Remarks on code availability)

Referee #3

(Remarks to the Author)
In James et al, they show that a subset of MOR positive ACC neurons are responsive to pain and are capable of mediating affective analgesic responses to pain induced by 0.5 mg/kg of morphine. To demonstrate this, the authors develop an automated pain detection system (LUPE) and use this with genetic manipulation of ACC neurons while mice undergo morphine treatment. On the basis of this data the authors claim that these behavioral analyses represent an improvement on the assessment of pain, compared to models such as the von Frey test. In addition, the paper also includes transcriptomic data of pain-responsive ACC neurons. Although the amount of data provided is immense and impressive, the advance in scientific knowledge this paper provides seems rather incremental for reasons provided below, which leads the reviewer to believe that this manuscript may be better suited as a methods paper.

Major comments:

1. The main strengths of the paper are both its methodological merit and the extensive characterization, detailed and clear instructions on how to construct LUPE, which is to be highly commended.

2. The involvement of MOR in ACC neurons in mediating affective analgesic responses (albeit with a cruder methodology) of 0.5-1 mg/kg doses of morphine, without affecting mechanical paw withdrawal thresholds in von Frey tests, has been characterized pharmacologically in past literature (Experimental neurology, 197(1), 22-30.). The scientific merit of this manuscript seems limited to using more advanced methodology (AI behavioral analysis + genetic manipulation) to otherwise recapitulate past findings on the involvement of the ACC in mediating morphine-induced analgesia. Additional insights such as a functional role for any of the genes identified in their transcriptomic data or projection-specific manipulation/functional circuit dissection of MOR painTRAP ACC neurons would have dramatically improved the scientific merits of the paper.

3. How specific are these neurons to mediating analgesic responses? Is it possible that the mice are undergoing deficits in decision making, thus making this a more generalized impairment of action selection or are these neurons specific for pain

coping behaviors? Measuring the activity of these neurons (Eg. Miniscope single-cell calcium imaging or, single unit-recordings) when mice are engaged in pain coping behaviors and/or using negative controls (Non-pain related decision making/action selection tasks) during chemogenetic inhibition, may be able to address this question.

4. The painTRAP method involves exposure to 55°C water, compared with homecageTRAP. Since the anterior cingulate cortex (ACC) is involved in multiple processes, including emotions and memory functions, using a non-painful stimulus (e.g., room-temperature water) as a control would help rule out emotional changes caused by the water exposure itself.

5. Previous studies ([Neuron. 2024 Apr 17;112(8):1328-1341.e4.], [Science. 2021 Jan 8; 371(6525): 153–159.], [Nat Commun. 2018 Sep 14;9(1):3751.], [Science. 2010 Dec 3;330(6009):1400-4.]) have shown that chronic pain induced by inflammatory or nerve injury increases spontaneous ACC activity. However, in Figure 2F-G, the SNI model showed reduced FOS expression compared to the uninjured group, suggesting decreased spontaneous ACC activity in chronic pain conditions. Additionally, in the SNI model, painTRAP ACC neuron activity appeared suppressed (Figure H-J). Nevertheless, in Figure 5B-C, additional optogenetic inhibition of painTRAP-MOR neurons induced place preference in the SNI model. This inconsistency needs to be addressed.

6. It is unclear if these neurons are capable of mediating affective analgesia towards mechanical pain. In Figure 2J, both acute thermal stimuli (painTRAP) and mechanical allodynia (touchFOS) activated the same neurons in the pain matrix, including the ACC. However, in Figures 4 and 5, ACC modulation only affected thermal stimuli-induced pain behavior and not mechanical thresholds. This discrepancy requires further exploration.

7. Numerous studies have sought to establish homologies between the mouse and human brain. However, identifying corresponding regions has proven challenging. In Figure 2J, they note that the mouse brain regions were selected based on human pain fMRI literature (NPS and SIIPS). While NPS and SIIPS include numerous cortical regions rather than subcortical areas, there are significant differences between the mouse and human cerebral cortex. And many of the selected areas are subcortical regions. More detailed information on the selection criteria for the human & mouse brain regions is needed to substantiate these claims.

8. The authors claim no change in chamber selection during the active optogenetic sessions in the uninjured group (Figure 5C). However, since the data lacks a GFP control group, this conclusion is unsupported. The uninjured mice may have shown significant avoidance of the light-paired chamber compared to their baseline. The experiment should ideally be redone with proper controls.

Minor comments
1. Naming conventions for experimental treatments are confusing at times. For example, in Figure 3P-Q, the text describes this as uninjured vs SNI. However, when reading figure S15, the uninjured group also received 55C water drops whereas the SNI group received 0.16g-filament pokes. These differences need to be made clear in the main text.

2. Related to the above comment, naming is not consistent at times. Mice with SNI are referred to as the SNI group, whereas in other figures (Figure 5C) they are referred to as the neuropathic pain group.

3. Figure 2B is a little puzzling given that there are numerous reports on morphine being able to paradoxically cause hyperalgesia (OIH) ([Pain physician, 14(2), 145.], [Brain Research, 888(1), 75-82.]). One may expect touchTRAP neurons to possibly be separate from painTRAP neurons given this, can the authors speculate on how to resolve this discrepancy, especially given that they did not find any changes in OPRM1 expression at any of the SNI timepoints (Fig S15)?

4. The methods section appears to contain errors, likely from copying and pasting from other papers. For example, it references fiber photometry testing and naloxone-precipitated withdrawal, neither of which are included in the paper. Additionally, the Hotplate thermal test section mentions using CNO in Figure 3C, but Figure 3C is clustering of transcriptomic data.

5. The calculation of "Max Possible Effect" in Figure 4 is not described and should be included in the methods section.

6. Figures 4E and 4J show no significant changes in left hindpaw licking behaviors. It would be beneficial to discuss possible reasons for these results.

7. There is a possible typo on page 8 regarding "Fig. 2D," which may need correction.

8. Figure 4J is not mentioned in the results section.

9. Figure legends for Figure 4 contain some typographical errors that need to be corrected.

10. Figure S4 focused on comparing LUPE to Noldus-based Ethovision. These experiments demonstrated differences in tracking distance, showing ‘LUPE’ may be more suited for tracking the entire area. However, Ethovision has options beyond the center-point tracking that was used in the figure, i.e. 3-point tracking. Using Ethovision’s more complex tracking options may mitigate differences with ‘LUPE’-generated results.

11. Typos were found on: Page 23, column 1, “LUPE – Light aUtoamted Pain Evaluator.” AND Page 23, column 2, paragraph 3, line 4, “imgaug”.

(Remarks on code availability)

Version 1:

Reviewer comments:

Referee #2

(Remarks to the Author)
We appreciate the authors' thoughtful and thorough response to our initial critiques. It is evident that a considerable amount of care, time, and additional experimentation went into addressing the concerns raised, and we commend the authors for their responsiveness and rigor.

In particular, we recognize and appreciate the significant reorganization of the manuscript, which has greatly improved clarity, conceptual coherence, and alignment between the data presented and the overarching goals of the study. The decision to center LUPE-based analyses throughout the primary figures and to relocate traditional evoked assays to the supplement was a particularly effective revision that clarified the authors' emphasis on spontaneous affective pain behaviors and underscored LUPE's methodological contribution to the field. Similarly, the removal of underdeveloped or tangential data (e.g., tracing studies) has sharpened the manuscript's focus.

Importantly, the authors addressed a major conceptual concern about the use of mating as a positive valence comparator by performing new calcium imaging experiments with more behaviorally matched appetitive and aversive stimuli (sucrose vs. quinine). These new data are well-conceived, align with prior literature on valence coding in frontal cortex, and convincingly support the conclusion that discrete ACC populations encode opposing affective states.

With these revisions, the manuscript presents a compelling set of scientific and methodological innovations. The chemogenetic dissection of MOR-expressing nociceptive ACC neurons stands out as a particularly powerful demonstration of circuit-specific modulation of affective pain, with exciting translational implications.

That said, some broader considerations remain, though they do not detract from the overall quality of the work. The manuscript is ambitious in scope, combining circuit-specific manipulations, transcriptomic profiling, and behavioral automation. While each component is strong and contributes meaningfully, the integration of these distinct methodological approaches occasionally feels complex, and at times the breadth of data may obscure the central narrative. Additionally, while the LUPE system represents a promising advance in automated behavioral analysis, we encourage some caution in interpreting its readouts as purely affective, particularly given the reliance on licking behavior as a core metric. Further validation of the behavioral specificity will be valuable as this platform gains wider adoption.

Nonetheless, these concerns do not detract from the substantial merits of the manuscript. The authors have reasonably addressed each of our comments, provided new data where appropriate, and made considerable improvements in organization, clarity, and justification. The work will undoubtedly stimulate valuable discussion in the field about how to define and measure affective pain, and how to target it therapeutically.

Conclusion: We support publication of this manuscript in its current revised form and commend the authors on a rigorous and impressive body of work. We look forward to the discourse it will inspire.

(Remarks on code availability)

Referee #3

(Remarks to the Author)
We appreciate the extensive revisions made by the authors and acknowledge the significant effort in addressing the previous reviewer comments. The revised manuscript is substantially improved in clarity and focus, particularly with respect to its central conceptual advance: the development of a circuit-targeted, cortical opioid-mimetic intervention for chronic pain. The streamlined presentation and expanded experimental data, including calcium imaging, transcriptomics, and behavioral modeling, substantially strengthen the overall narrative. We recognize that many of the original concerns stemmed from an unintended evaluation of an earlier preprint version, and we apologize for this oversight. That said, a few important points remain.

1. The authors repeatedly refer to their chemogenetic approach as “gene therapy.” While this usage is not unprecedented, the term “gene therapy” is traditionally reserved for interventions correcting disease-related genetic defects. Here, the approach is more accurately described as chemogenetic inhibition using an MOR promoter. Unless the field has broadly adopted this expanded use of the term, the manuscript should consider clarifying this terminology or providing justification for calling this intervention a “gene therapy” beyond its cell-type targeting.

2. The authors assert that their manipulation of MOR+ neurons in the ACC specifically targets affective pain-processing ensembles. However, their own data suggest that MOR expression spans multiple ACC layers and includes a substantial population of neurons not directly linked to pain-related licking behavior. This broad expression raises concerns about off-target modulation of other ACC functions, such as working memory, contextual fear, and general decision-making processes. The authors acknowledge that only a subset of MOR+ neurons are involved in pain behaviors, but they do not experimentally exclude the possibility that chemogenetic inhibition affects unrelated behavioral or cognitive domains. Additional experiments are warranted to address this potential limitation of the proposed therapeutic approach.

3. In chronic pain conditions, reductions in grooming, rearing, and walking behaviors are commonly interpreted as diminished motivation or increased affective burden. Figures 3D and 4G show that chemogenetic inhibition of ACC MOR+ neurons restores these non-licking behaviors. This suggests that the functional impact of the manipulation extends beyond licking and affects a broader behavioral repertoire. However, the manuscript continues to emphasize licking as the primary indicator of affective pain. We suggest the authors more explicitly acknowledge that changes in spontaneous non-licking behaviors are also likely manifestations of affective pain, and that their reversal by MOR+ inhibition may reflect more global improvements in motivational state.

4. In the revised Figure 2 and Supplementary Figure S13, ACC ensemble activity predicts licking behavior during acute pain with high fidelity, but this decoding performance appears to decline in chronic pain states. Interestingly, morphine seems to improve decoding performance in chronic pain. This raises intriguing questions about how chronic pain alters ACC circuit function and how opioidergic modulation reshapes ensemble dynamics. The manuscript would benefit from a more detailed discussion of these dynamics, particularly how chronic pain alters the functional encoding properties of the ACC and how opioid treatment may restore or reconfigure those properties.

(Remarks on code availability)

Referee #4

(Remarks to the Author)
Major comments:
The authors demonstrated that there is a population of ACC neurons that encode spontaneous pain-related behaviors which are selectively modulated by morphine. To do this they used a combination of TRAP2 reporter mice, Fos-driven Cre recombination, immunohistochemistry, ISH, and single-cell sequencing in the context of acute and chronic pain with and without morphine. They identify Oprm1 as stable across all cell types and conditions and timepoints in the ACC-making it a feasible target for MOR-promoter based cell-type specific therapies independent of chronic pain inducted transcriptomic changes. Reduction of Oprm1 following morphine administration did not alter mechanical withdrawal thresholds; however, it did diminish morphine's ability to alleviate affective-motivational pain behaviors.
They go on to posit that manual grading of pain-related behaviors is too subjective and costly when done by humans and hence created hardware and associated software to better analyze mouse behavior in the context of pain (LUPE). The software is accessible and usable on GitHub. Six different states were identified to assess pain and non-pain states in the animals and using deep learning, these newly identified states validate the system, its reproducibility, and further tested variations in the model.
Neural dynamics in the ACC is then assessed by calcium imaging (using GRIN lens implants). Variables included chronic pain through neuronal injury, heat, acute pain pricks, or other noxious stimuli in the context of morphine or without. The identify paw-licking as main activity to study. Using LUPE and the knowledge gathered thus far, they infer pain as a latent cognitive-affective state by analyzing patterns of sparse, spontaneous, and complex behaviors that may not overtly indicate the animal's pain. Moreover, they discover that latent cognitive-affective states are sensitive to morphine. They ultimately produce ARMS, a data-driven, continuous index of pain-related behavioral states which responds bidirectionally to both injury and analgesia.
After all of this, they use inhibitory DREADDs to target only the mu-opioidergic ACC neurons using the Oprm1 promoter region. This way, giving the activating drug inhibits these neurons, ameliorating pain and associated behaviors the same way (or better) than morphine without the tolerance and without the potential for (hopefully) addiction.
This manuscript represents an impressive body of research and innovation for a single paper. The work is dense and could easily be divided into several high-impact publications; however, I appreciate the authors' intention to present the complete story in one comprehensive manuscript. Despite the substantial amount of data spanning numerous experiments and technologies, the authors have done an admirable job consolidating their findings and maintaining a clear focus on their central message: the development of an opioid-mimicking gene therapy for chronic pain.
Overall, the narrative flows well. However, there are several inconsistencies within the figure legends, statistical representations, and supplementary tables—please see my detailed suggestions below. Given the paper's size and the likely involvement of many contributors, further polishing is needed to ensure that readers can easily follow and absorb all of the information presented.
Minor inconsistencies:
Line 52: the words "to that" should be resolved. One of the words will suffice. Examples below:
Current analgesic drugs, such as opioids, bathe the body in chemical compounds, acting on widely expression molecular targets that reduce pain unpleasantness but also can promote serious and fatal side effects.
Current analgesic drugs, such as opioids, bathe the body in chemical compounds, acting on widely expression molecular targets to reduce pain unpleasantness but also can promote serious and fatal side effects.
Line 114: you reference Supplementary Figure 2, wherein you use the abbreviation "SNI" for the first time, which is not

spelled out in the figure legend of Supplementary Figure 2, nor in the main text until Line 131. It may be helpful to explain what SNI is in the figure legend of Supplementary Figure 2 and still keep it in the main text later, at Line 131.
Line 166: The authors say that they injected Cre delivered by AAV9 which only floxed Oprm1 in the ACC but left all other cells intact. What does was delivered? AAV9 is historically very promiscuous and may have transduced cells beyond their target. Only images from the ACC are shown in Supplemental Figure 4S. Did the authors look at other regions in the brain known send or receive synapses to or from the ACC respectively? The authors did not say the total dose they delivered of the virus, but it would behoove them to show regions that could have been transduced, to verify negative signal.
Line 192: “machine-vison” should be “machine-vision”, yes?
Line 447: I believe “vesical” should be “vesicle”
Lines 871-884: It is appreciated that the authors have identified the viruses and their respective titers in the methods, however it is never stated what volume is delivered to the animals, and thus, the ultimate dose of each virus is unknown. It would be extremely helpful to know what doses of virus the authors administered for each of the treatments.
Supplemental Figure S5 panel K; the last point reads: “LUPE: ~$2,000 for chamber build + $2,500 computer = $5,300 total + GPU (market value)” Your math doesn’t add up. Be consistent with the prices given and make the total “~$4,500”.
Figure 2 panel a: The color of text “Lick left hindpaw…” needs to be a lighter color- its extremely difficult to read on the black background.
Finally, I believe the discussion could be improved upon by going more in depth describing how this technology could feasibly translate into clinic.

COMMENTS ON STATISTICS AND FIGURE LEGENDS:
In general, please consolidate all the statistics to one a single Supplementary Table and please make sure they are in order with Main Figures listed first and all Supplemental Figures following. For consistency, make sure all statistics in the legends also have a corresponding location in the tables. Please standardize the names of the statistics that you perform throughout the manuscript, legends and tables (examples: 1-way ANOVA or One-way ANOVA; t-Test or T-Test or t-test; always state whether t-tests are paired or unpaired, one-tailed or two-tailed and what post-hoc tests were used in all situations). Either use a star graphic or spell out “stars” but please be consistent throughout. Be consistent in how you describe the error bars and how they are represented (SEM vs. s.e.m.)
This is the best version for all applicable legends: “(star symbol)= $P < 0.05$. Bars, lines, or dots are mean; error bars and shaded areas are SEM. See Supplementary Table 1 for statistics.” As seen in figure 1 legend.
I found rows in the supplementary tables for Main Figures 1, 2, 3, 4 and Supplemental Figures S1, S7, S8, S9, S10, S13, S16, S17, and S18. Below are some inconsistencies that should be clarified.
S1g: Supplemental Figure 1 legend says that a Two-way ANOVA (Line 425) was executed on data shown in panel g, however, there are no statistics to show for it, either in the supplemental nor, nor the supplemental data table that expands on all statistics run. Please remove the reference to “Two-way ANOVA” or amend all legends and tables to reflect the statistics performed and the results.
Supplemental Figure 4: the statistics are only in this figure legend. For consistency, the authors should add the specifics of statistics to the Supplementary Data Tables. The statistics are fine, but there is also an inconsistency in the authors annotation of “t-Test” vs. “T-Test”; please make consistent and remove to data table.
Supplemental Figure S7h; in the Data Table with respective statistics, I do not believe that Line 21 should include a multiple comparisons value. It is listed as a permutation test comparing real vs. shuffled; then additionally states that there was a multiple comparisons analyses for Uninjured vs. Capsaicin. Panel S7h does not address capsaicin: Please Revise
Supplemental Figure S9: In panel C for everything, are the Kolgomorov-Smirnov Test plots supposed to be in color? The Supplemental Table for the statistics state at the KS = values between 0 and 1, and the p-values are mostly 0 or very small. Based on the p-value scale it looks like colors should fluctuate between black/dark purple and light yellow/white; however, I only see blocks of filled black ink or outline of boxes. Is this an accurate representation of the images? For panel F, there are no associated rows in the Supplemental Table for these statistics: Please Add.
Supplemental Figure S10: In the legend, you reference permutation t-tests for panels g) and j) however, there are no values listed nor are there rows in your supplementary table. Panel I) is missing from the legend entirely, although that panel and its associated statistics are in the Supplementary Table. Please correct and make consistent; add missing information and values.
Supplemental Figure S11 panel f: there is no color or block outline for the middle row of column “SNI1” and the 4th row down for column “Ctrl4”- is this correct? Or is the image incomplete. For consistency, please add rows to the Supplemental Table(s).
Supplemental Figure S12 contains the language “Stars indicate $p < 0.05$. Lines are mean; shaded areas are SEM.” There are no stars in this figure- please remove the associated language.
Supplemental Figure S13- Panel a has an unfilled while spot for Baseline, if the subpanel is truly white, could you please provide an outline of the box, just to confirm that it is not a bad image. Same for in panel h “Day 21 + morphine non-pain states, SNI1”.
Supplemental Figure S13- The legend for panels (c,g) state that there was a Mixed effects model, Tukey correction with a p value less than 0.0001; but no corresponding rows in the supplementary table; please amend for consistency.
Supplemental Figure S13- panel l legend states that its p value = 0.046, yet there is no “star” in the panel; is this correct? It says that stars indicate $p<0.05$.
Supplemental Figure S17- there are no associated statistics in the supplemental table for panel c; please amend for consistency.

(Remarks on code availability)
We were able to open it and run it. It looks more robust than previous pain-behavior software.

Version 2:

Reviewer comments:

Referee #3

(Remarks to the Author)
We appreciate the authors' thoughtful and extensive efforts in revising the manuscript and addressing the concerns raised in the previous round of review. The inclusion of additional analyses, clarifications, and discussion points—particularly regarding the behavioral classifier, ACC circuit function, and translational framing—reflects a genuine attempt to engage with reviewer feedback and improve the scientific clarity and rigor of the manuscript.

-Comment 1:
While we understand that the regulatory framework has broadened its definition of gene therapy, in the context of a scientific journal article rather than commercial or marketing material, this still seems to be an expansion of the term, for what otherwise is normally referred to in scientific journals as chemogenetic inhibition.

We appreciate that the authors have provided numerous examples in commercial contexts where chemogenetic-based therapies are referred to as "gene therapy". However, we note that even in these commercial contexts, the term "gene therapy" typically includes conditional qualifiers such as "**Chemogenetic** gene therapy" or "**Optogenetic** gene therapy", presumably to differentiate it from gene therapy that corrects diseases due to genetic defects.
In the one academic document cited: Kang, H. J., Krumm, B. E., Tassou, A., Geron, M., DiBerto, J. F., Kapolka, N. J., ... & Roth, B. L. (2024). Structure-guided design of a peripherally restricted chemogenetic system. Cell, 187(26), 7433-7449., nowhere in the paper is the term "gene therapy" used and their technology is referred to in the manuscript as a "peripherally restricted chemogenetic system/platform".
We believe terminological drift in a scientific journal article risks compromising mechanistic accuracy in scientific communication.

The authors' proposed solution to clarify their use of "gene therapy" in the discussion section represents a well-intentioned compromise. However, we believe that incorporating the conditional qualifier "**chemogenetic** gene therapy" **THROUGHOUT** the manuscript would better serve communication to a basic science audience, while also helping place emphasis on the potential translational relevance of the intervention, particularly given that this is a publication in a scientific journal rather than a commercial document.

We suggest revising the proposed clarification as:
"Throughout this study we use the term **chemogenetic** gene therapy in the regulatory sense—AAV delivery of a therapeutic transgene—implemented here with chemogenetic effectors.",
or any alternative phrasings that achieve the same terminological precision, with the same qualifying term.

-Comment 4:
The authors assert that their classifier generalizes well to chronic pain behavior. However, this claim remains only partially substantiated. The model was trained using acute pain conditions, and licking was used as the behavioral ground truth—limiting the potential to fully capture chronic pain states, which often involve reduced motivation, altered emotional tone, and passive coping.

The authors acknowledge that decoding accuracy from ACC signals is degraded in chronic pain (Figure S13), and that morphine partially restores this ability. However, this could reflect restoration of acute-like pain processing rather than true affective relief. Thus, it remains unclear whether the classifier captures chronic-specific affective signatures or merely reflects a shift in behavioral visibility.

To improve interpretability, the authors should more clearly discuss the limitations of using an acute pain-trained model to classify chronic pain behaviors. If feasible, retraining the model on chronic pain data or validating it against chronic-specific behavioral features would better support the generalizability of the approach.

(Remarks on code availability)

Referee #4

(Remarks to the Author)
The authors identified a population of anterior cingulate cortex (ACC) neurons that encode spontaneous pain-related behaviors and are selectively modulated by morphine. Using TRAP2 reporter mice, Fos-driven Cre recombination, histological methods, in situ hybridization, and single-cell sequencing in acute and chronic pain models, they found that Oprm1 expression remains stable across cell types, conditions, and time points—supporting its feasibility as a MOR-promoter target for cell-type–specific therapies regardless of chronic pain–induced transcriptomic changes. While morphine-induced Oprm1 reduction did not affect mechanical withdrawal thresholds, it impaired morphine's relief of affective-motivational pain behaviors. To improve behavioral assessment, they developed LUPE, an open-source hardware–software system using deep learning to classify six behavioral states distinguishing pain from non-pain conditions, validated through

reproducibility tests. Calcium imaging via GRIN lens implants revealed paw-licking as a key activity, and LUPE analyses enabled inference of latent cognitive-affective pain states that were morphine-sensitive, culminating in ARMS—a continuous index of pain-related states responsive to injury and analgesia. Finally, inhibitory DREADDs driven by the Oprm1 promoter selectively suppressed mu-opioidergic ACC neurons, reducing pain behaviors as effectively as or better than morphine, but without tolerance or addiction risk.

I think the authors revisions have continued to improve this extremely dense manuscript. The supplementary notes are a wonderful addition that address many of the open-ended discussion issues. The statistics are more uniform now. I think this is a spectacular ambitious body of work.

(Remarks on code availability)
I accessed the code in the last round of review and it was functional.

April 1, 2025

Dear Editors and Reviewers,

We sincerely appreciate the time and effort the reviewers have taken to provide detailed and constructive feedback on our manuscript, ***Mimicking opioid analgesia in cortical pain circuits.*** Their comments have been invaluable in helping us refine the study, clarify the key conceptual advances, and improve the overall presentation.

Following our prior submission (Manuscript #2024-08-17007), we consulted with then-editor Dr. Dave Rowland, who encouraged a resubmission after significant refinement. In this revised manuscript, we now clearly present the central conceptual advance: *the development of a cortical, opioid-mimicking gene therapy for chronic pain*.

As Dr. Rowland observed, the sheer amount of data in our initial submission likely "obscured this main message". We agree, and this was reflected in the reviews—none of the three referees offered critiques of the primary opioid-mimicking chemogenetic gene therapy data, questioned its novelty, or addressed its translational significance. Instead, comments focused primarily on secondary elements such as opioid cell-type mapping, c-Fos mapping and morphine pharmacology, which we have now moved to the Supplement or removed entirely.

We have fully addressed all reviewer concerns through extensive clarifications, new supporting analyses, and a more streamlined presentation that highlights the key insight: a cingulate and opioid cell-type-targeted therapeutic strategy for chronic pain.

Summary of manuscript's major advances:

- Our study introduces a targeted gene therapy for chronic pain that uses a synthetic mu-opioid receptor (MOR) promoter (*mMORp*) to deliver chemogenetic inhibition specifically to cortical MOR+ neurons. This intervention was guided by LUPE (Light aUtomated Pain Evaluator), a deep-learning system we developed to track spontaneous, naturalistic pain-related behaviors in freely behaving mice. Combining LUPE with *in vivo* calcium imaging, we identified anterior cingulate cortex (ACC) neuronal ensembles encoding ongoing pain states that are rapidly suppressed by morphine. Single-nucleus transcriptomics of these pain-responsive, opioid-receptor-expressing ensembles informed the design of our MOR promoter-based gene therapy, which, when delivered to the ACC, produced robust analgesia in a mouse model of chronic neuropathic pain. This integrative framework links behavior, neural activity, and gene expression to enable circuit-specific pain relief.
- These findings advance both basic pain neurobiology and translational precision pain medicine. LUPE captures real-time spontaneous pain states and reveals how cortical affective circuits encode the affective-motivational responses during chronic pain. By isolating endogenous analgesic circuits and modulating them with a cortex-targeted gene therapy, we pave the way towards future selective, non-addictive neuromodulation. This approach establishes a roadmap for identifying, imaging, and manipulating the specific brain circuits driving chronic pain—offering a new platform for safe, effective, brain-targeted treatments for chronic pain.

We received the following positive feedback on the initial review:

- **Referee 1** commended the overall scope and rigor of the study, stating, "This paper represents an impressive body of work that incorporates many state-of-the-art technologies." They appreciated the integration of multiple cutting-edge techniques—ranging from behavior quantification and in vivo calcium imaging to gene therapy—and highlighted the value of the chemogenetic pain gene therapy approach, calling it "quite novel and valuable." Additionally, they found the technical clarity and methodological execution to be strong, noting that the methods and tools were well-documented and effective.
- **Referee 2** was highly enthusiastic about the manuscript, describing it as "a tour de force… a major advance in the field of pain research." They specifically praised the emphasis on spontaneous behavior tracking over traditional evoked measures, highlighting the innovative direction of the LUPE system. The reviewer acknowledged the breadth and depth of the data collected, stating, "The amount of data collected is remarkable and includes an impressive combination of behavioral, imaging, and genetic techniques." They also viewed the behavioral analytics platform LUPE as a promising new tool, commending the thoughtful integration of supervised and unsupervised learning methods.
- **Referee 3** was more critical overall but still offered positive feedback on several aspects of the work. They acknowledged the utility of the LUPE system, calling it "a nice tool that could be useful for the field," and "the

data are immense and impressive," While raising concerns about novelty, they still recognized the value in the comprehensive scope of behavioral and circuit analyses performed in the chronic pain context.

Reviewers had the following concerns :

Referee 1 questioned the novelty and conceptual framing of the study. They were skeptical about whether LUPE offers a substantive advance over previous tools (e.g., Bohic et al.) and noted overlap with prior mapping of MOR+ ACC neurons. They challenged the use of licking as an affective-motivational pain measure and recommended a different neural decoding analyses and clearer framing of ACC's role in pain processing. Additional concerns included a missing code file and an overstatement of spinal vs. supraspinal opioid distinctions.

Author Revisions:

- Clarified LUPE's unique technical/conceptual features and analysis pipeline compared to prior methods.
- Cited prior MOR+ ACC studies and moved mapping data to Supplement to avoid redundancy.
- Provided theoretical and empirical support for licking as an affective-motivational behavior, per the original Gate Control Theory model of pain; added new LUPE analyses.
- Adopted auROC and permutation tests in neural decoding analyses.
- Updated text to frame ACC to remove interpretations of "selectivity" for affective pain.
- Re-uploaded the corrected code file with raw data.
- Revised Discussion to present a more nuanced view of opioid pharmacology.

Referee 2 felt the manuscript was overly dense and difficult to interpret. They found the figures and terminology confusing, requested clearer definitions of behavioral metrics (e.g., "attending," %MPE), and raised concerns about pseudoreplication in the transcriptomic data. They questioned the anatomical alignment of imaging and labeling experiments and whether ACC activity reflects pain-specific motivation. The *mate*TRAP study was seen as confounded, and overall, the reviewer called for streamlined presentation and clearer justification of novel behavioral constructs.

Author Revisions:

- Reorganized manuscript structure and reduced Supplementary Figures from 31 to 18.
- Removed LUPE icons and clarified all figure legends and layouts.
- Added behavioral definitions in Methods and moved human-scored behaviors to Supplement.
- Provided pseudobulk strategy explanation and statistical validation for snRNASeq.
- Clarified GRIN lens targeting of TRAP2-labeled ACC hotspots.
- New data on minimal single-neuron overlap across modalities; population-level decoding emphasized.
- Removed *mate*TRAP data; added new sucrose/quinine imaging data to support valence dissociation.
- Simplified and clarified novel behavioral metrics and their ethological relevance.

Referee 3 felt some findings—such as morphine's effect on pain behavior—were already known. They stated pharmacological follow up on transcriptomic data would be more impactful, questioned whether behavioral changes reflected analgesia, and noted the absence of a fluorophore control group in one optogenetic experiment.

Author Revisions:

- Complete re-write of all texts and figure structures to highlight the main biological advances—the first CNS gene therapy for pain and real-time tracking of endogenous pain states.
- Explained that snRNAseq showed limited viable targets, reinforcing the need for a circuit-based therapy.
- Added new analysis linking neuron modulation to analgesia rather than global behavioral suppression.
- Removed outdated claims regarding species homology and TRAP labeling from main text.
- Explained omission of GFP group based on design and lack of behavioral changes in the control group.
    - ***Importantly*, we are concerned that Reviewer 3 evaluated an outdated *bioRxiv* preprint (April 2024) rather than the submitted August 2024 manuscript; and thus missed many key conceptual advances in the *Nature* submission**. Many of their critiques (e.g., the absence of ACC calcium imaging vs. c-FOS mapping) refer to figures and content only present in the preprint, not the *Nature* submission. The submitted version included these requested experiments (*e.g.,* Main Figure 2 showed sub-second ACC neural dynamics modulated by morphine).
    - **Of the critical comments, 3 of 6 Major Comments and 6 of 10 Minor Comments were in direct reference to the *bioRxiv* preprint text and figures, and not the *Nature* submission**
    - **We respectfully believe this error led to a mischaracterization of the manuscript as primarily methods-focused during the original review.** The August 2024 *Nature* submission represented a major conceptual and translational advance beyond the preprint, and we hope the reviewer will re-evaluate the work considering these data that answered most of their original comments. Any remaining comments from Reviewer 3 have been fully addressed in this revision.

In general, to address reviewers comments directly we have made the following key revisions:

- Structural refinement across the *Introduction* to *Discussion* to emphasize the conceptual significance of cortical gene therapy for chronic pain.
- Condensed the Main Figures from 5 to 4, and the Supplementary Figures from 31 to 18.
- Focused data presentation by removing or relocating peripheral elements and replication data (*e.g.,* evoked pain measures, opioid cell-type FISH and single nuclei RNAseq) to the Supplement.
- Enhanced visualization, with simplified figures, standardized metrics *(e.g.,* LUPE-based deep-learning modeling for spontaneous pain behaviors), and new schematics to clearly convey each figure's message.
- Expanded methods and figure legends to ensure reproducibility.
- New experiments, including:
    - Calcium imaging with synchronized LUPE tracking across chronic pain development (Figure 3).
    - A *Mouse Pain Scale* to quantify analgesia and test our gene therapy (All figures).
    - Imaging of single-neuron responses to pain and appetitive/aversive stimuli (Supp. Figs. 11–12).

In this revised submission, *we have carefully addressed 100% of comments from all 3 Reviewers* in the following point-by-point response, including additional clarifications, new data, new analyses, and improved explanations where applicable. Below, please find **all previous reviewer comments in Black Text**, and **our responses in Blue Text**.

Referees' comments:

Referee #1 (Remarks to the Author):

Summary: The authors aimed to study mu-opioid receptor related sub circuits in the anterior cingulate cortex and their role in nociceptive and motivational (affective) pain behavior, physiology and activity dependent genetics in mice. They claim 4 main findings: 1) They designed a dark chamber for observation of naturalistic pain behaviors combined with deep learning algorithms that they term 'LUPE' 2) using calcium imaging, immunohistochemistry, FISH, and single nucleus RNA sequencing they identify cohorts of ACC neurons that express mu opioid receptors, and characterize their role in morphine analgesia (focusing on painTRAP network) 3) they characterize this subcircuit in acute to chronic pain transition, and evaluate the transcriptomic changes in chronic pain over 3 months 4) they used a novel synthetic opioid promoter approach with optogenetics and DREADD techniques to stimulate opioid-relevant sub circuits in the ACC. This opioid mimicry approach reproduced the apparent activity dependent physiology, gene expression profiles and analgesic behavioral effects seen with morphine.

Overall, the authors should be commended and congratulated for undertaking such a massive project that uses multiple levels of analysis with RNA genetics, behavior (with multiple pain models), calcium imaging, whole brain clearing, optogenetics, synthesizing new viral promoters for MOR activation and DREADD techniques. This paper could have been split into 3 different papers and each would still have contained significant new findings or important extensions of prior findings.

This study is significant in that it adds rigorous characterization of structure and function of mu opioid-related subcircuits of ACC in mice, and its potential role in mediating pain acute vs chronic pain processing over previously unreported timescales using newer methods. However, conclusions regarding nociception versus affective dimensions of pain were less convincing. Further, while their results on mimicking opioid activation using a synthetic virus and DREADD methods is very novel and rigorous, the authors appear to have replicated both the LUPE method and anatomical/ physiological results of MOR subcircuits in acute pain from prior studies/ related methods that were not appropriately credited. As a whole this impressive study ties together a replication of many prior findings with new rigorous loss and gain of function experiments to further understanding of how opioid sub circuits in ACC mediate acute and chronic pain and the transition between them.

AUTHORS: We appreciate the reviewer's thoughtful and thorough evaluation of our work, as well as their recognition of the rigor, scale, and multi-level approach of our study. We acknowledge suggestions that this research could have been divided into multiple papers, yet we believe that presenting these findings together provides a unified and conceptually strong case for the therapeutic potential of an ACC-targeted gene therapy approach for chronic pain—which is the core focus of our manuscript.

While we value the reviewer's positive assessment, we respectfully disagree with the interpretation that our findings are primarily an extension or replication of prior work. We agree with former editor Dr. Rowland that the previous high

density of the figures obscured the primary conceptual advancements, and we have taken the last 5.5 months to carefully re-work every aspect of the figures and text to improve clarity and have workshopped this revision with numerous other colleagues within and outside our fields.

Our study introduces several key conceptual and mechanistic advancements, particularly in support of our central premise—ACC MOR+ neurons can be selectively targeted for gene therapy to provide non-opioid analgesia:

Key Conceptual Advances Beyond Prior Work

1. **Deep-Learning Behavior Tracking Reveals New Temporal Organization of Affective-Motivational Pain States (Figure 1)**
   - Unlike traditional reflexive assays or Conditioned Place Preference learning/memory assays, our LUPE deep-learning system enables an unbiased, experimenter-free assessment of real-time ***spontaneous chronic pain behaviors***, which are more relevant for evaluating chronic pain states and translational validity of new analgesics.
   - Using this approach, we identified consistent latent behavior states across multiple acute and chronic pain models, which led to the development of a new ***Affective-Motivational Pain Scale***.
   - By focusing on spontaneous behaviors, we bridge the gap between reflexive responses and complex affective-motivational pain assays, directly addressing reviewer concerns about distinguishing these pain dimensions.
2. **Opioids Act on ACC Neurons Encoding Affective-Motivational Pain Behaviors (Figure 2 and 3)**
   - While prior studies have recorded ACC neurons during the application of noxious stimuli, our miniscope recordings provide the first real-time neural activity data showing how **ACC neurons dynamically encode spontaneous pain states and pain-recuperative behaviors, as well as the sub-second effects of morphine on this cortical process**.
   - This represents a major advance over behavioral pharmacology studies as we now demonstrate direct functional engagement of these neurons during spontaneous pain and analgesia—with sub-second and single-cell resolution during freely moving behavior.
3. **A Novel CNS Gene Therapy Strategy for Chronic Pain (Figure 4)**
   - We developed and validated a **synthetic mu-Opioid Receptor (MOR) promoter (*MORp*) driven gene therapy**, enabling precise, cell-type-specific modulation of MOR-expressing ACC neurons
   - This approach directly mimics the pain-relieving effects of morphine by alleviating affective-motivational pain states without systemic opioid exposure, presenting a fundamentally new, circuit-targeted strategy for chronic pain treatment.
   - **To our knowledge there have never been proposed brain-targeted gene therapies for chronic pain in any capacity; especially none that have used existing biological machinery to reverse engineer morphine analgesia in a circuit-specific manner to more selectively mimic the anti-nociceptive functions**.

Extensive Revisions to Improve Clarity and Emphasize These Advances. To ensure that these conceptual contributions are clearly articulated, we have conducted an extensive revision of the text and figures to:

- Better emphasize the translational significance of the *MORp* gene therapy strategy.
- More explicitly distinguish our findings from prior work and clarify novel contributions.
- Enhance clarity in differentiating nociceptive vs. affective-motivational pain processing in the ACC.
- Improve methodological explanations to highlight the importance of spontaneous pain measures via LUPE.

We deeply appreciate the reviewer's constructive feedback, which has helped us significantly refine the presentation of our findings. We hope that these revisions more clearly highlight the significant new conceptual advances this study provides beyond prior work, particularly in supporting the ACC-targeted gene therapy approach for chronic pain relief.

Referee 1, Comment 1. While the LUPE chamber is novel for observing naturalistic pain behavior, the power of this method stems from the deep learning method to characterize behavioral sequences and extract important component behaviors related to pain. This neuroethological approach to characterize behavioral sequences in pain has previously been published and elaborated in detail in the context of acute pain in mice by the Abraira lab (including one of the corresponding authors Yttri).

The authors cite this important paper in passing but fail to acknowledge that the first result of this paper basically involves modifications on this prior method. (Bohic M et al., Neuron. 2023 Sep 20;111(18):2811-2830.e8. doi: 10.1016/j.neuron.2023.06.008.; PMCID: PMC10697150) See figures S27,28 and31 for comparison to the original paper.

AUTHORS: We appreciate the reviewer's recognition of LUPE's novelty in observing naturalistic pain behavior and the importance of deep-learning approaches in characterizing pain-related behavioral sequences. We also acknowledge the significant contributions of prior studies, particularly the work by Bohic et al. (Neuron, 2023), in advancing machine-learning-based behavior tracking in acute pain models, hence why we cited it in the original manuscript, along with many other recent examples of applied machine-learning to different types of pain-related behaviors (e.g., *PainFace* for facial grimaces, etc.). We will now provide points to clearly illustrate that LUPE is, in no way, a modification of the Bohic et al. method. Below we explain some of the key differences and their implications - but most importantly, as a last point we identify how we have revised the manuscript to directly address these concerns.

Since the first submission in August 2024, we have significantly advanced the post-hoc analyses pipeline, and are excited to present our new LUPE-based behavioral state modeling for a new *Affective-Motivational Pain Scale (AMPS)* (Figure 1)

- We developed a data-driven, unsupervised method to identify spontaneous pain-related behavioral states. From high-resolution LUPE tracking data, we generated sliding-window transition matrices that captured the probabilities of switching between defined behaviors across time. These were transformed into 36-dimensional vectors and clustered using 100-fold cross-validated k-means, revealing six reproducible behavioral states that generalized across animals, pain models, and conditions.
- To classify each moment in time into one of these six states, we calculated the Euclidean distance between the current transition matrix and each centroid, assigning the closest match. Our model was robust to the removal of individual behaviors and showed strong internal validity. To distill these behavioral states into a single pain index, we performed principal component analysis (PCA) on the state distributions from uninjured and injured animals (capsaicin and formalin, no morphine). The second principal component (PC2) was increased by painful stimuli and decreased by analgesia, and we defined this as the AMPS Scale—a continuous, unsupervised measure of spontaneous pain intensity. Finally, we assessed within-state dynamics to determine how individual behaviors (e.g., licking) evolved over time within a state, revealing structured trajectories that may reflect feedback regulation.
- Our modeling approach combines transition probability modeling, unsupervised clustering, and dimensionality reduction to extract latent behavioral states and derive a real-time index of pain. While transition matrices are Markovian at the local level, the overall model captures non-Markovian structure across time, enabling the detection of sustained behavioral states and context-dependent dynamics. We used no prior labels or assumptions about what pain looks like—allowing the structure of the data to reveal latent pain states de novo.
- Our model is well-suited to capture subjective states of pain unpleasantness that drive behavior selection. The spontaneous behavioral states we identified reflect internal affective-motivational processes that evolve over seconds-to-minutes, closely mirroring the timescale of reported human pain experiences. Importantly, we found that licking—an ethologically grounded, recuperative behavior—was embedded in specific high-pain states, and that it often ramped up just before the termination of these states, suggesting that licking may act as a behavioral modulator of internal pain state, consistent with a negative feedback loop such as that proposed by Gate Control Theory. In this framework, licking inhibits spinal pain transmission—effectively closing the gate and ending the pain state. Our data-driven identification of such state transitions and their resolution by goal-directed behaviors provides a computational link between internal affective states, behavior selection, and feedback regulation of nociception.
- In sum, we present a novel framework for studying spontaneous pain states and their modulation—bridging behavioral dynamics, internal state modeling, and translational insights into how organisms select actions to self-regulate pain.

LUPE's Unique Strengths and Advantages Over Prior Methods. A critical point, as noted by the reviewer, is that Senior Author Eric Yttri was also an author on the Bohic et al. study, making him uniquely positioned to directly provide this comparison between the LUPE platform (combined workflow of many custom-trained algorithms: DLC → A-SOiD → B-SOiD → Markov Model + K-Means clustering → PCA) versus the MoSeq algorithm. Given Dr. Yttri's expertise in neuroethological analysis, he can fully attest to LUPE's unique strengths, improved ease of use, and direct applicability to chronic pain research over the methods used for acute inflammatory pain in Bohic et al., as well as other published systems.

While Bohic et al. made important contributions, their MoSeq-based approach uses an existing algorithm from 2015 to hyper-segment behaviors, identifying 90+ "syllables" including 16 different types of rearing behaviors, but without a clear framework for distinguishing which of these are relevant to pain processing. No new analysis methods were developed in that paper, only an application of pre-existing pipelines.

- For example, they emphasize rearing module 15 as a pain-relevant movement, but it remains unclear why module 15 is pain-relevant while rearing module 16 or the other 14 rearing modules are not relevant to pain.

- Moreover, a closer look at their dataset shows that multiple rearing modules were reduced in controls, which seems to contradict their own interpretation.
    - We did not feel that our research article was the place to critique other methods, given how distinct they are from LUPE, but in response, we have added more direct points to the revised manuscript.

MoSeq's Depth Camera vs. LUPE's High-Resolution Bottom-Up Camera. A key difference that fundamentally sets LUPE apart from MoSeq-based tracking is the camera type, camera placement and camera resolution:

- MoSeq relies on a top-down depth camera, which cannot resolve mouse limbs or detailed body parts and is thus incapable of detecting fine pain-related movements such as paw licking.
- LUPE uses high-definition resolution cameras positioned below the mouse, offering a totally unique and distinct advancement over the Bohic paper by allowing us to:
    - Precisely resolve limb movements and paw positioning, which are critical for assessing pain-related behaviors.
    - Detect fine motor behaviors such as licking (a key recuperative pain response), which MoSeq completely misses due to its depth-camera limitations.

How the LUPE Platform Provides a More Intuitive and Behaviorally Relevant Approach. Beyond the crucial, standardized LUPE chamber that provides more robust DLC tracking than other models due to the consistent ambient lighting and stable environment (**Fig. S6**), LUPE's analysis algorithm is derived from combining multiple existing programs, that were specifically and uniquely trained for pain behavior, something that no other approach has done:

- **DeepLabCut** key-point tracking of 20 body points
- **Supervised A-SOiD**, developed by Dr. Yttri (*Nature Methods*, 2024; PMID: 38383746), wherein our paper is the first to apply A-SoiD to expert experimenter-defined pain behaviors. This is a crucial step, as pure unsupervised methods often either "lump" or "split" complex behavior patterns that have received consensus agreement from the pain field as being highly important for drug-screening, such as directed licking of injured tissues, in line with Gate Control Theory.
- **Unsupervised B-SOiD** clustering, also developed by Dr. Yttri (*Nature Comms*. 2021; PMID: 34465784), is then used on the A-SOiD output to further subcluster behaviors within that "class" or repertoire of supervised behaviors.
- **Six core behavior repertoires were identified**: Still, Walk, Rear, Groom, Lick Left Hindpaw, Lick Right Hindpaw.
    - Unlike prior studies that arbitrarily segment behaviors into indistinguishable subtypes, we take a holistic approach by collapsing micro-types into broad, functionally relevant categories. For example, rather than distinguishing n=16 rearing micro-types (e.g., left- or rightward rear at 5°, 10°, 20° etc.), we classify them all as "Rears" to focus on the organization of the different core behaviors rather than arbitrary subtypes.
    - This dimensionality reduction enables mathematical analysis of temporal dynamics between behavior repertoires, forming the foundation of the new Mouse Pain Scale.
- **Pain States and the Mouse Pain Scales (new Figure 1, Supp. Fig 7-9):**
    - We developed an automated post-hoc analysis pipeline (B-SOiD→ Markov Model + K-Means clustering) to generate ethograms of spontaneous behaviors and assess behavior-switching probabilities, enabling **unsupervised clustering** to reveal n=6 high-dimensional behavior states:
        - Two "Pain States": Enhanced by acute and chronic pain.
        - Four "Non-Pain" or "Pain-Suppressed States": Unaffected or reduced by injury.
    - **Principal Component Analysis** (PCA) on state probabilities across uninjured, acutely injured (capsaicin, formalin), and chronic pain (nerve injury) mice revealed:
        - PC1: Represents general mouse behavior, unaffected by injury or drugs.
        - PC2: Captures behavior patterns that increase with pain and decrease with analgesics (e.g., morphine, opioid-mimicking gene therapy).
            - This **AMPS scores** enables objective, real-time evaluation of pain affective-motivational behavior intensity and analgesic efficacy—eliminating the need for human-applied stimuli and subjective behavior scoring

Why the LUPE Platform is an important innovation and a conceptual advance supporting the primary advancement for our biologically-inspired gene therapy for pain:

- Traditional pain assays rely on predefined behavioral measures, such as reflexive withdrawal or manually scored latency from stimulus to response, which fail to capture the dynamic, multidimensional nature of pain as it unfolds

naturally over multiple timescales (from milliseconds to seconds to minutes). Our approach advances preclinical pain research by uncovering latent behavioral states—hidden organizational patterns of spontaneous behavior that differentiate healthy, injured, and analgesic-treated mice.

- This technique not only allows us to model behavior as a probabilistic sequence of transitions rather than a set of isolated actions but also describes how their relationships change as the animal progresses through a slower sequence of discrete states, providing a rich representation of how injury reshapes behavioral organization over time.
- Unlike previous methods that focus on individual behaviors, our state-space approach detects pain-motivated behavioral regimes, enabling the identification of states that are sensitive to injury and responsive to morphine.
- By assessing the probability of transitioning between states, rather than merely counting the frequency of specific behaviors, we provide a more nuanced, unbiased, and interpretable assessment of pain that accounts for behavioral context and variability between subjects.
    - **This is not just a unique and novel behavior analysis for the pain field, but to the best of our knowledge, this represents a *first-of-its-kind* analysis for freely moving behavior dynamics across all fields of ethological behavior science.**

- This latent state modeling approach should also greatly impact preclinical drug discovery by offering a high-resolution, experimenter-free measure of pain relief that is more sensitive to low-dose analgesics than traditional, experimenter delivered, subjective scored, reflexive metrics, or other low-dimensional behavior tracking systems.
    - By constructing a continuous Affective-Motivational Pain Scale (AMPS) based on the second principal component of state occupancies, we establish a quantitative metric that tracks pain severity and its alleviation with pharmacological intervention, such as morphine.
    - By assessing the within-state dynamics of licking, we tested specific predictions following from Gate Control Theory. Specifically, **that licking/rubbing/touching injured tissues is an innate recuperative behavior for endogenous antinociception that is driven by internal negative affective-motivational states (i.e., aversion/unpleasantness) that lead to related neural activities for behavior selection in the ACC**.
    - By linking these behavioral states to single-neuron and ensemble activity in the ACC via calcium imaging, we use spontaneous pain behavioral expression to investigate underlying neural circuits, revealing how analgesics modulate pain-encoding neuronal ensembles over multiple timescales.
    - **Crucially, this framework is not limited to pain research**—it provides a generalizable method for decoding motivational and affective states from spontaneous behavior in a way that is free from anthropomorphic assumptions. By enabling the unbiased discovery of latent cognitive-affective states in rodents, this approach advances neuroscience beyond conventional task-based paradigms, allowing preclinical researchers to extract meaningful insights from the complex and otherwise intractable structure of naturalistic behavior.

Revisions to Address This Concern. We have now revised the manuscript to:

- Explicitly differentiate LUPE from Bohic et al. and similar behavioral tracking studies.
- More clearly articulate LUPE's key advantages, particularly paw-specific tracking, experimenter-free assessment, 24-hour monitoring, and its direct relevance for drug testing.
- Ensure that our citations appropriately credit prior work while still emphasizing LUPE's substantial conceptual and practical improvements.

Referee 1, Comment 2. The authors omit mention of significant prior work that has been done characterizing Mu opioid receptor sub circuits in the interestingly cortex using immunohistochemistry, patch clamp recording, and other tools. Their topographic results of mu opioid receptor mapping Appears to replicate these prior findings (here using RNA-seq methods) and extend them by comparing them to nociceptive activated neurons across multiple new time points.

a. See a brief list below of prior studies:

1.Qiu, X.-T. et al. Transcriptomic and proteomic profiling of the anterior cingulate cortex in neuropathic pain model rats. Front Mol Neurosci 16, 1164426 (2023).

2. Zamfir, M. et al. Distinct and sex-specific expression of mu opioid receptors in anterior cingulate and somatosensory S1 cortical areas. PAIN 164, 703 (2023).

3. Yao, Z. et al. A taxonomy of transcriptomic cell types across the isocortex and hippocampal formation. Cell 184, 3222-3241.e26 (2021). *This was cited by the authors in methods but the paper contains data that was replicated by this paper - no mention was made in the results or discussion.
4. Ochandarena, N. E., Niehaus, J. K., Tassou, A. & Scherrer, G. Cell-type specific molecular architecture for mu opioid receptor function in pain and addiction circuits. Neuropharmacology 238, 109597 (2023).

AUTHORS: We appreciate the reviewer's careful review of the literature and their effort to provide relevant citations. We would like to clarify that we did cite papers 1 and 3 in our manuscript. We apologize for the oversight in not citing the Zamfir et al. study, it is now cited in the revised manuscript. We did not cite paper 4 (Ochandarena et al., 2023) because it is a review article that does not present new experimental findings beyond what had already been covered in our own review on the same topic published the year prior (Kimmey et al (Corder), PMID: 33314372).

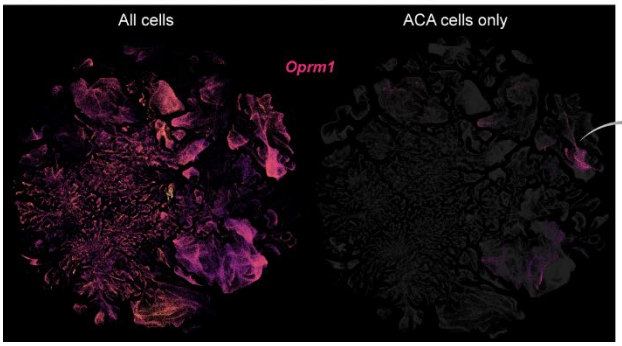
While we appreciate the importance of these previous studies, we respectfully disagree with the characterization that our work merely replicates prior findings. **Our study presents the *first ever* cell-type-resolved transcriptomic analysis of neuropathic pain (SNI model) in the ACC, providing an unprecedented level of insight into molecular adaptations within functionally relevant neuronal populations**.

To address the specific papers referenced by the reviewer, we have now added new discussion in the Main Text:

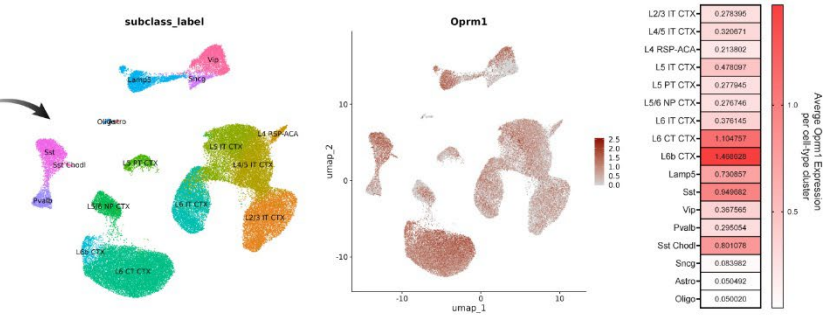
1. Qiu et al., 2023 (Front Mol Neurosci)
    - This study performed bulk RNA sequencing of rat ACC in a neuropathic pain model.
    - Bulk RNA-seq does not resolve transcriptomic changes at the single-cell level, meaning it cannot distinguish which neuronal subtypes contribute to observed transcriptional changes, thereby constraining what meaningful information can be learned via gene ontology / pathway analyses.
    - In contrast, our study uses single-nucleus RNA sequencing (snRNA-seq), allowing us to map pain-induced transcriptional changes to specific neuronal subclasses, particularly L5 PT-2 neurons.
        - **Our approach far exceeds the resolution of Qiu et al., providing previously unattainable insights into cell-type-specific adaptations in chronic pain.**
2. Zamfir et al., 2023 (PAIN)
    - This study used a MOR-mCherry reporter mouse to characterize the regional expression of MORs in the ACC and somatosensory cortex.
    - However, it did not include any transcriptomic data and did not assess how MOR cell-types are altered in pain states.
    - **Our study not only maps MOR-expressing neurons in the ACC but also all opioid receptor and opioid peptide-expressing neurons, plus analyzes their transcriptional adaptations in response to neuropathic pain, *something that has never been done before*.**
    - From the results of our *Oprm1* FISH and snRNAseq analysis, we provide data that differ from the results presented in Zamfir et al., wherein we find that many more cell-types express *Opmr1*/MORs than are detected with the GCPR-fused-fluorescent tag in the MOR-mCherry mouse. One possible explanation is that mCherry fluorescence is only detectable when the tag aggregates in the cell soma—a well-documented limitation noted by multiple labs, including the creator of this mouse line, Dr. Brigitte Kieffer (PMID: 24623156): "*MOR-mCherry was more readily visualized in cell bodies than neural processes*." This limitation has also been reported in studies by Dr. Corder (*Corder et al., Nature Medicine, 2017, PMID: 28092666; Wang et al. (Corder), Neuron, 2018, PMID: 29576387*).
        - In comparison to our multi-approach dataset—including snRNAseq for *Oprm1*, FISH for *Oprm1*, IHC for MOR, AAV-*MORp*-eYFP expression, and *Oprm1*-Cre;Ai9 tdTomato expression—we conclude that Zamfir et al. significantly underestimates the cell-type and cortical layer distribution of MORs in the ACC. **Furthermore, our study extends beyond mere mapping ACC MOR+ neurons, providing the first characterization of transcriptomic changes in different MOR+ neuron subtypes throughout chronic pain development**.
3. Yao et al., 2021 (Cell, Allen Institute RNA Atlas)
    - This study is a landmark resource paper cataloging transcriptomic cell types across the cortex and hippocampus.
    - However, it is a reference database and contains no experimental manipulation related to acute nor chronic pain.
    - **We cited and used this dataset as a comparative reference, but our study provides the first direct assessment of how chronic pain alters the molecular landscape of ACC neurons, which cannot be inferred from the Allen Atlas alone.**

- To further address this point, *see the response-letter figure below* where we conducted a re-analysis of *Oprm1*-expressing cell types using the updated Allen ABC Atlas, which includes 10X Genomics single-cell RNAseq data from over 4 million neurons. After downloading the FASTQ files for the labeled ACA (Anterior Cingulate, aka ACC), we first QC'd the cells and then applied the same analysis pipeline used for our own 10X Genomics single-nucleus RNAseq dataset (Supp. Fig. 3). Our re-analysis confirms a similar *Oprm1*+ expression pattern across ACC neuronal clusters, aligning with our findings. However, this dataset provides only basic expression patterns and, to our knowledge, has not been previously examined for this purpose until this figure. In contrast, our study represents the *first-of-its-kind* single-cell transcriptomic dataset characterizing the effects of chronic pain development on ACC cell-types, as well as the *first* nociceptive immediate early gene (IEG) mapping onto molecular cell-type identity.
    - **Noxious IEG Mapping in Pain-Responsive ACC Neurons.** To highlight a major advancement in knowledge, and a completely unique aspect of our study is our noxious IEG mapping approach:
        - We identified neurons that were functionally engaged by an acute noxious stimulus and then analyzed their transcriptomes at multiple timepoints during the development of chronic neuropathic pain.
        - This allows us to directly link gene expression to noxious stimulus-evoked neuronal activation, something no prior study, including those cited, has achieved.

ABC Whole Brain Atlas, 10X Genomics single-cell RNA seq



Re-clustered ACA cell data from Allen ABC Atlas using same parameters as Supp. Fig. 3



4. Ochandarena et al., 2023 (Neuropharmacology, Review Article)
    - This is a review article that provides an overview of MOR-expressing neurons in pain and addiction circuits.
    - As stated, we chose not to cite it because it does not contain new experimental data, and our own review from the previous year had already covered much of the same ground.
    - Thus, given that this review does not provide new information over other citations, it is not cited in this revised manuscript.

Revisions to Address This Concern. To ensure that our contributions are more clearly articulated, we have revised the manuscript to:

- Explicitly highlight the key technical and conceptual advances over prior work (*e.g.,* single-cell resolution, pain-state-specific transcriptomics, and IEG-based molecular profiling).
- Clarify how our study fundamentally differs from bulk RNA-seq approaches (like Qiu et al.), MOR-mCherry mapping (like Zamfir et al.), and general cortical cell atlases (like Yao et al.).
- Ensure that our citations appropriately credit prior work while emphasizing the significant new information our study provides for the pain field.
- **Additionally, we have now moved all transcriptomic and anatomical mapping of these cell-types to the Supplementary Materials, to place greater emphasis on the primary advancements of the manuscript—the development of a biologically-inspired, opioid-mimicking gene therapy for pain**.

Referee 1, Comment 3. There is an overly simplistic notion in much literature, including in the introduction to this paper, that the ACC is specific or selective for mediating the affective component of pain, while significant evidence exists that nociceptive signals are processed among ACC neurons (including in this paper). The authors "(line 498) conclude that opioids as well as our opioid mimicry therapy inhibit cortical nociceptive functions to reduce the integration of negative valence information within the ACC resulting in reduced aversive arousal and attentional processes that bias the selective of nocifensive behaviors ." This sentence / claim is confusing and highlights the confusion around this point in the field. For example 1) what cortical nociceptive functions are the authors referring to ? 2) Attentional processes were not rigorously studied in this paper 3) 'Nocifensive behaviors' are not necessarily related to affective processing of pain. The following

conclusion is another confusing example: Line 507) "We find that ACC nociception preferentially engages animals in affective-motivational pain attending and escape behaviors. "

a. The authors make the assumption that paw licking behavior reflects affective processes, while the gold standard in prior literature studying affective pain processing has been to use conditioned place preference protocols. It is possible that mice perform less paw licking responses because they experience less pain – and this reviewer is not aware of a clear way to dissociate this confound to support author's assumption above. A more convincing demonstration of affective behaviors would have tested some metrics related to depression or task that required significant motivation beyond licking a paw (such as walking to another chamber).

AUTHORS: To clarify, nowhere in the introduction or elsewhere in the paper do we make any claim that the ACC is selective or specific to pain. We generally agree with reviewer's testament that there is a simplistic view of ACC function in the literature, and we outright addressed this exact point in the original Discussion section "*theories of ACC function*" in an attempt to place our results in the broad context of "*what does the ACC do?*", in particular surrounding cognitive functions and not just pain. Perhaps our phrasing on the subset of ACC neurons that encode the behavior-selection of pain-related behaviors was misinterpreted. We apologize for any confusion and have updated the text almost entirely to drive home the main conceptual advancements of our work toward the development of a new ACC-targeted opioid mimicking gene therapy for chronic pain.

We respectfully disagree with the reviewer's assertion that paw licking is not a valid metric for assessing affective-motivational pain processing. **Licking and rubbing injured tissue have long been recognized as key affective-motivational pain responses, dating back to the original Gate Control Theory of Pain** (Melzack & Wall, 1965) and refined in 1968 by Melzack and Casey when they fully developed the first concepts of the affective-motivational components of pain perception (*Melzack R, Casey KL. Sensory, motivational and central control determinants of pain: a new conceptual model. In: Kenshalo D, editor. The Skin Senses. Springfield, IL: Charles C Thomas; 1968. pp. 423–443*)

These behaviors are not merely reflexive but serve a critical recuperative function in pain modulation. When we experience pain in a part of our body, we naturally tend to rub, massage, or shake the affected area. This response, known as touch-mediated analgesia, refers to pain relief triggered by physical touch.

- **Affective Component:** As originally defined by Ronald Melzack and Ken Casey in 1968, this is the subjective aversive or unpleasant quality of a pain perception
- **Motivational Component:** This is the behavioral response aimed at minimizing the aversive or unpleasantness affective state, which has the functional value of reducing harm, promoting recovery, and avoiding future injury. This involves neural circuits that translate pain-induced distress into need-state-driven behaviors, such as escape, avoidance, resting, or recuperation. Importantly, as predicted by Gate Control Theory, touching/rubbing/licking activates Aβ peripheral afferents that limit ascending spinal nociception, which alleviate discomfort and reduce nociceptive input (aka recuperation)

This conceptualization aligns with the broader framework of affective pain behaviors, which extend beyond conditioned place preference (CPP) paradigms. **While CPP is a widely used approach, it primarily assesses learned associations with pain relief rather than in-the-moment, spontaneous pain-related affective-motivational behaviors**. Notably, affective-motivational responses to pain can manifest in diverse behavioral expressions, including the recuperative / self-soothing behaviors observed in our study.

Moreover, **our prior work has explicitly defined and operationalized these behaviors in preclinical pain** models (Corder et al., Science 2019, PMID: 30655440; Corder et al., Nature Medicine 2017, PMID: 28092666; Manglik et al. (Corder) Nature 2016, PMID: 27533032; Salimando et al (Corder). Nature Communications. 2016, PMID: 37704594; and multiple recent bioRxiv preprints, PMIDs: 3980354 and 38405972). These studies demonstrate that spontaneous pain behaviors, including paw licking, correlate with neural activity in known affective pain circuits such as the BLA and PAG, reinforcing their relevance in assessing the affective-motivational dimension of pain. Equally, many other pain researchers use these operationalized behaviors to assess the affective-motivational component of pain, including as examples Drs. Allan Basbaum, Quifu Ma, Clifford Woolf, etc.

Nevertheless, to directly test the reviewer's claim, we leveraged our behavioral state-space model to test whether licking is both driven by pain and analgesic by assessing the dynamics of lick over bouts of State 4, a state specific to injured animals, generalized across injury types, dose-dependently reduced by morphine, and contained about three quarters of all licks (Supp. Fig. 9). By generating 500 simulations of behavior based on the transition matrix describing this state, we first showed that our model predicts that licks should be uniformly distributed over time during State 4. However, if licking is pain-driven, licks should in fact ramp over State 4, and if licking is analgesic, they should then fall preceding State 4 offset. We find that in all injury models, licking follows the distribution predicted by gate-control theory as described above, significantly deviating from the null hypothesis that it is an epiphenomenon of pain (Fig 1n, S9). While it is certainly true that not every instance of paw licking is due to pain, the vast majority only occur in a pain state, increase with time in that pain

state, and accumulate prior to the end of that pain state. Therefore, we empirically show that licking is an affective-motivational pain-behavior.

To further advance the conceptual framework of pain measurement, we have employed our LUPE deep-learning behavior tracking system to analyze high-dimensional behavioral transitions, allowing us to quantitatively derive pain and non-pain states. This approach led to the development of an **Affective Motivational Pain Scale,** which provides a new, objective, data-driven measure of pain state classification beyond traditional ethogram scoring. This innovative framework enhances the resolution of affective-motivational pain behaviors, offering a more comprehensive assessment of pain-related states in freely behaving mice that we used to assess morphine analgesia and efficacy of our opioid-mimicking gene therapy.

Lastly, to address the suggestion to assess motivation by "walking to another chamber", **we did provide this exact data in our original submission via a multi-day, optogenetic real-time place preference (RTPP) task (former Main Figure 5, now moved to Supp. Fig. 20)**, which we designed to transition into a conditioned place preference (CPP) test. Here, we demonstrated that optogenetic inhibition of ACC MOR+ cell-types increased preference for the chamber environment where these ACC neurons were being inhibited. ACC MOR+ neuron inhibition also reduced licking behaviors, decreased pain state engagement, and lowered high-dimensional Pain Scale Scores relative to controls. These findings provide direct evidence that inhibiting ACC MOR+ neurons modulates both spontaneous pain behaviors and preference-based measures of pain relief, which complement our LUPE measurements.

Thus, while we acknowledge that CPP and other paradigms involving significant motivated behavior (e.g., chamber crossing) provide additional insight into affective pain processing, recuperative paw licking remains a well-established and ethologically valid measure of pain's affective-motivational components. Furthermore, our RT-PP/CPP findings and LUPE-driven high-dimensional pain state analysis **provide converging evidence that our behavioral assessments robustly capture the affective-motivational aspects of pain, both of which are reduced via our MORp chemogenetic therapy.**

b. The discussion of 'specificity' of ACC circuits to pain unpleasantness, but also their key role in nociceptive should be made more conceptually clear in the discussion.

AUTHORS: We appreciate the reviewer's request for clarification regarding the term "selective" in our description of pain-responsive neural ensembles. To be precise, we are not claiming that these neurons respond exclusively to pain. Instead, our data indicate that *a subset of ACC neurons is selectively activated* during behavior-selection of affective-motivational or recuperative behaviors such as licking the injured tissue. Meaning that this subset is preferentially engaged compared to the broader ACC population.

To support this claim, we have incorporated additional clarification in the revised manuscript, explicitly referencing the following findings:

1. Pain Trap and Pain FOS Studies – Our data demonstrate that a subset of ACC neurons exhibit significant activation during noxious stimuli, distinguishing them from the non-responsive ACC population.
2. Calcium Imaging and Behavioral Correlations – We have collected an updated dataset combining LUPE behavior tracking with single cell calcium imaging to track changes in encoding of pain behaviors in the ACC in the days and weeks following SNI and the development of chronic pain. Together with the original capsaicin experiment, our imaging data shows that a subset of ACC neurons exhibit increased spontaneous calcium transient rates under capsaicin (**Fig. 2i,j**). This neuronal subset is time-locked with the selection of *Lick* behaviors only at the injured tissues over other general behavior. Specifically, this subset of recuperative Lick-encoding neurons shows blunted increased activity over the development of chronic pain post-SNI (Fig. 3g) and are modulated by morphine analgesia (**Fig. 3g-l**). These neurons, unlike others, track acute pain, chronic pain, and their reversal by morphine, and, thus, we call this the selective population for pain.
3. Within-Day Registration of Noxious, Aversive, and Appetitive Responsive Neurons. – For this revision, we collected a novel dataset wherein noxious hot water, aversive quinine, and appetitive sucrose-responsive neurons were identified and their responses to other stimuli were tracked (**Supplementary Figure 12**). A subpopulation of approximately 14% of ACC neurons responded only to 55°C hot water, suggesting selective engagement during noxious stimuli. This functional dissociation suggests separate neural circuits are dedicated to processing noxious vs. aversive vs. appetitive stimuli within the ACC.

Revisions to address comments. To address the reviewer's concern, we have refined our language in the manuscript to clarify that responsive ACC neurons are functionally selective rather than exclusively "pain-specific". That is, these neurons exhibit preferential engagement during noxious stimuli, are distinct from reward- or aversion-responsive populations, and contribute to pain-driven behavioral states, and we acknowledge that these neurons may still participate in broader affective and motivational processes unrelated to pain, which far exceed the scope of this revision.

We thank the reviewer for highlighting the need to clarify this key point, and we believe these revisions strengthen the manuscript by providing a more precise definition of pain-selective neural ensembles in the ACC.

Referee 1, Comment 4. The classification of pain behaviors using Fisher decoder from neural data are very impressive and a notable finding. A small point: calculation of p-value does not require t-tests, but should be calculated directly from the shuffled label models (by calculating the probability that true values from models occur in shuffled models). Also, authors should report AUC-ROC instead of accuracy, which is less robust.

AUTHORS: We appreciate the reviewer's positive feedback on our Fisher decoder analysis and agree with their suggestion regarding the calculation of p-values and the use of auROC. T-tests here were performed to show that behavior encoding was significantly above chance across animals, as compared to each of their chance level accuracies, so these comparisons are retained. However, the reviewer is absolutely correct, and we included auROCs and permutation tests in the supplement (Fig S10).

Referee 1, Comment 5 + Remarks on code availability. The software code provided by the authors appeared to be well commented but could not successfully be run because a data file that should have contained raw data was empty.

The code appears well commented and in theory should run. However, the raw data (pkl) file is empty, so the code does not successfully run and so results are not able to be reproduced.

AUTHORS: We apologize for this oversight and appreciate the reviewer's effort in testing our provided software code. We have now corrected the issue by ensuring that the necessary raw data file is properly included and accessible. Thank you for bringing this to our attention, and we appreciate your patience. We have also retested the code to confirm functionality and have provided updated instructions to prevent any future issues.

Referee 1, Comment 6. Some, points in the discussion should be adjusted to account for uncertainty in the literature. For example Line 468 "While opioids can be administered to the spinal cord..." This statement is not necessarily true, as to what is the 'primary' site of action that provides relief for opioids., most likely depends on what the pain generator is (spinal infusions can help completely when pain is primarily peripheral or spinally driven).

AUTHORS: We appreciate the reviewer's suggestion to refine certain statements in the discussion to better reflect the complexity and variability in opioid mechanisms of action. Specifically, we acknowledge that the primary site of opioid analgesia is context-dependent and route-of-administration-dependent, influenced by the source and nature of pain (e.g., peripheral, spinal, or supraspinal mechanisms). To address this, we have revised the relevant discussion points to remove any overly definitive or general language and instead acknowledge the variability in opioid action depending on the pain generator. We now state that opioids can act at multiple levels of the nervous system, including the spinal cord, brainstem, and cortex, with their primary site of action varying depending on the pain condition being treated. Thank you for highlighting this nuance, as it allows us to present a more balanced and accurate discussion of opioid pharmacology.

Referee #2 (Remarks to the Author):

This is an exciting, impactful, well execute set of studies, where James, Oswell, McCall, Hsu et al. propose that distinct mu opioid receptor expressing nociceptive neurons in the anterior cingulate cortex mediate the affectively aversive components of pain. To assess pain behaviors without bias of experimenter presence and manual behavior scoring, the authors develop a new experimental arena and computational behavioral analysis pipeline, collectively called LUPE. Using this technique, the authors determine the effective dose of morphine to produce "affective" analgesia in contrast to previous doses used to induce reflexive sensory analgesia in mice. Then they further uncover the neural basis of this effect through activity based "trapping" of pain responsive ACC neurons. Using whole brain imaging and single nucleus RNA sequencing, they identify the anatomical and genetic characteristics of these neurons as well as their projections. Lastly, using a custom intersectional genetic strategy, the authors demonstrate that manipulation of these nociceptive, mu opioid receptor expressing neurons in the ACC specifically attenuates affective pain behaviors in both acute and chronic pain models.

The authors have both developed major experimental and technological innovations which they use make unique observations about the complex pain encoding properties of the ACC. The development of an unbiased behavioral assay

and analysis pipeline addresses core issues of experimenter bias that have plagued pain research for decades. Importantly, they thoroughly characterize nociceptive ACC neurons, providing critical insights into the anatomical, genetic and circuit characteristics of these neurons and demonstrate that precision targeting of these neurons could be used to alleviate affective components of pain.

Overall, this is a tour de force, with an incredible and impressive amount of work included. Unfortunately, the amount of data is at times overwhelming and sparingly/inadequately described and the display of the data is confusing in several instances. This combination leaves the reader confused and wondering whether inclusion of less data would have given additional room for a more detailed and thorough description and synthesis of the major findings and impact. Further, in some instances the methods and logic for the interpretation of the data (or choice of analysis and display) is unclear or lacking. Finally, the authors should reconsider the order in which the figures are displayed and described- e.g. starting with LUPE felt slightly misleading since LUPE was then rarely used in the rest of the paper and/or if it was, the results were not clearly meaningful and other readouts were provided (e.g. attending). Additionally, ending on the tracing experiments felt anticlimactic/open ended. Overall, this paper presents important, impactful technical and scholarly contributions to the field of pain neuroscience; however, several recommendations are necessary for publication. These recommendations are detailed below to further clarify the behavioral readouts, operational definitions, and other methodological details throughout the paper. The authors base their primary conclusions on these novel readouts and analyses, but it is unclear how reliable, replicable and meaningful they are currently. In addition, reorganization and simplification of the paper should be strongly considered for maximum and widespread impact.

AUTHORS: We genuinely value the reviewer's thoughtful and gracious evaluation of our work, as well as their recognition of the technical and conceptual contributions this study makes to the field of pain neuroscience. It is gratifying to hear that the reviewer found this study to be a well-executed, impactful, and exciting body of work. We are especially grateful for the reviewer's positive remarks regarding:

- The development of LUPE as an experimenter-free behavioral assay that addresses long-standing biases in pain research.
- The rigorous characterization of nociceptive, mu-opioid receptor (MOR)-expressing ACC neurons, including their anatomical, genetic, and circuit properties.
- The demonstration that targeting these neurons through a novel genetic strategy can selectively alleviate affective pain behaviors.

At the same time, we fully acknowledge the reviewer's concern that the breadth of data, while valuable, can at times be overwhelming and would benefit from clearer presentation, interpretation, and synthesis. This is very useful feedback, and we have taken this to heart in our extensive revisions to improve clarity, focus, and organization.

Key Revisions Included to Address the Reviewer's Suggestions:

1. **Refining the Organization and Flow of the Manuscript**
    - We have reorganized the manuscript to create a more natural and conceptually coherent flow, emphasizing core biological discoveries while maintaining LUPE as a central methodological advancement, now featured in four of the five main figures. All non-LUPE metrics, including human-experimenter-evoked assays (e.g., von Frey), have been moved to the Supplementary Materials. **This refinement further clarifies that our primary focus is on spontaneous pain in acute and chronic injury models, as these are the most clinically relevant features of pain, which support the use of the opioid-mimicking gene therapy**.
    - **The axonal output tracing and the monosynaptic rabies tracing experiments have been removed.**
2. **Clarifying and Streamlining Data Presentation**
    - We have simplified all figures and moved some details to the Supplementary Materials to ensure that each main figure conveys a clear and interpretable finding without unnecessary complexity.
        1. For example, all FISH and snRNAseq analyses of opioidergic and nociceptive cell types have been moved to the Supplementary Materials, ensuring that the Main Figures focus on morphine's analgesic mechanisms and how we can mimic its antinociceptive effects using an ACC-targeted chemogenetic gene therapy.
    - We have revised the descriptions of key results to better synthesize the major takeaways and clarify their significance.
3. **Ensuring Operational Definitions and Readout Validity**
    - We have now explicitly defined our behavioral metrics (e.g., how LUPE-derived readouts relate to affective pain, nociception, and attentional components) and have strengthened the justification for their reliability and relevance.

- We have also included additional validation of our novel behavioral metrics to demonstrate their reproducibility and robustness.

4. **Refining and Strengthening the Discussion**
   - We have revised the Discussion section to better integrate and synthesize findings across the various methodologies used.
   - We have more clearly articulated how each component of the study contributes to the broader understanding of cortical pain processing and analgesia.

Referee 2, Comment 1. Methodological detail in the Methods and in the Figures and legends – Insufficient methodological detail throughout the main text, methods and figure legends make it very difficult to discern necessary experimental details throughout the entire paper. Without describing ever instance, here are some examples:

AUTHORS: We appreciate the reviewer's feedback on methodological clarity and have made every effort to address these concerns to ensure that the manuscript is as clear and accessible as possible. Our primary goal is to advance the conceptual foundation supporting the development of CNS gene therapy for chronic pain, and we recognize that clear methodological descriptions are essential for effectively communicating our findings.

We have attempted to add as much methodological detail as possible to the Main Text; however, we ask the reviewer to consider the constraints of formatting in Nature making it extremely difficult to include every methodological detail within the main body. This limitation is why we wrote an extensively detailed Methods section (8 pages in length) and provided didactic illustrations and timelines throughout the Supplementary Figures.

We firmly believe that scientific replication is essential, and we want our tools and approaches to be widely adopted by the field. For this reason, we have prioritized being as comprehensive as possible in detailing our methods. However, we also needed to balance this level of detail with Nature's broad readership, which includes many scientists who are not pain researchers or even neuroscientists.

To improve clarity, consistency, and transparency, we have made the following revisions:

- Expanded the Methods section and figure legends to provide more explicit methodological details.
- Ensured consistency in behavioral readouts and data presentation across figures.
- Added missing experimental details, including AP coordinates, scale bars, viral serotypes, and sex distribution of subjects.
- Clarified the purpose and rationale behind specific experimental choices, especially in the tracing experiments.

Referee 2, Comment 1.1 In figures 2,4,5 there is a little black box/mouse symbol to display the use of LUPE (I gather, though this is not explicitly stated anywhere), but this symbol is difference from the symbol used for LUPE in fig 1- making it confusing to follow.

AUTHORS: We acknowledge the inconsistency in LUPE's graphical representation across figures. Since LUPE is now the primary behavioral readout in 4 of 5 figures, we no longer need to use cartoon symbols to label different test panels. This revision ensures a more consistent and streamlined presentation of our data.

Referee 2, Comment 1.2 The behavioral readouts and pain testing are consistent in Fig 1-2, then are different in the following Figures, but the justification is unclear, and the data was difficult to follow for this reason.

AUTHORS: We agree with the reviewer and appreciate the opportunity to significantly revise our data presentation to improve clarity and consistency. We have now made LUPE the primary behavioral readout for both morphine and our gene therapy experiments, reflecting our focus on spontaneous pain rather than human-experimenter evoked pain. To ensure a clearer and more logical flow in the manuscript, we have standardized the behavioral readouts in the Main Figures to include only LUPE-derived measures of spontaneous behaviors related to affective-motivational pain responses. All traditional, experimenter-delivered evoked pain tests (e.g., von Frey, Hargreaves, acetone tests) have been moved to the Supplementary Figures.

This revision enhances the conceptual focus of the Main Figures on spontaneous, unbiased pain behaviors, while still making evoked measures available for reference in the Supplement. It also better highlights the novelty and strength of LUPE—specifically, its ability to capture fast behavioral dynamics of spontaneous pain behaviors without human scoring bias or experimenter intervention.

Referee 2, Comment 1.3 The exact viruses used in Fig 5i-m (including serotype etc) is unclear. It is also unclear why the representative photomicrographs are included for k and m but not l. Further, the text describing the purpose for each of these tracing experiments is not clear and took a long time to figure out.

AUTHORS: We appreciate the reviewer's feedback and have made several revisions to improve clarity and organization. To address these concerns, we have:

- **The axonal output tracing and the monosynaptic rabies tracing experiments have been removed to better focus the revision on the primary advancement—development of the opioid-mimicking gene therapy for pain.**
- Added methodological details, including viral serotypes and injection parameters to the Methods, to ensure full transparency and reproducibility.

Referee 2, Comment 1.4 the AP coordinates and scale bars are not included for photomicrographs, making interpretation difficult.

AUTHORS: We apologize for this omission. AP coordinates and scale bars have now been added to all relevant photomicrographs to facilitate interpretation.

Referee 2, Comment 1.5 It is unclear when males or females are being used (e.g. Fig 3a – how many of each sex?) – considering the known differences in pain thresholds and responses in males and females, this is critical information. There are additional examples throughout the manuscript. I suggest that the authors consider more consistency, simplicity and explicitly communicated methods throughout the manuscript.

AUTHORS: We acknowledge that clarity on sex distribution is essential, especially given known sex differences in pain processing. We have now explicitly reported the number of male and female subjects used in each figure in both the figure legends, and have created Supplementary Tables with such statistical details..

Referee 2, Comment 2. Affective behavioral definition and readouts

o In the main text, the authors reference " formalin-induced affective-motivational licking behavior" (line 124) and "reduces the selection of attentional recuperative behaviors (e.g., directed licking the effected somatic tissue)" (line 136). Multiple figures plot attending and licking behavior separately (e.g. Figure 4d, 4e). Clarifying how exactly these "licking" behaviors differ from each other and/or the licking behavior that is standardly used in the field. It is unclear how this relates to affect or motivation.

o The methods for quantifying "attending" behavior is not specified, nor are any references given for this scoring. It is briefly mentioned in the main text that "attending, guarding and escape" behaviors are quantified- but there are no specific details on what exactly this refers to. It is impossible to assess the validity of the data in the paper without explicit description of "attending" behavior and how this and licking equates to affective components of pain.

AUTHORS: We apologize for any confusion caused by our data visualization and appreciate the reviewer's request for greater clarity in defining and distinguishing licking and attending behaviors.

Clarifying "Licking" vs. "Attending" Behaviors

- The metric "attending" is a human-scored summation of directed licking at the injury site, which we have previously published as a measure of affective-motivational pain behaviors (Science 2019, Nature 2016, Nature Medicine 2017).
- In contrast, the "licks" shown in our figures were high-speed, LUPE-tracked behaviors, recorded with millisecond precision.
    - We have made an updated Methods subsection clarifying all operationalized methods.

To simplify data interpretation and avoid confusion, we have moved all human-scored data from evoked stimuli and hotplates, etc. to the Supplementary Figures. Additionally, we have explicitly explained in the text why human scoring is subjective and inadequate for capturing sub-second behavioral dynamics and transitions, which are crucial for linking real-time neural activity to pain states. This limitation was one of the key reasons we developed LUPE, which provides objective, automated, and high-resolution quantification of spontaneous pain behaviors.

By emphasizing LUPE-derived results in the Main Figures, we ensure that our primary analyses are unbiased, reproducible, and better aligned with high-resolution neural recordings. This physical separation of behavior types between the Main and Supplementary Figures should further alleviate confusion for readers unfamiliar with different behavioral classifications.

We sincerely appreciate this feedback, as it has allowed us to improve the clarity, rigor, and accessibility of our behavioral definitions while reinforcing the importance of LUPE as an essential methodological advancement in pain research.

Referee 2, Comment 3 Figure 1-5 -The authors quantify pain responses using the "% maximum possible effect" however this metric is not described anywhere in the main text. What is the % MPE of VF Mechanical Threshold? Where is the raw data for these graphs, can it be included in the supplement? It is difficult to compare these data to previously published work when it is displayed in this way.

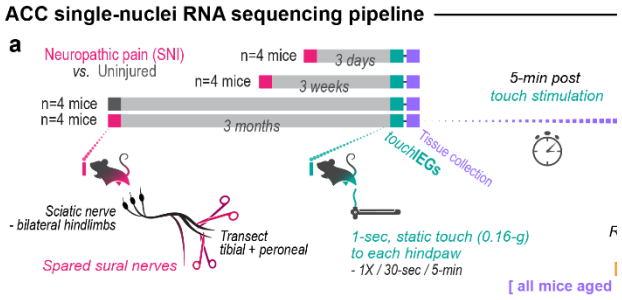
AUTHORS: We have included all requested raw data for these measurements in the Supplemental Figures and updated the Methods section to explicitly describe the calculation of Maximum Possible Effect (MPE) for all relevant behavioral measures. MPE is calculated as:

$$\%MPE = 100 \times \left(\frac{\text{drug response time} - \text{baseline latency}}{\text{cutoff time} - \text{baseline latency}} \right)$$

MPE normalizes behavioral responses across individual mice, accounting for baseline differences and ensuring that pain responses and analgesic effects can be compared across groups in a standardized way. This approach is widely used in pain and analgesia research, particularly in studies assessing drug-induced changes in pain thresholds (PMID: 22543045).

Referee 2, Comment 4. Fig 3 – Displaying this data using the N as the number of cells rather than N as the number of mice (with a single data point for each animal representing the average number of cells) may be displaying pseudoreplication rather than a meaningful outcome. It is unclear how many animals were used for this analysis. Have the authors analyzed the data and display as the average per animal? If there is not a high enough N, this should be clearly stated as a more descriptive finding rather than quantitative.

AUTHORS: We respectfully note that the number of mice per group were written in the Main Text at the start of the paragraph on the snRNAseq results, and it was displayed in Figure 3a (**now Supp. Fig. 3a**) alongside the experimental timeline (n=4 male mice per condition, with 4 conditions = 16 mice total). **We apologize if this was not clear and have now added these group numbers to the Figure Legend, in addition to the Main Text and Figure illustration.**



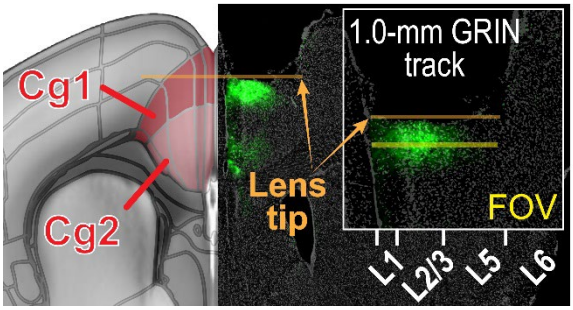
Our pseudobulk analysis follows the field's gold standard for single-cell and single-nucleus transcriptomic studies and we are fully powered for this quantitative experimental design. For pseudobulk analysis, we:

- First group individual nuclei by mouse/sample ID (i.e. n=animals) and cell type cluster (i.e. one cell-type group/animal/cluster).
- Then, average the counts of each gene within each group (i.e. pseudobulk counts).
- This gives you a "pseudobulk" gene expression profile for each sample-cell type combination, resembling a bulk RNA-seq dataset — but resolved by cell type.
    - **Better Statistical Robustness**. Single-cell differential expression analysis testing can be noisy due to zero inflation and cell-to-cell variability. Pseudobulk treats the *sample* (mouse) as the unit of replication, which aligns better with differential expression tools (DESeq2, edgeR, etc.).
    - **Controls for Sample-Level Variability**. True biological replication happens at the level of *samples* (not individual cells), as cells from any sample are likely to be transcriptomically closer to other cells from that same sample, rather than any other sample, so pseudobulk respects this by allowing differential expression testing across groups of samples — not across individual cells, which are not independent replicates.
    - **Avoids Overinflated p-values**. Studies using single-cell/nuclei approaches often suffer from overly optimistic p-values due to inflated n-values (n-value of cells rather than true biological replicates),failure to correct for multiple testing (which we utilize), and selection of less stringent differential expression

calculations (using a Wilcoxon Rank-Sum test vs. a linear mixed model or negative binomial distribution (of which we used the later). Our pseudobulk approach mitigates these, leading to less false positives.

Referee 2, Comment 5 Mapping the nociceptive hotspot- Here the authors state that Cg1 layers 2/3 followed by 5/6 are the "hotspot" with variable tagging in Cg2. However, the calcium imaging basically ablates Cg1 and is examining Cg2 due to grin lens placement (understandable, but needs to be addressed in the text somewhere and considered when drawing conclusions about the imaging and the other TRAP quantification).

AUTHORS: We apologize if our data visualization was not clear. The yellow lines in our figures indicate the Field of View (FOV), which represents the imaging plane located ~100–200 microns below the tip of the GRIN lens. To improve clarity, we have now updated all figures to explicitly show both the FOV (yellow lines) and a newly added orange line to mark the GRIN lens tips. These updates clearly demonstrate that we are imaging from the middle of Cg1, precisely within our defined "nociceptive hotspot."



We appreciate the reviewer's concern regarding potential tissue damage from GRIN lens implantation and its implications for interpreting our calcium imaging data in Cg1 vs. Cg2. To clarify:

- As with any invasive neuroscience procedure, some minor tissue damage is unavoidable, but we disagree with the characterization that the lens "basically ablates" Cg1.
- Miniscope 1p calcium imaging records signals from approximately 100–200 microns below the lens, meaning that neuronal activity from Cg1 layers 2/3 to 5 is still captured.
- Histological verification confirms appropriate lens placement in every mouse we run, allowing us to image a relevant portion of Cg1.

Referee 2, Comment 6.1 Figure 4 -The authors conclude that because chemogenetic targeting of the nociceptive MOR+ ACC population prolongs latency to "attending" (?) behaviors, these neurons specifically encode the affective component of pain. Figure 4o Results show that chemogenetic inhibition of these neurons induces analgesia to both acetone and 55-degree water droplet but does not affect von Frey thresholds. An alternative hypothesis to the author's proposition that nociceptive MOR+ ACC neurons are necessary for the affective dimension of pain (in contrast to sensory-discriminative aspect of pain) is that these neurons are modality specific (i.e. are responsible for analgesia to thermal pain stimuli and not mechanical). Because the Von Frey assay does not allow for an equivalent measure of pain "affect" to the thermal assay, the encoding of pain modality versus pain dimension is not addressed by the current set of experiments. Can you quantify and show the "attending" behavior following mechanical stimulation so that the behavioral readouts are the same?

AUTHORS: We appreciate the reviewer's suggestion to further explore whether ACC MOR+ nociceptive neurons encode the affective dimension of pain or if they are instead modality-specific (thermal vs. mechanical pain).

To address this question, we have now included new calcium imaging data from the ACC in response to heat, cold, mechanical touch, pinprick, sucrose, and quinine. In the **new Supp. Figure 11**, we find that:

- Single neurons show minimal modality overlap, but at the population level, ACC activity can reliably decode different external stimuli, reinforcing its role in integrating multiple pain modalities rather than encoding one specific type.
- ACC activity is more strongly linked to salient behavioral selection, such as engagement in recuperative licking, further supporting its role in spontaneous affective-motivational pain.

Thus, while modality coding may play a role, our findings do not support a modality-specific function for ACC neurons but instead reinforce their role in spontaneous affective-motivational behavior engagement after injury.

Revised Presentation of Evoked vs. Spontaneous Pain Data. To enhance clarity and conceptual focus, we have moved all evoked behavioral and calcium imaging data to the Supplementary Figures. This ensures that the Main Figures emphasize the most clinically relevant outcome—spontaneous neuropathic pain—rather than evoked, modality-specific responses. Given that spontaneous pain is the primary concern for chronic pain patients, its neural encoding in the ACC is the most translationally significant measure.

Additionally, lesion studies in humans do not suggest that the anterior ACC encodes sensory modality per se, but rather that ACC neural signals are primarily linked to pain affect. This aligns with our findings that ACC circuits exhibit broad population-level discrimination of external stimuli without strict modality specificity at the single-neuron level.

We have revised the text to explicitly address the distinction between modality specificity and pain affect encoding and to ensure that our findings are clearly interpreted as supporting affective-motivational encoding rather than simple sensory modality specificity.

Referee 2, Comment 6.2. Figure 4q the authors use unconventional methods to quantify affective pain such as "Delta time withdrawal to attend" and "% Attending post 1st attending bout." Further rationale should be provided to justify the use of this metric, as it does not appear to be validated with LUPE, previous literature or standard practice. In the absence of other readouts – it is unclear what these readouts really mean? Were there other parameters that were explored that were not significant (but maybe provide justification or context to the parameters that were chosen for display)?

AUTHORS: We appreciate the reviewer's inquiry regarding the use of "Delta Time Withdrawal to Attend" and "% Attending Post 1st Attending Bout" as behavioral metrics in Figure 4q and agree that these are less commonly reported in the pain literature. However, **these measures provide meaningful insights into the sequence and selection of pain-related behaviors, which is critical for understanding affective-motivational engagement with pain.**

Rationale for These Metrics

- While these specific measures may not be commonly used in pain studies, measuring the timing and transitions between different behaviors is a well-established approach in ethological behavioral science.
- "Delta Time Withdrawal to Attend" captures the latency to engage in pain-directed recuperative behaviors after an initial reflexive behavior, providing a quantitative measure of behavioral prioritization and decision-making following a noxious stimulus.
- "% Attending Post 1st Attending Bout" helps differentiate between initial behavioral selection or reflexive behaviors (which may or may not involve the brain) vs. sustained engagement in pain-directed actions, allowing us to investigate the role of the ACC in both cases separately.

In our original submission, we interpreted these findings as follows:

- Chemogenetic inhibition of ACC nociceptive MOR+ neurons prevents the cortical integration of valence, sensory, and motivational information, thereby deprioritizing engagement in recuperative nocifensive behaviors relative to other possible action selections.
- However, once an animal does engage in attending/licking, the behavioral sequence is maintained regardless of the inhibition of ACC neurons, suggesting that the ACC is involved in the initial behavior selection, but downstream circuits (e.g., BLA, PAG, NAc, VTA, or dorsal medial striatum) sustain the response, at least over the short time frames measured.
- This interpretation aligns with human cingulotomy studies, where patients exhibit pain indifference, reduced recuperative behaviors, and impaired pain recall. These findings highlight that affective-driven attentional processes to pain over time are the primary phenomenological aspect of the pain experience that the ACC is likely computing.

LUPE Validation and Other Parameters Considered

- LUPE-derived spontaneous behaviors and the transitions between those behaviors support the same conclusion that ACC manipulation alters pain-related behavior sequences.
- We did explore other parameters related to pain-directed behavioral transitions, but the metrics presented were chosen because they best captured the observed dissociation between nociceptive engagement and reflexive sensory responses.
- **To improve clarity**, we have moved this data into the Supplement and now explicitly state in the revised manuscript how the human-scored behavior transition data implies unique pain behavior patterns that cannot be captured by human eye, which justifies the development of LUPE. LUPE allows us to capture sub-second behavioral dynamics automatically and more objectively than human scoring, which is essential for sync'ing with calcium imaging to determine how rapidly neural processes are linked to behavior. These automated, high-resolution analyses provide a more precise and unbiased assessment of affective-motivational pain engagement, complementing traditional measures.

Referee 2, Comment 7 Figure 5 The authors conclude that because chemogenetic targeting of the nociceptive MOR+ ACC population diminishes attending behavior after acetone and 55-degree hot water droplet assays in chronic pain mice but not mechanical withdrawal threshold, manipulation of these neurons specifically modulates the affective dimension of pain.

AUTHORS: We appreciate the reviewer's feedback on Figure 5, particularly regarding the interpretation of modality vs. pain dimension specificity, the clarity of behavioral metrics, and the figure organization. Below, we address each point and outline our revisions to improve clarity and readability.

Referee 2, Comment 7.1 Figure 5E Lack of effect on withdrawal threshold compared to attending behavior following acetone and 55-degree heat stimuli may represent modality specificity rather than pain dimension specificity.

AUTHORS: See response to Referee 2, Comment 6.1.

Referee 2, Comment 7.2 Figure 5F Though the authors demonstrate a clear effect on lick instances (supporting the claim that these neurons are necessary for affective pain analgesia), there are no results displaying effects on withdrawal – which is needed to support their claim about the pain dimension specificity of this analgesic effect.

AUTHORS: We apologize that our data visualization/presentation was not clear in the original submission. We had multiple graphs in Main Figures 4 and 5 demonstrating no alteration in mechanical thresholds or withdrawal frequency to noxious heat and cold, and these results were provided for the *Oprm1* knockout, *Oprm1* re-expression, and the primary *mMORp*-DREADD experiments which supports our conclusion that ACC MOR+ neurons modulate the affective-motivational dimension of pain rather than sensory-discriminative reflexes.

To improve clarity, we have now moved all these results to **Figures S4, S16, and S17**, since the revised manuscript is focused on the more clinically relevant spontaneous pain rather than evoked sensory thresholds.

Referee 2, Comment 7.3 Figure 5F-H The design of these plots makes it incredibly difficult for the reader to follow and interpret results. "Lick left/lick right" and "groom/rear" imply this is a ratio (?) and it is unclear why these data are shown on the same graph and split into separate graphs for controls and DREADD groups. This would be much easier to understand if separate plots for each behavior with control and treatment groups were displayed on the same plot to allow for clear comparison between groups.

AUTHORS: Thank you very much for this suggestion. We sincerely aimed to simplify and clarify the figure presentation to ensure accessibility for all readers. To achieve this, we sought feedback from multiple faculty and graduate students, including non-pain experts and non-neuroscientists at Penn, Harvard, UCSF, UC Irvine and UCSD, to ensure that the updated graphs were clear, intuitive, and easy to interpret. This approach was met with great success, as these readers were able to follow the data presentation without confusion.

To directly address the reviewer's concern that the presentation of "Lick left/lick right" and "Groom/rear" may be difficult to interpret, we have made the following improvements:

- Separated each behavior into its own plot.
- Displayed control and treatment groups on the same plot to allow for direct comparisons between groups.
- Clarified all figure legends and axis labels to ensure that the data presentation is intuitive and easy to follow.

We greatly appreciate this constructive feedback and believe these updates significantly enhance the clarity and readability of our figures for all audiences.

Referee 2, Comment 7.4 In the main text, references to this figure do not follow along with the alphabetical order of the panels- making it difficult to follow.

AUTHORS: We have now revised the text to ensure that all references to panels follow the correct alphabetical order for improved clarity.

Referee 2, Comment 7.5 Figure 5I-K are very difficult to follow.

AUTHORS: To improve clarity, we have removed all input-output tracing experiments and revised the description of the DREADD FOS mapping experiment.

Referee 2, Comment 8 Fig. S10. Authors identify a 'hot spot' of nociceptive ACC neurons using TRAP2 activity dependent genetic capture and permanent fluorescent capture following exposure to a noxious thermal stimuli.

Referee 2, Comment 8.1 C-D- While the additional experiment in f and g showing activity in this population is valence specific subsequent calcium imaging and single cell RNA sequencing experiments use coordinates from this "identified" hotspot, however these experiments use 2 different forms of pain, capsaicin induced pain and sciatic nerve transection induced pain. Perhaps authors can provide additional justification for why we would expect these populations to overlap, or alternatively authors may perform an additional TRAP/cFos pain experiment to demonstrate the overlap in activity across these different pain modalities.

AUTHORS: We sincerely appreciate the reviewer's thoughtful suggestion and their interest in further validating the overlap between capsaicin-induced pain and sciatic nerve injury (SNI)-induced pain in ACC nociceptive neurons. While we recognize the explicit request to examine the overlap between capsaicin- and SNI-activated neurons using TRAP2 methods, we made the decision to prioritize developing the use of calcium imaging over TRAP2, since TRAP2 only provides a static snapshot, whereas calcium imaging allows us to examine dynamic, sub-second neural activity linked to behavior. As part of this shift, we refocused the paper's main figures to highlight calcium imaging data, while TRAP experiments have been removed from the main text to maintain the emphasis on real-time neural dynamics.

To directly address the reviewer's concern, we have performed extensive new calcium imaging in the ACC of both uninjured and SNI neuropathic pain mice to investigate neural responses to a panel of sensory stimuli, similar to our approach in Corder et al., Science 2019. Specifically, we tested:

- Noxious 55°C hot water
- Room temperature (30°C) water
- Acetone (cooling stimulus)
- Von Frey 0.16g light touch
- Pinprick (noxious mechanical stimulation)

In uninjured mice, we found that 6.3% of recorded cells were activated only by the three noxious stimuli: pin prick, acetone, and hot water. In comparison, 36% of neurons responded to noxious, but not non-noxious stimuli. Thus, 18% of all neurons that were selective for noxious stimuli were activated by all 3 stimuli of different modalities. This percentage was not significantly changed by SNI, with 19% of noxious-selective neurons responding to all three modalities. Additionally, while the individual neurons that respond to individual stimuli may not always be the same, all stimuli are readily decodable from ACC activity, suggesting that the ACC successfully encodes a wide variety of noxious modalities.

Further addressing the reviewer's concern, we performed calcium imaging before and in the days and weeks following SNI in addition to our original capsaicin experiment. We observed similar results as to our capsaicin experiment. Specifically, we identified neurons encoding lick probability that exhibited lick-encoding activation and inhibition. In both groups, morphine enhanced lick-encoding inhibition, and reduced calcium transient rates in these neurons overall in a manner predicting reduction in pain behaviors. While not performed in the same animals, this agreement between the capsaicin and SNI experiments suggests that there are functionally similar computations carried out in morphine-sensitive neurons within the same region following capsaicin- and SNI-induced pain and subsequent analgesia.

Referee 2, Comment 8 FigS12 The authors conclude that independent populations in the ACC encode valence
o Using activity dependent trapping, the authors compare populations activated by mating opportunity vs. those activated after pain stimulus administration. It is unclear if duration of stimulus, social quality of stimulus (i.e. mating opportunity requires presence of a conspecific while pain stimulus does not) and other properties of these stimuli are equivalent between the negative valence (pain) and positive valence (mating) stimuli. More equivalent positive valence stimuli should be used to properly test this hypothesis.

AUTHORS: We appreciate the reviewer's concern regarding the difficulty of testing valence and the need for appropriate comparisons between positive and negative stimuli. We fully acknowledge the challenges associated with valence testing, as we encountered similar difficulties when publishing the first study on pain valence calcium imaging in the BLA (doi: 10.1126/science.aap8586).

To strengthen our findings and provide an additional positive valence control, we have now added new calcium imaging experiments comparing sucrose (appetitive), quinine (aversive), and 55°C noxious hot water responses in the ACC. These data reveal:

- Opposing modulation of neuronal activity, consistent with the literature supporting separable valence-coding neurons and population codes in the ACC.

- Minimal overlap between sucrose- and noxious stimulus-responsive populations compared to overlap of aversive and noxious stimulus-responsive neurons suggesting a strong functional segregation of positive and negative valence encoding.
- Near-perfect decoding accuracy when classifying neurons based on their response to sucrose vs. noxious stimuli, reinforcing the idea that ACC neurons are able to discriminate valence.

These findings align well with our published results (Ye et al (Deisseroth), Cell. 2019; PMID: 27238022), where we observed clear valence segregation in the mPFC (ACC/PL) for cocaine vs. noxious shock-responsive populations, further supporting the notion that distinct neural ensembles encode appetitive vs. aversive stimuli.

Additionally, based on the reviewer's concern, **we have removed the TRAP2 data on mating opportunities to avoid potential confounds related to differences in social and temporal properties between mating and pain stimuli.** Instead, we now focus on a more behaviorally equivalent positive valence comparison using sucrose.

These new calcium findings, now included in **Supplementary Figure 12** to keep the Main Figures focused on pain, further strengthen our argument that separable populations of ACC neurons encode positive versus negative valence.

Referee #3 (Remarks to the Author):

In James et al, they show that a subset of MOR positive ACC neurons are responsive to pain and are capable of mediating affective analgesic responses to pain induced by 0.5 mg/kg of morphine. To demonstrate this, the authors develop an automated pain detection system (LUPE) and use this with genetic manipulation of ACC neurons while mice undergo morphine treatment. On the basis of this data the authors claim that these behavioral analyses represent an improvement on the assessment of pain, compared to models such as the von Frey test. In addition, the paper also includes transcriptomic data of pain-responsive ACC neurons. Although the amount of data provided is immense and impressive, the advance in scientific knowledge this paper provides seems rather incremental for reasons provided below, which leads the reviewer to believe that this manuscript may be better suited as a methods paper.

AUTHORS: We appreciate the reviewer's assessment of our work and their recognition of the technical rigor and depth of parts of our dataset. However**,** it appears that the reviewer may have assessed our earlier *bioRxiv* preprint (April 2024) rather than the substantially updated manuscript submitted to *Nature* (August 2024). The reviewer makes multiple comments on TRAP2 activity labeling data in a Figure 2, but Figure 2 is miniscope calcium imaging; there is no TRAP2 data in the main figures.

Thus, we believe that many of the key conceptual advances may have been overlooked, and we respectfully disagree with the characterization of the submitted study as primarily "a methods paper". We do agree that this characterization could be gleaned from a reading of the earlier preprint which had substantially different experimental goals, text, methods, etc.

Importantly, we are happy to report that the *Nature*-submitted manuscript from August 2024 included the exact *in vivo* miniscope calcium imagining experiments requested by the reviewer, as well as gene therapy behavioral data that directly addresses many of the comments below. Furthermore, this revision has addressed the remaining few clarification points.

We are eager to get on the same page with the Reviewer.

While LUPE is an innovative behavioral analysis tool**, our manuscript is fundamentally focused on biological and conceptual advances that provide entirely new insights into ACC nociceptive circuits and opioid-mimicking gene therapy for pain**. Below, we summarize the **major conceptual discoveries** presented in our revised work that address the comments of Reviewer 3:

1. ***Primary Advancement:*** CNS Gene Therapy for Circuit-Specific Analgesia ***(Concept never reported before)***

- We developed and validated a mu-opioid receptor promoter-driven (MORp) gene therapy that enables precise, circuit-specific modulation of opioid-responsive ACC neurons.
- This CNS gene therapy mimics morphine's analgesic effects without systemic opioid exposure, presenting a potentially transformative approach for pain management that currently is dominating by ingested/injected pharmacological drugs that act widely throughout the body.

2. Discovery of High-Dimensional Pain States Unfolding Across Seconds to Minutes

- We used high-dimensional behavior analysis from LUPE to quantify pain and non-pain states and develop an Affective Motivational Pain Scale, an objective measure of spontaneous pain engagement.

- Principal Component Analysis (PCA) revealed that Pain States cluster separately from pain-suppressed behaviors, demonstrating a data-driven classification of pain states.
- These pain states are composed of individual behaviors that unfold over timescales significantly longer than traditional reflexive withdrawal responses. We found that the **average duration of a spontaneous pain state bout is ~30 seconds, which closely parallels human data from Dr. Vania Apkarian reporting that the average perceptual bout of spontaneous pain is ~22.5 seconds** (Baliki et al. JNeurosci. 2006; PMID: 17122041).
    - Notably, this human study on backpain fluctuations also identified ACC activity as correlating with spontaneous pain, consistent with our findings that State-4 pain states exhibit increased affective-motivational behaviors such as licking, which are encoded by subsets of ACC neurons. **Thus, our study provides the first direct evidence of spontaneous pain in a preclinical model, which we further identified was encoded at the level of single neurons and ensembles within the ACC**
        - → Having identified **a new cellular target for spontaneous pain** (i.e., a functional population of ACC neurons), that is sensitive to morphine (Figure 2 and Figure 3), **we leveraged this new knowledge for a totally novel, biologically-inspired chemogenetic gene therapy** that directly interferes with this neural populations to alleviate spontaneous pain (Figure 4).

3. *In Vivo* Calcium Imaging of ACC Neurons During Evoked Stimuli and Pain Valence Panels

- To determine to what degree single neurons show modality overlap, we performed in vivo calcium imaging of ACC nociceptive neurons responding to a panel of evoked stimuli, including heat, cold, mechanical touch, pinprick, and innocuous water.
- These data demonstrate that single neurons show minimal modality overlap, but at the population level, ACC activity can reliably decode different external stimuli, reinforcing its role in integrating multiple pain modalities.
- We also investigated pain valence encoding using sucrose, quinine, and hot water stimuli, showing that ACC circuits encode pain-related affect rather than general valence.

4. Chronic Pain Development: Imaging Neural Activity Across Pain Chronification *(Concept never reported before)*

- We tracked the evolution of ACC nociceptive activity from acute to chronic pain states, providing the first longitudinal *in vivo* calcium imaging dataset of the ACC spanning chronic pain development over weeks.
- These findings reveal that chronic pain does not induce a persistent hyperactive state in ACC neurons but rather results in episodic engagement of pain-encoding circuits, challenging previous assumptions.

5. Morphine's Effects on Sub-second Neural Dynamics of Spontaneous Pain *(Concept never reported before)*

- We investigated how morphine alters neural activity in real time, revealing that morphine selectively suppresses sub-second neural dynamics linked to spontaneous pain episodes, while leaving non-pain behaviors intact.
- These finding highlight morphine's analgesic specificity for affective-motivational pain states, rather than a general suppression of behavior or neural activity.

Final Perspective. While LUPE is a powerful methodological framework, the core scientific contributions of our study lie in:

1. Mechanistic discoveries about ACC nociceptive circuits and their real-time function in pain behaviors.
2. A novel high-dimensional analysis of pain states, including an automated Mouse Pain Scale.
3. The first ACC imaging data tracking chronic pain development and opioid modulation in real time with spontaneous pain behavior.
4. The first brain circuit-targeted gene therapy strategy for precision analgesia.

Given the strength of these conceptual and translational advances, we believe the manuscript represents a major step forward in pain neuroscience rather than a methods-focused study.

Major comments:

Referee 3, Comment 1 The main strengths of the paper are both its methodological merit and the extensive characterization, detailed and clear instructions on how to construct LUPE, which is to be highly commended.

AUTHORS: We appreciate the reviewer's recognition of the novelty, methodological merit, and potential impact of our work. Our goal was to develop a rigorous, unbiased, and scalable approach to studying pain behavior and its neural underpinnings, and we are pleased that the reviewer finds LUPE to be a valuable contribution to the field.

Referee 3, Comment 2.1. The involvement of MOR in ACC neurons in mediating affective analgesic responses (albeit with a cruder methodology) of 0.5-1 mg/kg doses of morphine, without affecting mechanical paw withdrawal thresholds in von Frey tests, has been characterized pharmacologically in past literature (Experimental neurology, 197(1), 22-30.). The scientific merit of this manuscript seems limited to using more advanced methodology (AI behavioral analysis + genetic manipulation) to otherwise recapitulate past findings on the involvement of the ACC in mediating morphine-induced analgesia.

AUTHORS: We appreciate the reviewer's reference to the *Experimental Neurology* paper from Dr. Perry Fuchs and fully acknowledge the foundational contributions of prior studies demonstrating that low-dose morphine (0.5 mg/kg) selectively alleviates spontaneous pain without affecting mechanical withdrawal thresholds. In our *Nature* submission, we did not claim to be the first to make this observation; **this was merely a supporting observation, that we acknowledged with multiple citations of past literature, to our primary goal of mimicking this analgesic effect with the *MORp*-hM4 chemogenetic gene therapy**.

Indeed, *we cited this exact paper*, as well as multiple other key studies on opioid dosing to selectively alter affective-motivational pain behaviors, including two additional papers from Dr. Perry Fuchs (using the Place Escape/Avoidance Paradigm, PEAP), as well as work by Dr. Frank Porreca (Conditioned Place Preference), Dr. Clifford Woolf (home-cage wheel running), Dr. Bill Cowder (morphine reinforcement), and Dr. Rob Gereau (paw weight-bearing and CatWalk gait analysis). Each of these studies supports the distinction between affective-motivational and reflexive or motor side-effect measures in opioid pharmacology.

Our current study builds on this literature by showing that low-dose morphine (0.5 mg/kg) also produces analgesic effects as measured by LUPE—specifically, reductions in licking duration, Pain States probabilities, and Pain Scale scores. **We were pleased to find that our LUPE-based findings are consistent with the existing literature and further validate LUPE's ability to capture affective-motivational aspects of pain**.

However, **our intention was not merely to replicate these behavioral findings using more advanced methods. Rather, we sought to extend this foundational work by providing the first-ever real-time, single-neuron and population-level recordings of morphine's effects in the ACC linked to spontaneous pain behavior.** This unprecedented new data provides the biological rationale for developing our opioid-mimicking gene therapy approach.

We apologize if the structure of our previous manuscript placed undue emphasis on this replication, which is a minor aspect of our studies. **In this revised version, we retain all references to low-dose morphine analgesia but now focus more directly on the novel aspects of our work—specifically, the imaging results and their implications for the development of new, circuit-targeted analgesic strategies.**

Referee 3, Comment 2.2 Additional insights such as a functional role for any of the genes identified in their transcriptomic data or projection-specific manipulation/functional circuit dissection of MOR painTRAP ACC neurons would have dramatically improved the scientific merits of the paper.

AUTHORS: We understand the reviewer's suggestion to further explore the functional role of genes identified in our transcriptomic analysis and to incorporate projection-specific manipulation of MOR painTRAP ACC neurons, based on the written results text from the *bioRxiv* preprint version of our work. So, while potentially interesting, this is a far tangent from the submitted Nature manuscript's primary focus to develop an opioid mimicking chemogenetic gene therapy.

Important to the reviewer's comment, a critical takeaway, from our snRNA-seq dataset—discussed in depth in the submitted *Nature* manuscript, but not the *bioRxiv* preprint—is that transcriptional changes in the ACC at 3 weeks and 3 months post-SNI are remarkably limited. Despite sequencing over 100,000 nuclei, only eight genes showed significant upregulation, and these were restricted to a single neuronal subclass (L5 PT-2 neurons). The absence of widespread and enduring transcriptional remodeling suggests that chronic pain does not result in large-scale, long-term molecular shifts in the ACC, making it difficult to identify strong candidate genes for functional validation.

Overall, we found a lack of strong drug targets from our transcriptomic data. Among the eight genes identified, only *Htr1f* and *Hdac9* have been previously implicated in pain mechanisms and have existing pharmacological agents. However:

- *Htr1f* (5-HT1F receptor) is widely expressed across the brain and targeting it pharmacologically would not be ACC-specific, reducing its relevance to studying ACC circuits as a translational target. Cannulation might be interested but this again is not a translationally viable option and far outside our goal of developing an ACC targeted gene therapy.
- *Hdac9* (Histone Deacetylase 9) is also broadly expressed, and HDAC inhibitors have systemic effects, making them unsuitable for precise circuit-level pain modulation.

- Other genes, such as *Sorcs2* and *Tafa1*, have some associations with pain modulation, but there are currently no drugs that selectively target them.
- The remaining genes (*Mgat4c, Gm17167, Gm30382, Schip1*) have no known links to pain and no available pharmacological agents to test.

While we agree that functional validation of transcriptomic findings can be valuable, the lack of a strong, ACC-restricted druggable target severely limits the feasibility of a pharmacological approach. Given this limitation, further pharmacological exploration would be extraneous to our extensive efforts to develop an ACC-targeted gene therapy, which remains the central translational focus of this study. **To remove ambiguity for readers, we have moved all snRNAseq data to the Supplementary Figures and have kept the Main Figures focused on our gene therapy approach**.

Why a Circuit-Based Gene Therapy Paves the Way for Precision Pain Medicine. The failure of traditional drug development for chronic pain stems largely from the lack of viable and specific molecular targets that change consistently across individuals or pain conditions. This challenge motivated our development of the MORp gene therapy platform, which bypasses the need for disease-specific molecular targets and instead targets the functionally defined neural circuits that mediate analgesia. Unlike conventional pharmacological strategies, which indiscriminately affect multiple receptors and brain regions, our MORp-based gene therapy offers circuit-level precision through a synthetic mu-opioid receptor (MOR) promoter designed to restrict expression to endogenous opioid-sensitive neurons in the anterior cingulate cortex (ACC).

Key advantages and a look toward future impacts on effective and safe clinical pain relief:

- **Precision targeting of opioid-responsive neural circuits.** MORp gene therapy selectively modulates the same ACC neurons involved in morphine-induced analgesia, enabling cell-type and circuit-specific therapeutic intervention.
- **Minimization of systemic side effects.** By confining therapeutic effects to a defined brain circuit, this approach may circumvent the widespread off-target effects (e.g., respiratory depression, constipation, sedation) commonly associated with systemic opioid administration. Efforts stemming from our critical first development of a CNS gene therapy will need to verify these possible massive benefits of such a targeted system.
- **Bypassing the need for chronic pain-altered molecular targets.** Many chronic pain conditions lack consistent transcriptional signatures, limiting traditional drug discovery. Our approach leverages circuit function, rather than disease-specific molecular changes, as the therapeutic substrate.
- **Supports long-term, non-addictive pain relief.** This strategy opens the door to sustained pain relief without the addiction liabilities and tolerance associated with repeated opioid dosing.
- **A platform for modular control.** The *MORp* system is compatible with chemogenetic or optogenetic actuators or other neuromodulatory systems, allowing dynamic, on-demand control of pain relief—tailored to the individual's pain state or daily activity pattern.
- **Applicable across diverse pain conditions.** Because the therapy targets generalizable circuits for pain affect and not condition-specific peripheral pathology, it could be broadly effective across multiple chronic pain diagnoses, including neuropathic, inflammatory, and centralized pain disorders.
- **Improved quality of life and reduced health system burden.** Chronic pain is a leading cause of disability and healthcare utilization. A precision therapy that avoids opioid side effects could significantly reduce hospital visits, improve function, and decrease dependence on polypharmacy.
- **Scalable and translatable therapeutic platform.** *MORp* gene therapy lays the groundwork for a new class of CNS-targeted, circuit-specific gene therapies that could be adapted to other conditions involving dysregulated affective-motivational processing, such as depression or addiction.

Referee 3, Comment 3 How specific are these neurons to mediating analgesic responses? Is it possible that the mice are undergoing deficits in decision making, thus making this a more generalized impairment of action selection or are these neurons specific for pain coping behaviors? Measuring the activity of these neurons (Eg. Miniscope single-cell calcium imaging or, single unit-recordings) when mice are engaged in pain coping behaviors and/or using negative controls (Non-pain related decision making/action selection tasks) during chemogenetic inhibition, may be able to address this question.

AUTHORS: We thank the reviewer for this excellent and insightful comment, but **we believe this is response to the April 2024 *bioRxiv* preprint, and not to the manuscript submitted to *Nature* in August 2024**. However, we're especially pleased that our original miniscope calcium imaging data aligns well with the reviewer's suggestion to examine the activity of specific neurons during pain coping behaviors. Our original submission included evidence that anterior cingulate cortex (ACC) neurons show selective responses—quantified by the **d-prime** metric (see just below and **Supp. Figure 10**)—for licking behavior, and that morphine enhances this selectivity by suppressing activity during grooming (another self-directed behavior modulated by pain).

- d-prime (d′) is a measure from signal detection theory that quantifies how well two distributions (such as neural responses to two different behaviors) can be distinguished from each other. It reflects the separation between signal and noise, taking into account both the mean difference and the variability of the responses. Here, d′ is used to assess how selectively a single neuron or population responds to one behavior over another. A higher d′ value indicates greater selectivity or discriminability.
  - **Formula**: $d' = (\mu_1 - \mu_2) / \sqrt{(0.5(\sigma_1^2 + \sigma_2^2))}$ where μ and σ are the means and standard deviations of the two distributions.
  - **Simply put**: d′ = 0 means no discriminability (responses are indistinguishable), whereas d′ > 0 means increasing ability to distinguish between conditions (e.g., licking vs grooming)

Moreover, population-level decoding of behavior remains robust under morphine, which would likely degrade if there were a general impairment in action selection. These findings suggest that low-dose morphine sharpens behavioral representations rather than impairing decision-making processes.

To further emphasize this point, in a new experiment imaging across days and weeks following spared nerve injury (SNI), we included nine SNI and nine uninjured control mice to ensure a sufficient sample of non-pain-associated hindpaw licking events. Strikingly, we observed significant adaptations in lick-evoked activity in neurons encoding lick probability after SNI that were not present in uninjured controls. Most directly addressing the reviewer's question, we found that the extent to which morphine restored lick-encoding activity to baseline levels predicted the reduction in pain behaviors across individual SNI mice—but not in uninjured animals. This result strongly implies that morphine's effects on these neurons are related to analgesia, in a manner that involves action selection but cannot be fully explained by a generalized impairment in it.

While we fully agree that calcium imaging during chemogenetic inhibition of ACC MOR+ neurons would be a powerful next step, this experiment would require a prohibitively large number of GRIN-lens implanted mice due to the need for appropriate viral and behavioral controls, essentially doubling our current cohort of 18 animals. However, supporting the connection between our imaging and chemogenetic results, we found that spontaneous transient rates in lick-probability encoding neurons are sensitive to morphine, suggesting that these neurons may be MOR+ and potentially the same population we manipulate in our chemogenetic experiments.

Taken together, our data support the conclusion that the reduction in pain-related behaviors we observe following chemogenetic inhibition of MOR+ ACC neurons mimics an analgesic effect rather than inducing a generalized deficit in action selection. Specifically:

1. Morphine either preserves or enhances the neural discriminability of behaviors in both injured and uninjured mice, arguing against a global disruption of action selection at 0.5 mg/kg.
2. SNI induces adaptations in lick-evoked responses in morphine-sensitive ACC neurons that are not seen in controls, suggesting these neurons track pain state and overlap with the MOR+ population we later manipulate.
3. The degree to which morphine normalizes activity in these neurons predicts behavioral analgesia in SNI but not uninjured mice, linking morphine's neural effects directly to pain relief rather than nonspecific behavioral changes.

Referee 3, Comment 4 The painTRAP method involves exposure to 55°C water, compared with *homecage*TRAP. Since the anterior cingulate cortex (ACC) is involved in multiple processes, including emotions and memory functions, using a non-painful stimulus (e.g., room-temperature water) as a control would help rule out emotional changes caused by the water exposure itself.

- **AUTHORS:** We appreciate the reviewer's suggestion to include a non-painful control stimulus to distinguish pain-specific activation from general emotional or stress-related responses. To address this, we performed miniscope calcium imaging data examining ACC neural responses to a panel of stimuli including non-noxious controls, including the room-temperature water stimulus. **Please see Referee 2, Comment 6.1 and Supplementary Figure 11 for details.**

Referee 3, Comment 5 Previous studies ([Neuron. 2024 Apr 17;112(8):1328-1341.e4.], [Science. 2021 Jan 8; 371(6525): 153–159.], [Nat Commun. 2018 Sep 14;9(1):3751.], [Science. 2010 Dec 3;330(6009):1400-4.]) have shown that chronic pain induced by inflammatory or nerve injury increases spontaneous ACC activity. However, in Figure 2F-G, the SNI model showed reduced FOS expression compared to the uninjured group, suggesting decreased spontaneous ACC activity in chronic pain conditions. Additionally, in the SNI model, painTRAP ACC neuron activity appeared suppressed (Figure

H-J). Nevertheless, in Figure 5B-C, additional optogenetic inhibition of painTRAP-MOR neurons induced place preference in the SNI model. This inconsistency needs to be addressed.

AUTHORS: While we appreciate this interesting line of reasoning, **we believe this comment is in reference to the out-dated information in the April 2024 *bioRxiv* preprint that had a FOS mapping dataset in Figure 2**. As this is not a part of this submission, we cannot directly address this comment.

Referee 3, Comment 6 Numerous studies have sought to establish homologies between the mouse and human brain. However, identifying corresponding regions has proven challenging. In Figure 2J, they note that the mouse brain regions were selected based on human pain fMRI literature (NPS and SIIPS). While NPS and SIIPS include numerous cortical regions rather than subcortical areas, there are significant differences between the mouse and human cerebral cortex. And many of the selected areas are subcortical regions. More detailed information on the selection criteria for the human & mouse brain regions is needed to substantiate these claims.

AUTHORS: This comment on "Figure 2J" appears to be in reference to our out-of-date *bioRxiv* preprint and not the submitted manuscript to *Nature*, since Figure 2 was miniscope calcium aiming in the submitted manuscript. Since that preprint draft was written, we have removed this data and cannot directly address this comment.

Referee 3, Comment 7 The authors claim no change in chamber selection during the active optogenetic sessions in the uninjured group (Figure 5C). However, since the data lacks a GFP control group, this conclusion is unsupported. The uninjured mice may have shown significant avoidance of the light-paired chamber compared to their baseline. The experiment should ideally be redone with proper controls.

AUTHORS: We sincerely appreciate the reviewer's careful consideration of our experimental design and the suggestion to include a GFP control group to rule out potential avoidance of the optogenetic-paired chamber in uninjured mice. We acknowledge the importance of appropriate controls in optogenetic experiments and fully agree that behavioral interpretations must be made cautiously.

To clarify, we did not observe any evidence of avoidance behavior in uninjured control mice. If optogenetic inhibition had been aversive in uninjured animals, we would have expected a significant reduction in time spent in the optogenetic-paired chamber; however, this was not the case. Instead, uninjured mice spent equal time in both chambers before and during the optogenetic session, supporting the conclusion that the manipulation itself did not induce an aversive response.

Why a GFP control group was not included. We fully acknowledge the rationale behind using a GFP control to **rule out non-specific effects of optogenetic stimulation**, but we believe our existing design already accounted for these concerns:

1. **The experiment was designed to test the effect of nerve injury, not a direct effect of light exposure.**
    - Since uninjured mice showed no avoidance or preference, we did not observe any evidence that light exposure itself influenced behavior.
    - A GFP control would typically be necessary if uninjured animals had shown avoidance, which was not the case.
    - Furthermore, given that we have already demonstrated that optogenetic inhibition does not affect chamber preference in uninjured animals, we believe repeating the experiment for this additional group would not be a justified use of research funds, time, or live animals.

Revised Placement of the Experiment in the Supplement. Recognizing that this experiment was only intended to support the translational MORp-hM4 dataset, we have now moved this analysis to **Supplementary Figure 20** to better reflect its supporting role in the manuscript.

We greatly appreciate the reviewer's suggestion, as it prompted us to further clarify our rationale for experimental design. While we acknowledge the value of a GFP control in some contexts, we believe our existing data sufficiently control for non-specific effects, and adding this group would not be a responsible use of resources or live animals. That said, we remain open to discussing any additional ways to further improve the clarity of our conclusions.

Minor comments

Referee 3, Minor 1 Naming conventions for experimental treatments are confusing at times. For example, in Figure 3P-Q, the text describes this as uninjured vs SNI. However, when reading figure S15, the uninjured group also received 55C water drops whereas the SNI group received 0.16g-filament pokes. These differences need to be made clear in the main text.

AUTHORS: We believe this comment may refer to our April 2024 *bioRxiv* preprint rather than the submitted version of the manuscript, as the Figure 3 referred to was not included in the submission but was partly shown in a Supplementary Figure with major aesthetic/design updates. Nonetheless, we have carefully reviewed the text and have prioritized clarity in the revised manuscript, ensuring that all experimental conditions are clearly defined and consistently labeled throughout the figures and main text.

Referee 3, Minor 2 Related to the above comment, naming is not consistent at times. Mice with SNI are referred to as the SNI group, whereas in other figures (Figure 5C) they are referred to as the neuropathic pain group.

AUTHORS: We have carefully reviewed the text and have prioritized clarity in the revised manuscript, ensuring that all experimental conditions are clearly defined and consistently labeled throughout the figures and main text.

Referee 3, Minor 3. Figure 2B is a little puzzling given that there are numerous reports on morphine being able to paradoxically cause hyperalgesia (OIH) ([Pain physician, 14(2), 145.], [Brain Research, 888(1), 75-82.]). One may expect touch-TRAP neurons to possibly be separate from painTRAP neurons given this, can the authors speculate on how to resolve this discrepancy, especially given that they did not find any changes in OPRM1 expression at any of the SNI timepoints (Fig S15)?

AUTHORS: Since Figure 2 of the submitted paper was on miniscope calcium imaging, this appears to be a reference to our April 2024 *bioRxiv* preprint. Given that the specific figure and analysis in question are not included in the submission, this point does not seem directly relevant to our revised study. Additionally, while opioid-induced hyperalgesia (OIH) is an important phenomenon in pain research, it is not something we have investigated or discussed in this manuscript. As a result, we are unable to fully address the specifics of this concern within the context of the current study. Nonetheless, we appreciate the reviewer's engagement with our work and remain open to discussing any aspects of the submission that may require further clarification.

Referee 3, Minor 4 The methods section appears to contain errors, likely from copying and pasting from other papers. For example, it references fiber photometry testing and naloxone-precipitated withdrawal, neither of which are included in the paper. Additionally, the Hotplate thermal test section mentions using CNO in Figure 3C, but Figure 3C is clustering of transcriptomic data.

AUTHORS: This comment appears to be in reference to our April 2024 *bioRxiv* preprint rather than the submitted manuscript, as the mentioned methodological inconsistencies do not pertain to the current version. That said, we have carefully checked all methods in the revised manuscript to ensure accuracy and clarity. We appreciate the opportunity to refine this section and will make any necessary corrections to maintain consistency between the text and figures.

Referee 3, Minor 5 The calculation of "Max Possible Effect" in Figure 4 is not described and should be included in the methods section.

AUTHORS: We have updated the **Methods section** to include a clear description of this calculation to ensure transparency and reproducibility. Additionally, please see Referee 2 comment 3 for an explanation of how MPE was used.

Referee 3, Minor 6 There is a possible typo on page 8 regarding "Fig. 2D," which may need correction.

AUTHORS: This comment appears to be in reference to the April 2024 *bioRxiv* preprint and not the submitted manuscript to *Nature*. Nonetheless, we have carefully reviewed the revised text to ensure all figure references are accurate and correct any potential errors.

Referee 3, Minor 7 Figure 4J is not mentioned in the results section.

AUTHORS: The results for **Figure 4J** were described in the text; however, we acknowledge that we **missed adding the "J" at the end of the sentence**. Nonetheless, **we have removed these data from the revised manuscript to avoid the impression that this is a methods-focused study.**

Referee 3, Minor 8 Figure legends for Figure 4 contain some typographical errors that need to be corrected.

AUTHORS: Thank you for catching these typographical errors. We have carefully reviewed and **updated all typographical errors in the figure legends for Figure 4** to ensure clarity and accuracy in the revised manuscript.

Referee 3, Minor 9 Figure S4 focused on comparing LUPE to Noldus-based Ethovision. These experiments demonstrated differences in tracking distance, showing 'LUPE' may be more suited for tracking the entire area. However, Ethovision has options beyond the center-point tracking that was used in the figure, i.e. 3-point tracking. Using Ethovision's more complex tracking options may mitigate differences with 'LUPE'-generated results.

AUTHORS: We appreciate the reviewer's suggestion regarding the use of Ethovision's 3-point tracking option. We did test this method and found that it produced similar results to center-point tracking since the three points—nose tip, center of body, and tail base—are all aligned along the body median axis and do not capture paw movements as LUPE does. Because LUPE is specifically designed to track fine-scale pain-related behaviors, such as paw guarding and licking, Ethovision's 3-point tracking remains less precise for our primary behavioral assessments.

We have removed these data from the revised manuscript to avoid the impression that this is a methods-focused study.

Referee 3, Minor 10 Typos were found on: Page 23, column 1, "LUPE – Light aUtoamted Pain Evaluator." AND Page 23, column 2, paragraph 3, line 4, "imgaug".

AUTHORS: 'Imgaug' is the correct spelling for the name of the augmentation method. We have updated and corrected the LUPE typographical error. We appreciate the reviewer's attention to detail.

Manuscript # 2024-08-17007A-Z — Mimicking opioid analgesia in cortical pain circuits

Oswell, Rogers, James *et al.*

Author Response Letter

July 25, 2025

We thank the editor and referees for their continued engagement and feedback during this revision. We are especially grateful for the thoughtful and rigorous evaluations provided during this second round, which helped us sharpen the focus and improve the clarity of our manuscript. We deeply appreciate the recognition of the conceptual and technical innovations in our study, and we are pleased to have addressed the remaining concerns.

Several comments from the reviewers were particularly encouraging and affirming:

- **Referee #2** praised the "*compelling set of scientific and methodological innovations*," commended the new calcium imaging data using appetitive and aversive stimuli and highlighted the strength of the chemogenetic dissection of ACC MOR+ neurons as having "*exciting translational implications.*"
- **Referee #3** acknowledged that the revised manuscript is "*substantially improved in clarity and focus*," and emphasized the central advance as "*the development of a circuit-targeted, cortical opioid-mimetic intervention for chronic pain."*
- **Referee #4** described the manuscript as "*an impressive body of research and innovation for a single paper*" and appreciated our effort to present a comprehensive story that integrates behavioral modeling, calcium imaging, transcriptomics, and gene therapy in a single coherent framework.

In response to the second round of comments, we have made several targeted edits and clarifications that further improve the manuscript:

- We clarified our use of the term "gene therapy," providing context and rationale for this usage within the scope of targeted chemogenetic interventions in preclinical and clinical practice.
- We added commentary in as new Supplemental Notes to address changes in ACC ensemble decoding performance across acute and chronic pain states, and how morphine reshapes these dynamics, as well as clinical translation avenues.
- We completed a full consistency check and updated figure legends, supplementary tables, and statistical annotations to meet formatting standards, improve clarity, and address all remaining minor issues raised.
- To meet the editorial word limit requirements, we have streamlined the main text and moved several detailed sections into 6 Supplemental Notes, ensuring that all important points raised during review are preserved and accessible to readers.
- The **major text edits can be found in the Main Text document, in blue font**. Smaller edits are not colored.

Regarding **Referee 3, Comment 2**, we respectfully opted not to perform supplementary behavioral assays related to general decision-making. Our current data address multiple potential off-target effects of ACC MOR+ cell-type inhibition through complementary lines of evidence. Specifically, we found:

I. No changes in general locomotion, grooming, rearing, or behavioral state transitions following chemogenetic inhibition of the broad MOR+ population in uninjured mice.
II. No analgesic tolerance following repeated DCZ dosing in chronic pain mice.
III. Restricted manipulation of nociception-activated, mu-opioid–expressing neurons effectively facilitates analgesia in chronic pain mice with our dual intersection strategy (*pain*TRAP ∩ *mMORp*).
IV. Preservation of associative learning in real-time place preference acquisition in *pain*TRAP ∩ *mMORp* mice with chronic pain.
V. No reinforcement effects in uninjured animals, suggesting reduced addiction liability.

Additional cognitive testing would prolong the revision by 6–7 months and add substantial core-facility costs yet would not change the manuscript's primary conclusions. To meet the editor's four-week resubmission timeline, we respectfully defer these assays. The revised Discussion now notes that broader cognitive assessments are planned for future rodent and non-human-primate studies. *See our full reply on Page 4.*

We believe the manuscript is now significantly improved and presents a clear, cohesive, and rigorous account of the development of a targeted cortical intervention for affective-motivational, spontaneous chronic pain.

Below, please find the **Referee Comments in black text** with **our Author Responses in blue text**.

Best regards from all Senior Authors,

Greg Corder, Eric Yttri, Karl Deisseroth, and Ben Reiner

Referee #2 (Remarks to the Author):

Ref. 2, Comment 1: We appreciate the authors' thoughtful and thorough response to our initial critiques. It is evident that a considerable amount of care, time, and additional experimentation went into addressing the concerns raised, and we commend the authors for their responsiveness and rigor.

In particular, we recognize and appreciate the significant reorganization of the manuscript, which has greatly improved clarity, conceptual coherence, and alignment between the data presented and the overarching goals of the study. The decision to center LUPE-based analyses throughout the primary figures and to relocate traditional evoked assays to the supplement was a particularly effective revision that clarified the authors' emphasis on spontaneous affective pain behaviors and underscored LUPE's methodological contribution to the field. Similarly, the removal of underdeveloped or tangential data (e.g., tracing studies) has sharpened the manuscript's focus.

Importantly, the authors addressed a major conceptual concern about the use of mating as a positive valence comparator by performing new calcium imaging experiments with more behaviorally matched appetitive and aversive stimuli (sucrose vs. quinine). These new data are well-conceived, align with prior literature on valence coding in frontal cortex, and convincingly support the conclusion that discrete ACC populations encode opposing affective states.

With these revisions, the manuscript presents a compelling set of scientific and methodological innovations. The chemogenetic dissection of MOR-expressing nociceptive ACC neurons stands out as a particularly powerful demonstration of circuit-specific modulation of affective pain, with exciting translational implications.

That said, some broader considerations remain, though they do not detract from the overall quality of the work. The manuscript is ambitious in scope, combining circuit-specific manipulations, transcriptomic profiling, and behavioral automation. While each component is strong and contributes meaningfully, the integration of these distinct methodological approaches occasionally feels complex, and at times the breadth of data may obscure the central narrative. Additionally, while the LUPE system represents a promising advance in automated behavioral analysis, we encourage some caution in interpreting its readouts as purely affective, particularly given the reliance on licking behavior as a core metric. Further validation of the behavioral specificity will be valuable as this platform gains wider adoption.

Nonetheless, these concerns do not detract from the substantial merits of the manuscript. The authors have reasonably addressed each of our comments, provided new data where appropriate, and made considerable improvements in organization, clarity, and justification. The work will undoubtedly stimulate valuable discussion in the field about how to define and measure affective pain, and how to target it therapeutically.

Conclusion: We support publication of this manuscript in its current revised form and commend the authors on a rigorous and impressive body of work. We look forward to the discourse it will inspire.

AUTHOR RESPONSE: We're grateful for a fair and thoughtful review process that has measurably improved the clarity, focus, and impact of our work. We too look forward to the public discourse!

For public discourse, should these comments and responses be published alongside the manuscript in an online *Peer Review* file, we would like to include the following clarification regarding the interpretation of the state models, AMPS, and licking event structure within pain states as core metrics for affective-motivational pain behavior, as supported by the following points presented in the manuscript:

- **Discrete affective state mapping – Fig. S7c:** In our Markov model, "Pain State 4" (PS4) emerges as one of several distinct latent behavior states characterized *partly* by high-frequency licking—consistent with a dynamic motivational response rather than a simple, discrete reflex.
- **Rising–falling *lick* dynamics – Fig. S9b right column:** Within PS4, lick rates begin at or near zero, rise steadily to a clear peak, and then fall rapidly to zero as the mouse exits the state. This "peak-and-resolution" profile mirrors a noxious-initiated, affectively driven recuperative response designed to reduce ongoing pain. The same lick trajectory appears across all injury models—capsaicin, formalin, and SNI—indicating a stereotyped motif that fits Gate Control Theory's prediction of dynamically regulated recuperative behaviors.
    - Crucially, a mouse can enter PS4 without licking at onset. Entry is defined by a high-dimensional combination of other stereotyped actions that mark an internal neural state we interpret as an "affective signal"; this state then triggers the ensuing burst of recuperative licking. Hence, while licking is a discrete act (that can occur during injury, or drinking, or grooming fur, etc.), it is the injury context, the pattern and the timing of its engagement—not its mere presence—that signals the underlying affective-motivational state.
- **State-specific modulation by analgesia:** Both morphine and targeted MOR-promoter driven inhibition selectively suppress PS4 state occupancy and licking during PS4, without altering licking in other states—indicating that licking here reflects an affective-motivational drive rather than general motor output.

Referee #3 (Remarks to the Author):

We appreciate the extensive revisions made by the authors and acknowledge the significant effort in addressing the previous reviewer comments. The revised manuscript is substantially improved in clarity and focus, particularly with respect to its central conceptual advance: the development of a circuit-targeted, cortical opioid-mimetic intervention for chronic pain. The streamlined presentation and expanded experimental data, including calcium imaging, transcriptomics, and behavioral modeling, substantially strengthen the overall narrative. We recognize that many of the original concerns stemmed from an unintended evaluation of an earlier preprint version, and we apologize for this oversight. That said, a few important points remain.

We thank the reviewer for thoroughly re-evaluating the manuscript and recognizing the substantial improvements in clarity and narrative focus.

Ref. 3, Comment 1: The authors repeatedly refer to their chemogenetic approach as "gene therapy." While this usage is not unprecedented, the term "gene therapy" is traditionally reserved for interventions correcting disease-related genetic defects. Here, the approach is more accurately described as chemogenetic inhibition using an MOR promoter. Unless the field has broadly adopted this expanded use of the term, the manuscript should consider clarifying this terminology or providing justification for calling this intervention a "gene therapy" beyond its cell-type targeting.

We agree that "gene therapy" was once used almost exclusively for correcting monogenic defects. Today, however, both regulators and industry define it more broadly as *in vivo* delivery of genetic material to achieve a therapeutic effect. Below we explain why "gene therapy" is the most accurate, FDA-aligned descriptor for our approach and why simply calling it "chemogenetics" would obscure its translational context that we are framing as the prime conclusion for this study and the underlying IP.

1. Regulatory and clinical precedent

- FDA guidance calls a gene-therapy product "any product that mediates its effect by transcription or translation of transferred genetic material." Our AAV constructs fit this definition: benefit arises only after the MOR promoter drives expression of hM4Gi or iC++.
- Non-monogenic gene therapies already in clinic—e.g., AAV-VEGF (RGX-314) for macular degeneration and AAV-OPN1LW (AGTC-401) for color-blindness—modify cell physiology rather than "fix" a gene, paralleling our strategy of silencing hyperactive pain circuits.

2. Vector, capsid, and transgene continuity with human programs

- We use clinical-grade capsids AAV9 and AAV1—the same serotypes in FDA-approved Zolgensma and multiple CNS trials.
- hM4Gi and iC++ are on GMP-compatible backbones identical to those in trials.
- Thus, every element—transgene, capsid, delivery route—already resides in current clinical gene-therapy pipelines.

3. Cross-species validation of the human MOR promoter

- We previously showed that a human version of the *OPRM1* promoter (*hMORp*) drives selective expression in ACC MOR^+ neurons of mice and rhesus macaques (Salimando et al., *Nat. Commun.* 2023. PMID: 37704594).
- *hMORp* promoter labels human iPSC-derived nociceptors, confirming translational relevance.
- This cross-species activity underscores our goal of direct translational deployment that we hope to inspire as a result of this paper as the first CNS gene therapy to modulate cortical nociceptive activity in opioidergic cell-types.

4. Commercial momentum for functional gene therapies: *i.e., vectors intended to modulate cell activity—not repair a mutated gene*

Pain-related chemogenetic and optogenetic companies with portfolios calling these treatments "gene therapies"

- Circuit Therapeutics — AAV-hM4Di to silence dorsal-root-ganglion neurons in neuropathic pain; described as a "chemogenetic gene therapy that silences pain fibers".
- CODA Biotherapeutics — AAV-Gi-DREADD platform for chronic neuropathic pain and trigeminal neuralgia.
- Redpin Therapeutics (now part of Kriya) — AAV-encoded ligand-gated chloride channels for severe pain; Kriya portfolio labels it a "precision chemogenetic gene therapy."
- UNC / Scherrer–Roth — HCAD Gi-DREADD AAV for peripheral nociceptors (Cell 2024); authors call it a "peripheral chemogenetic gene therapy" and have filed IP for pain indications.

Vision-restoration optogenetic programs with portfolios calling these treatments "gene therapies"

- GenSight Biologics – LUMEVOQ (GS010): AAV2-ChrimsonR delivered to retinal ganglion cells for late-stage retinitis pigmentosa; marketed as an "optogenetic gene therapy" (Phase III, 2025 update).

- Nanoscope Therapeutics – MCO-010: AAV-multi-characteristic opsin to confer light sensitivity in inherited retinal degeneration; described in company materials and FDA Fast-Track designation (2024) as "gene therapy with optogenetic actuator."
- Ray Therapeutics: AAV-ChRmine to restore photosensitivity in retinal ganglion cells; public statements call it an "optogenetic gene therapy for vision-related pain and photophobia."
- Bionic Sight: AAV-Channelrhodopsin construct paired with a wearable camera for advanced blindness; SEC filings classify it as an optogenetic gene-therapy product.

These programs illustrate that industry routinely applies the term gene therapy to vectors that deliver chemogenetic or optogenetic effectors for functional modulation—whether the target is pain circuitry or visual processing—further supporting our use of the term in this manuscript.

5. Communication to interdisciplinary audiences

- Clinicians, patient advocates, and regulators already use "gene therapy" as the umbrella term for these AAV platforms, whereas "chemogenetics" is still niche jargon.
- Retaining "gene therapy" clearly signals the translational trajectory of the work.

6. Clarification added to the manuscript

- We added to **Discussion**: "Throughout this study we use the term *gene therapy* in the regulatory sense—AAV delivery of a therapeutic transgene—implemented here with chemogenetic effectors."
- The **Discussion** now distinguishes mechanism ("chemogenetic") from delivery platform ("gene therapy") and cites FDA guidance plus the commercial examples above.

For these reasons, we respectfully maintain use of "gene therapy" while providing the requested added context.

Ref. 3, Comment 2: The authors assert that their manipulation of MOR+ neurons in the ACC specifically targets affective pain-processing ensembles. However, their own data suggest that MOR expression spans multiple ACC layers and includes a substantial population of neurons not directly linked to pain-related licking behavior. This broad expression raises concerns about off-target modulation of other ACC functions, such as working memory, contextual fear, and general decision-making processes. The authors acknowledge that only a subset of MOR+ neurons are involved in pain behaviors, but they do not experimentally exclude the possibility that chemogenetic inhibition affects unrelated behavioral or cognitive domains. Additional experiments are warranted to address this potential limitation of the proposed therapeutic approach.

AUTHOR RESPONSE: We respectfully opted not to conduct additional cognitive testing, and we appreciate the opportunity to clarify our rationale. In addition to the lack of side-effects such as analgesic tolerance and motor effects, and lack of reinforcement observed with our intersectional cortical gene therapy—suggesting reduced addictive liability—we believe our current data already provide a robust assessment of potential limitations from targeting using two distinct gene therapy strategies: **(i)** the broader population of ACC MOR+ neurons (via AAV-mMORp-hM4), or **(ii)** the pain-active subpopulation of ACC MOR+ neurons (via AAV-*mMORp*-FlpO + AAV-*nEF*-Con/Fon-hM4 + *pain*TRAP^{FosCreERT2} and AAV-*mMORp*-DIO-iC^{++} + *pain*TRAP^{FosCreERT2}).

- **Broader ACC MOR+ neurons: AAV-*mMORp*-hM4**
  - **No evidence of analgesic tolerance**: We did not observe diminished efficacy following repeated DCZ administration over seven consecutive days in mice treated with AAV-*mMORp*-hM4: **Fig. 4d-h, Fig. 4l, and Fig. S17a-c**
    - Similarly, stable behavioral effects were observed in the INTERSECT AAV-*mMOR*p-DIO-iC++ real-time place preference paradigm across days: **Fig. S18e, right**
  - **No motor or general behavior alterations**: Automated LUPE tracking reveals no changes in general locomotion, grooming, rearing, or overall behavioral state transitions following DCZ administration, as confirmed by LUPE-AMPS (PC-2) and the *General Behavior Scale* (PC-1) scores (**Fig. S17d).**
- **Intersectional pain-active subpopulation of ACC MOR+ neuron:**
  - **High specificity for pain-encoding neurons**: Our dual-intersectional strategy (*pain*TRAP ∩ *mMORp*) ensures that chemogenetic and optogenetic manipulations are restricted to nociception-activated/*Fos*+ neurons that co-express *Oprm1*, thereby minimizing modulation of ACC populations involved in unrelated neural functions: **Fig. 4a *right* (blue diagram), 4c *right* (blue histology), 4k (blue group), Fig. S15d, Fig. S18**
  - **Preserved learning capacity**: Intersectional *pain*TRAP ∩ mMORp mice successfully acquire and recall a real-time place preference contingency via optogenetic inhibition of the target ensemble, demonstrating intact associative learning under the gene therapy conditions: **Fig. S18e**
  - **No reinforcement in uninjured mice**: Both real-time place preference (RTPP) and conditioned place preference (CPP) were observed only in mice with chronic neuropathic pain (SNI), and not in uninjured controls, suggesting that

the analgesic effects are state-dependent and carry reduced risk of off-target reward or addiction-like side-effects: **Fig. S18e.**

Implementing a comprehensive cognitive behavioral battery at this stage would delay resubmission by 6–7 months under optimal conditions, due to required IACUC amendments for new behavioral tests that we are not currently approved to perform at Penn (≥4+ weeks depending on IACUC panel approval speed), AAV vector production of new aliquots of AAV-*mMORp*-hM4 (4–6 weeks), and a ~11-week timeline for animal preparation and testing (including mouse ordering, AAV injection surgery, transgene expression incubation, SNI chronic pain development, behavioral assays, data analysis and figure making, etc.). Important to note, the Corder Lab, which is responsible for mouse behavior on this project, does not currently possess the necessary equipment for touchscreen or operant-based cognitive testing, or other tests of decision-making in mice. The Penn Neurobehavior Testing Core quoted $26,000–29,000 for a basic 2–3 task assessment in 40 mice once all IACUC protocols for the Corder Lab and the Core have been approved to use chronic pain procedures within the Core's testing space and vivarium (Category E) (https://www.itmat.upenn.edu/nbtc.html), further limiting feasibility during this revision cycle.

Crucially, even if performed, these additional experiments would not alter the main results or conclusions of the manuscript. At most, such data would be included in a future supplementary figure. As this is the first report to propose chemogenetic gene therapy for cortical pain relief, we take seriously our responsibility to establish a full safety profile and will pursue longer-term behavioral safety testing in both rodents and non-human primates as follow-up work.

Given these constraints, all co-Senior and co-First Authors convened and we all agreed to propose to address this comment by **(i)** explicitly stating in the revised *Discussion – Note S6* that future studies will include detailed cognitive assessments of the broader AAV-mMORp-hM4 intervention in mice and macaques (of which, the NHP studies are already underway since 2024 in collaboration with Dr. Michael Platt at Penn), and **(ii)** emphasizing the current behavioral and histological evidence supporting the pain-specificity, functional selectivity, and lack of reward liability of our approach.

Ref. 3, Comment 3: In chronic pain conditions, reductions in grooming, rearing, and walking behaviors are commonly interpreted as diminished motivation or increased affective burden. Figures 3D and 4G show that chemogenetic inhibition of ACC MOR+ neurons restores these non-licking behaviors. This suggests that the functional impact of the manipulation extends beyond licking and affects a broader behavioral repertoire. However, the manuscript continues to emphasize licking as the primary indicator of affective pain. We suggest the authors more explicitly acknowledge that changes in spontaneous non-licking behaviors are also likely manifestations of affective pain, and that their reversal by MOR+ inhibition may reflect more global improvements in motivational state.

AUTHOR RESPONSE: We believe the figures and accompanying text already align with the reviewer's recommendation and would like to clarify. **Figure S17c** quantifies individual occurrences of Lick, Groom, Rear, Walk, and Still behaviors before and after spared-nerve-injury (SNI)–induced neuropathic pain. SNI significantly increases Lick bouts and decreases Rear and Walk events, while Groom and Still remain unchanged.

The referee-cited **Figures 3d and 4g**, by contrast, show LUPE-AMPS state probabilities—high-dimensional, fast-dynamic patterns that define pain and non-pain states. These latent states, together with the *General Behavior Scale* and the *Affective-Motivational Pain Scale*, are the *primary readouts* we use to evaluate both morphine and our MOR-targeted gene therapy (**see also Fig. S17d**). Importantly, clustering methods to obtain the six behavior states did not rely disproportionately on any single behavior (including licking), as systematic removal of individual behaviors disrupted classification less than expected by chance (**Fig. S7c**).

By emphasizing state-level metrics, we move beyond any single behavior (e.g., reflex thresholds or isolated licking/grooming/rearing counts), because the probability structure and transitions between states track injury-induced sensitization and opioid analgesia with far greater fidelity.

Accordingly, we believe the figures and accompanying text already align with the reviewer's recommendation: the full constellation of spontaneous licking and non-licking behaviors, taken together, represents the behavioral manifestation of chronic pain in mice. We explicitly draw attention to morphine (**Fig. 3a-f**) or *mMOR*p-hM4 chemogenetic (**Fig. 4d-h**) modulation of the entire behavioral repertoire is captured in our state model and reflected in both the *General Behavior Scale* and the *Affective-Motivational Pain Scale* (AMPS; **Fig. 4l ← this is the last data panel of the paper, wherein we took advice from Reviewer 2 from the previous review to end the paper on the most exciting/compelling result that shows ACC *mMORp*-hM4 has comparable analgesia to systemic morphine**).

We do not believe that the current figures or text promote the idea of licking as the primary indicator of affective pain, Nevertheless, we explicitly acknowledge that spontaneous changes in the dynamics and patterns of multiple behaviors are the key behavior readout to assess chronic pain and analgesic efficacy.

See the existing Discussion for reference:
Developing precise, accurate, and reliable preclinical methods to capture behavioral biomarkers that reflect the complexity of pain is essential for identifying treatments that engage cognitive-emotional pain circuits. Traditional rodent measures—such as reflexive withdrawal thresholds—provide valuable information about stimulus detection but fail to capture the affective-evaluative dimensions of pain that shape motivational behavior. In this context, deep learning and machine vision have become powerful tools for automated, high-resolution behavioral analysis, enabling unbiased quantification of pain-related dynamics and responses to analgesics or neural

interventions [110–112]. LUPE advances this field by combining expert-guided semi-supervised classification with unsupervised clustering to robustly identify a core set of interpretable, ethologically grounded behaviors. Unlike fully unsupervised methods such as MoSeq[113]—which rely on fine-scale pose dynamics to define micro-behaviors that may not map cleanly onto cognitive-affective states[111]—LUPE explicitly captures high-level, volitional behaviors like paw licking that are historically linked to pain. By modeling transitions between these behaviors as Markov processes and clustering them using a k-means framework, LUPE identifies latent behavioral states that encode injury and analgesia, including a reproducible pain-associated state (*Pain State-4*). From these latent dynamics, we derive the mouse *Affective-Motivational Pain Scale* (AMPS)—a continuous, data-driven metric that tracks affective-motivational aspects of pain and their relief with opioids. Together, these features make LUPE a significant leap forward in behavioral phenotyping for pain neuroscience. It not only enables standardized and high-throughput behavioral analysis but also provides a new lens for decoding internal pain states—advancing our ability to evaluate therapeutics that target the affective burden of pain.

Ref. 3, Comment 4: In the revised Figure 2 and Supplementary Figure S13, ACC ensemble activity predicts licking behavior during acute pain with high fidelity, but this decoding performance appears to decline in chronic pain states. Interestingly, morphine seems to improve decoding performance in chronic pain. This raises intriguing questions about how chronic pain alters ACC circuit function and how opioidergic modulation reshapes ensemble dynamics. The manuscript would benefit from a more detailed discussion of these dynamics, particularly how chronic pain alters the functional encoding properties of the ACC and how opioid treatment may restore or reconfigure those properties.

AUTHOR RESPONSE: We agree that the observed reduction in *Lick*-encoding following induction of chronic pain in the ACC (**Fig. S13**, does warrant more discussion, and have added a new Supplementary Note S4 that is referenced in the Main Text:

Main Text reference:

> "Here, *p*Lick neurons were identified using the same approach as in **Fig. 2**. Consistent with prior findings[55], SNI impaired decoding accuracy of sensory stimuli, behaviors, and latent states relative to baseline and uninjured controls (**Fig. S10e–g, S13a–h, Supp. Table 3 Rows 14-17, Supplementary Note S4**)."

Supplementary Note:

> **Note S4: Chronic-pain reconfiguration of ACC ensemble coding and selective opioid rescue.** Spared-nerve injury (SNI) produces a broad degradation of information encoding in ACC ensembles. In the present data set (**Fig. 3 and S13**), decoders trained on population activity in the dorsal ACC predict paw-licking with high fidelity in acute pain but perform markedly worse after SNI. A similar loss of fidelity has been reported for both noxious and innocuous tactile stimuli in a recent calcium-imaging study of the mouse ACC [55]; there, stimulus decoding accuracy declined in parallel with neuropathic allodynia (Acuña Fig. 3N,O). Furthermore, these authors report both hyperexcitability of ACC neurons (Acuña, Fig. 3E) and increased responsiveness of nociceptive stimulus-responsive neurons to touch (Acuña, Fig. 3L). They found that decoding impairments were rescued by the non-opioidergic analgesic gabapentin (Acuña, Fig. 4E,F).
>
> In the current experiments, we did not find any significant change in population-level decoding of Lick following capsaicin (**Fig. 2e, S10h**) or following morphine in either condition (**Fig. S10h, Fig. S13b, c**). However, we identified improvements of single cell selectivity (d') for lick among *p*Lick neurons observed following morphine in both experiments (**Fig. S10l, S13i,j**), which suggest that a rescue of Lick-related information in particular morphine-responsive ensembles.
>
> It is important to note that direct comparison of effects of the gabapentin results from Acuña et al.[55] and those of our morphine treatments are not possible between these two studies, as sensory and behavioral information may not be represented by the same ensembles in the same manner. Indeed, we observed contrasting adaptations in *p*Lick neurons following chronic pain. Specifically, we did not see an increase in activity in *p*Lick neurons in SNI mice compared to controls (**Fig. 3k,l**), and, furthermore, these neurons reduced their activity during licking. These differences suggest – though not definitively, due to our large experimental differences – that the hyper-excitable and -responsive sensory neurons identified by Acuña et al. are a distinct ensemble, adapted uniquely following chronic pain.
>
> Nevertheless, the results of Acuña et al.[55] contextualize the degradation of behavior-encoding we observe following the induction of SNI. First, these data suggest that chronic pain broadly and multimodally degrades information encoding in ACC ensembles. Second, these results suggest that this information degradation results from complementary processes in the ACC: excessive and insufficient recruitment of sensory- and behavior-responsive neurons, respectively. Third, these show that this neural sensory hypersensitivity and behavioral hyposensitivity are associated with behavioral sensory hypersensitivity (Acuña, Fig. S13D) and perseverative, spontaneous motivational-affective behaviors (Fig. 3m), respectively. Together, our two studies suggest that persistent pain diminishes the signal-to-noise ratio of ACC population codes across multiple behavioral and sensory domains.

Referee #4 (Remarks to the Author):

Major comments:

Ref. 4, Comment 1: The authors demonstrated that there is a population of ACC neurons that encode spontaneous pain-related behaviors which are selectively modulated by morphine. To do this they used a combination of TRAP2 reporter mice, Fos-driven Cre

recombination, immunohistochemistry, ISH, and single-cell sequencing in the context of acute and chronic pain with and without morphine. They identify Oprm1 as stable across all cell types and conditions and timepoints in the ACC-making it a feasible target for MOR-promoter based cell-type specific therapies independent of chronic pain inducted transcriptomic changes. Reduction of Oprm1 following morphine administration did not alter mechanical withdrawal thresholds; however, it did diminish morphine's ability to alleviate affective-motivational pain behaviors.

They go on to posit that manual grading of pain-related behaviors is too subjective and costly when done by humans and hence created hardware and associated software to better analyze mouse behavior in the context of pain (LUPE). The software is accessible and usable on GitHub. Six different states were identified to assess pain and non-pain states in the animals and using deep learning, these newly identified states validate the system, its reproducibility, and further tested variations in the model.

Neural dynamics in the ACC is then assessed by calcium imaging (using GRIN lens implants). Variables included chronic pain through neuronal injury, heat, acute pain pricks, or other noxious stimuli in the context of morphine or without. The identify paw-licking as main activity to study. Using LUPE and the knowledge gathered thus far, they infer pain as a latent cognitive-affective state by analyzing patterns of sparse, spontaneous, and complex behaviors that may not overtly indicate the animal's pain. Moreover, they discover that latent cognitive-affective states are sensitive to morphine. They ultimately produce ARMS, a data-driven, continuous index of pain-related behavioral states which responds bidirectionally to both injury and analgesia.

After all of this, they use inhibitory DREADDs to target only the mu-opioidergic ACC neurons using the Oprm1 promoter region. This way, giving the activating drug inhibits these neurons, ameliorating pain and associated behaviors the same way (or better) than morphine without the tolerance and without the potential for (hopefully) addiction.

This manuscript represents an impressive body of research and innovation for a single paper. The work is dense and could easily be divided into several high-impact publications; however, I appreciate the authors' intention to present the complete story in one comprehensive manuscript. Despite the substantial amount of data spanning numerous experiments and technologies, the authors have done an admirable job consolidating their findings and maintaining a clear focus on their central message: the development of an opioid-mimicking gene therapy for chronic pain.

Overall, the narrative flows well. However, there are several inconsistencies within the figure legends, statistical representations, and supplementary tables—please see my detailed suggestions below. Given the paper's size and the likely involvement of many contributors, further polishing is needed to ensure that readers can easily follow and absorb all of the information presented.

AUTHOR RESPONSE: We sincerely thank Referee #4 for the thoughtful, detailed, and encouraging evaluation of our work. We are delighted that you found the integrated approach - from LUPE-based behavioral profiling and calcium imaging to cell-type-specific gene therapy - both comprehensive and compelling. Your recognition of the study's breadth, innovation, and clear narrative focus is greatly appreciated.

We also appreciate your constructive recommendation to improve consistency across figure legends, statistical reporting, and Supplementary Tables. We have carefully cross-checked all figures and legends, standardized statistical annotations (tests, n, and exact p values), and updated Supplementary Tables to align with the main text. These revisions are now incorporated to ensure clarity and ease of navigation for readers.

Thank you again for your positive assessment and valuable suggestions; they have helped us further refine the manuscript and strengthen its presentation.

Minor inconsistencies:

Ref. 4, Comment 2: Line 52: the words "to that" should be resolved. One of the words will suffice. Examples below:

> *Current analgesic drugs, such as opioids, bathe the body in chemical compounds, acting on widely expression molecular targets **that** reduce pain unpleasantness but also can promote serious and fatal side effects.*
> *Current analgesic drugs, such as opioids, bathe the body in chemical compounds, acting on widely expression molecular targets **to** reduce pain unpleasantness but also can promote serious and fatal side effects.*

AUTHOR RESPONSE: We have made the update to "… that …"

Ref. 4, Comment 3: Line 114: you reference Supplementary Figure 2, wherein you use the abbreviation "SNI" for the first time, which is not spelled out in the figure legend of Supplementary Figure 2, nor in the main text until Line 131. It may be helpful to explain what SNI is in the figure legend of Supplementary Figure 2 and still keep it in the main text later, at Line 131.

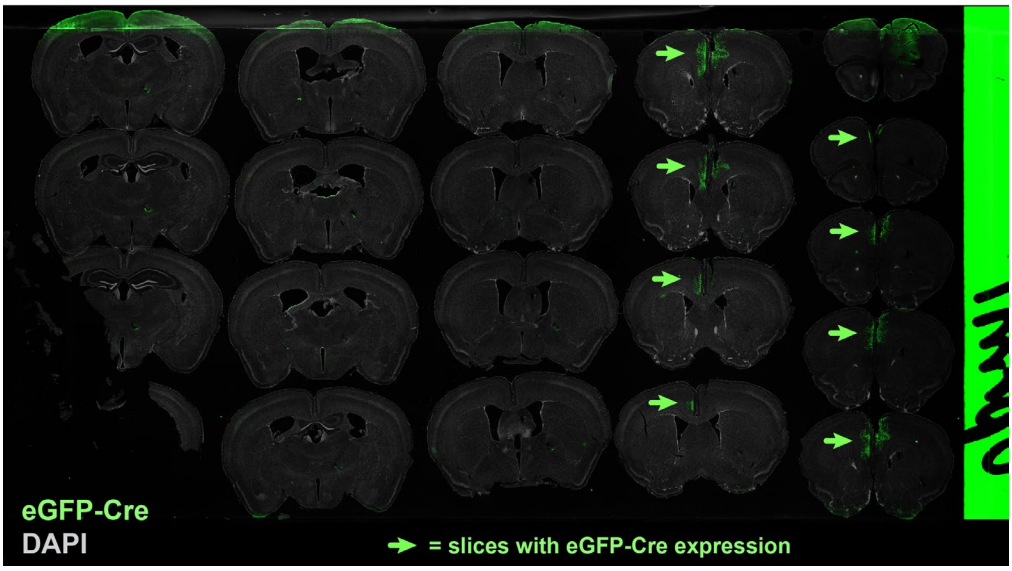
AUTHOR RESPONSE: The text has been updated such that the first reference to "spared nerve injury, SNI" happens on Line 94 when discussing the RNA-sequencing results: "Single-nucleus RNA-sequencing of this ACC nociceptive hotspot, before and after the development of chronic neuropathic pain using the spared nerve injury (SNI) model, resolved 23 cell types."

Additionally, we have updated the Fig. S2 legend to spell out SNI: "*(c) Oprm1 mRNA quantification across the left hemisphere (ipsilateral to the left hindlimb Spared Nerve Injury, SNI) of ACC, broken down by subregion and layers, in male and female mice with or without neuropathic pain.*"

Ref. 4, Comment 4: Line 166: The authors say that they injected Cre delivered by AAV9 which only floxed Oprm1 in the ACC but left all other cells intact. What does was delivered? AAV9 is historically very promiscuous and may have transduced cells beyond their target. Only images from the ACC are shown in Supplemental Figure 4S. Did the authors look at other regions in the brain known send or receive synapses to or from the ACC respectively? The authors did not say the total dose they delivered of the virus, but it would behoove them to show regions that could have been transduced, to verify negative signal.

AUTHOR RESPONSE: Thank you for raising this point. We injected AAV9-hSyn-eGFP-Cre-WPRE-SV40 (titer = 5.4×10^{11} vg ml^{-1}; 400 nl per hemisphere, ~2.2×10^{8} vg total) directly into the ACC. Because AAV9 can exhibit broad tropism, we routinely collect and examine serial coronal sections spanning a significant portion of the brain to screen for off-target transduction. For this experiment (performed in 2020–2021), archived 4× whole-slide scans confirm robust eGFP-Cre signal confined to Cg1/Cg2 with no detectable fluorescence in regions that project to, or receive input from, the ACC (e.g., thalamus, amygdala, somatosensory cortex or motor cortex). Here, we have added a representative slide-scan image from n=1 mouse to illustrate this point:

4X Keyence Microscope slide-scan:
slices left-to-right are posterior-to-anterior coronal sections



Ref. 4, Comment 5: Line 192: "machine-vison" should be "machine-vision", yes?

AUTHOR RESPONSE: Yes. We made the correction.

Ref. 4, Comment 6: Line 447: I believe "vesical" should be "vesicle"

AUTHOR RESPONSE: Yes. We made the correction.

Ref. 4, Comment 7: Lines 871-884: It is appreciated that the authors have identified the viruses and their respective titers in the methods, however it is never stated what volume is delivered to the animals, and thus, the ultimate dose of each virus is unknown. It would be extremely helpful to know what doses of virus the authors administered for each of the treatments.

AUTHOR RESPONSE: We have added the following updates to the Methods section on Titre in vg/mL, Volume in nL, Total Dose in vg, and Source, starting on Line 936:

Viral Vectors.
All viral vectors were either purchased from Addgene.org, or custom designed and packaged by the authors as indicated. All AAVs were aliquoted and stored at -80°C until use. The following viral vectors were used (Titre in vg/mL, Volume in nL, Total Dose in vg, Source):

*AAV9-hSyn-HI-eGFP-Cre-wpre-SV40 (5.44 × 10^{11} vg ml^{-1}, 400 nl, 2.18 × 10^{8} vg, Addgene 105540-AAV9); AAV9-hSyn-GFP (1.90 × 10^{11} vg ml^{-1}, 400 nl, 7.60 × 10^{7} vg, Addgene 50465-AAV9); AAVDJ-hSyn1-DIO-mCh-2A-MOR (1.13 × 10^{12} vg ml^{-1}, 500 nl, 5.65 × 10^{8} vg, custom from Banghart Lab); AAV5-hSyn-DIO-EGFP (1.30 × 10^{12} vg ml^{-1}, 500 nl, 6.50 × 10^{8} vg, Addgene 50457-AAV5); AAV9-hSyn-jGCaMP8m-WPRE (1.90 × 10^{12} vg ml^{-1}, 800 nl, 1.52 × 10^{9} vg, Addgene 162375-AAV9); AAV1-mMORp-hM4Di-mCherry (1.17 × 10^{12} vg ml^{-1}, 500 nl, 5.85 × 10^{8} vg, custom from Deisseroth Lab); AAV1-mMORp-eYFP (1.00 × 10^{12} vg ml^{-1}, 500 nl, 5.00 × 10^{8} vg, custom from Deisseroth Lab); AAV1-mMORp-Flpo (9.21 × 10^{11} vg ml^{-1}, 400 nl, 3.68 × 10^{8} vg [*Co-injected with Con/Fon-hM4D(Gi)], custom from Deisseroth Lab); AAV5-nEF-Con/Fon-hM4D(Gi)-mCherry (7.40 × 10^{12} vg ml^{-1}, 400 nl, 2.96 × 10^{9} vg [*Co-injected with mMORp-FlpO], custom from Deisseroth Lab); and AAV1-mMORp-DIO-iC++-eYFP (1.35 × 10^{12} vg ml^{-1}, 400 nl, 5.40 × 10^{8} vg, custom from Deisseroth Lab).*

Ref. 4, Comment 8: Supplemental Figure S5 panel K; the last point reads: "LUPE: ~$2,000 for chamber build + $2,500 computer = $5,300 total + GPU (market value)" Your math doesn't add up. Be consistent with the prices given and make the total "~$4,500".

AUTHOR RESPONSE: We made the figure correction.

Ref. 4, Comment 9: Figure 2 panel a: The color of text "Lick left hindpaw…" needs to be a lighter color- its extremely difficult to read on the black background.

AUTHOR RESPONSE: Thank you. We updated Fig. 2a text to a lighter purple hue. The idea of the color was to match 'Left Lick' across all figures, so we hope this purplish color is more visible.

Ref. 4, Comment 10: Finally, I believe the discussion could be improved upon by going more in depth describing how this technology could feasibly translate into clinic.

AUTHOR RESPONSE: To accommodate round 1 revisions on explanations of methods and broadening of the discussion, the 1st round version of the manuscript was ~2,000 words over the *Nature* guidelines for full Article word limits. Per instructions from the editor after the 2nd round, we have had to substantially cut text to bring the word count closer to the guidelines. To address this comment, we have added a brief sentence in the main text of the Discussion and added additional depth describing this technology's application as a Supplementary Note + references on how the AAV-MORp technology could be deployed clinically.

Discussion, final paragraph:

> "For translational neuromodulation, our human opioid receptor promoter-based gene therapy[43] could be adapted for non-invasive delivery using focused ultrasound blood-brain barrier opening[85], to access and control multiple pain-encoding opioidergic neurons simultaneously in cortical and subcortical circuits (**Supplementary Note 6**)."

Supplementary Note:

> **Note S6: Translating MOR-promoter chemogenetics to the clinic.** The present study shows that selectively silencing μ-opioidergic ensembles in the ACC can relieve the affective burden of chronic pain without producing tolerance or reinforcement. A logical next goal is to adapt this strategy for human use by pairing a human MOR promoter (*hMORp*) with AAV delivery and non-invasive blood–brain-barrier opening via focused ultrasound (FUS-BBBO)[85,116–118].
>
> We have previously demonstrated that *hMORp* restricts reporter expression almost exclusively to *OPRM1*+ pyramidal neurons in the macaque ACC after intraparenchymal AAV1 infusion, achieving >90 % cell-type specificity with minimal spread to neighboring cortices [43]. This primate validation suggests that promoter specificity, and therefore circuit selectivity, could be preserved in the human ACC, whose *OPRM1* regulatory sequence and laminar architecture are highly conserved.
>
> Direct stereotaxic AAV injection is already used clinically for movement disorders, yet it requires burr-hole drilling and needle tracks that carry hemorrhagic and infectious risks, especially if multiple cortical or subcortical pain hubs must be targeted. FUS-BBBO combines a systemically infused AAV with transient, MRI-guided ultrasound sonications to open the blood–brain barrier over millimeter-scale foci. The method has been applied in human patients[119,120] for antibody and chemotherapeutic delivery with an excellent safety record and no permanent tissue damage. In large-animal models, single FUS sessions enrich vector genomes 50- to 100-fold within the sonicated voxel while sparing the rest of the brain, making it an attractive, incision-free alternative for gene transfer to the ACC.
>
> A chronic pain treatment could involve a one-time intravenous dose of an AAV-capsid optimized for human neurons carrying *hMORp*-hM4Di. FUS-BBBO would then concentrate the vector within the dorsal ACC bilaterally; expression stabilizes within weeks. Patients would thereafter self-administer an orally bioavailable DREADD agonist such as deschloroclozapine on an as-needed basis. Because the ligand is inert at endogenous receptors and active only at the engineered *hMORp*-DREADD

ensemble, analgesia would be mediated by a single cortical node, circumventing the respiratory depression and reward pathway engagement that limit systemic opioids. Ligand dosing can be titrated, withheld, or re-initiated without loss of efficacy in pre-clinical models, offering a reversible and adjustable alternative to ablative surgery or continuous deep-brain stimulation.

Before human trials, several gaps must be addressed: (i) Dose optimization and biodistribution of systemic AAV under FUS-BBBO; (ii) Long-term safety of *hMORp*-driven DREADD expression, especially under repeated ligand exposure, including potential limitations such as deficits to decision-making and executive cognitive functions, analgesic tolerance and dependence, respiratory depression, motor impairment, and addiction-liability; and (iii) Ligand selection focusing on FDA-approved molecules with favorable pharmacokinetics and negligible off-target actions.

Combining promoter-level cell specificity with acoustic targeting offers a scalable path toward circuit-precise, addiction-sparing analgesia. As FUS-BBBO platforms mature and systemic AAV capsids advance in clinical pipelines, *hMORp*-based chemogenetics could be deployed as a single-session outpatient procedure that endows patients with on-demand control over their own cortical pain circuitry, thereby moving the field beyond broad opioids toward personalized, precision pain medicine.

COMMENTS ON STATISTICS AND FIGURE LEGENDS:

In general, please consolidate all the statistics to one a single Supplementary Table and please make sure they are in order with Main Figures listed first and all Supplemental Figures following. For consistency, make sure all statistics in the legends also have a corresponding location in the tables. Please standardize the names of the statistics that you perform throughout the manuscript, legends and tables (examples: 1-way ANOVA or One-way ANOVA; t-Test or T-Test or t-test; always state whether t-tests are paired or unpaired, one-tailed or two-tailed and what post-hoc tests were used in all situations). Either use a star graphic or spell out “stars” but please be consistent throughout. Be consistent in how you describe the error bars and how they are represented (SEM vs. s.e.m.)

AUTHOR RESPONSE: We have gone through all legends and table rows, and we updated statistics formats to be uniform throughout.

Ref. 4, Comment 11: This is the best version for all applicable legends: “(star symbol)= $P < 0.05$. Bars, lines, or dots are mean; error bars and shaded areas are SEM. See Supplementary Table 1 for statistics.” As seen in figure 1 legend.

AUTHOR RESPONSE: We adopted this format throughout all legends.

Ref. 4, Comment 12: S1g: Supplemental Figure 1 legend says that a Two-way ANOVA (Line 425) was executed on data shown in panel g, however, there are no statistics to show for it, either in the supplemental nor, nor the supplemental data table that expands on all statistics run. Please remove the reference to “Two-way ANOVA” or amend all legends and tables to reflect the statistics performed and the results.

AUTHOR RESPONSE: We have removed the reference to the Two-Way ANOVA in this legend.

Ref. 4, Comment 13: Supplemental Figure 4: the statistics are only in this figure legend. For consistency, the authors should add the specifics of statistics to the Supplementary Data Tables. The statistics are fine, but there is also an inconsistency in the authors annotation of “t-Test” vs. “T-Test”; please make consistent and remove to data table.

AUTHOR RESPONSE: We have added the statistics to the Supplementary Data Table (rows 50-65). Thank you for catching this oversight.

Ref. 4, Comment 14: Supplemental Figure S7h; in the Data Table with respective statistics, I do not believe that Line 21 should include a multiple comparisons value. It is listed as a permutation test comparing real vs. shuffled; then additionally states that there was a multiple comparisons analyses for Uninjured vs. Capsaicin. Panel S7h does not address capsaicin: Please Revise

AUTHOR RESPONSE: Thank you for pointing this error out! This was a typo. This entry has been deleted.

Ref. 4, Comment 15: Supplemental Figure S9: In panel C for everything, are the Kolgomorov-Smirnov Test plots supposed to be in color? The Supplemental Table for the statistics state at the KS = values between 0 and 1, and the p-values are mostly 0 or very small. Based on the p-value scale it looks like colors should fluctuate between black/dark purple and light yellow/white; however, I only see

blocks of filled black ink or outline of boxes. Is this an accurate representation of the images? For panel F, there are no associated rows in the Supplemental Table for these statistics: Please Add.

AUTHOR RESPONSE: We have updated the p-value heatmaps to logscale so that more variation in p-values can be shown. We have also added statistics for Panel F in row 110 of the supplementary table.

Ref. 4, Comment 16: Supplemental Figure S10: In the legend, you reference permutation t-tests for panels g) and j) however, there are no values listed nor are there rows in your supplementary table. Panel l) is missing from the legend entirely, although that panel and its associated statistics are in the Supplementary Table. Please correct and make consistent; add missing information and values.

AUTHOR RESPONSE: We added table rows for panels g (row 116) and j (row 117) and a legend entry for panel l.

Ref. 4, Comment 17: Supplemental Figure S11 panel f: there is no color or block outline for the middle row of column “SNI1” and the 4th row down for column “Ctrl4”- is this correct? Or is the image incomplete. For consistency, please add rows to the Supplemental Table(s).

AUTHOR RESPONSE: We have added outlines to the image and a row to Supplementary Table (row 120)

Ref. 4, Comment 18: Supplemental Figure S12 contains the language “Stars indicate $p < 0.05$. Lines are mean; shaded areas are SEM.” There are no stars in this figure- please remove the associated language.

AUTHOR RESPONSE: We have removed this language.

Ref. 4, Comment 19: Supplemental Figure S13- Panel a has an unfilled while spot for Baseline, if the subpanel is truly white, could you please provide an outline of the box, just to confirm that it is not a bad image. Same for in panel h “Day 21 + morphine non-pain states, SNI1”.

AUTHOR RESPONSE: These boxes have been outlined.

Ref. 4, Comment 20: Supplemental Figure S13- The legend for panels (c,g) state that there was a Mixed effects model, Tukey correction with a p value less than 0.0001; but no corresponding rows in the supplementary table; please amend for consistency.

AUTHOR RESPONSE: Statistics were added (panel c: rows 123-124, and panel g: rows 127-128) to the supplementary table.

Ref. 4, Comment 21: Supplemental Figure S13- panel l legend states that its p value = 0.046, yet there is no “star” in the panel; is this correct? It says that stars indicate $p<0.05$.

AUTHOR RESPONSE: We have added stars to the panel to indicate the significant effect of treatment.

Ref. 4, Comment 22: Supplemental Figure S17- there are no associated statistics in the supplemental table for panel c; please amend for consistency.

AUTHOR RESPONSE: We have added statistics for panel c in rows 149-153.

Referee #4 (Remarks on code availability):

We were able to open it and run it. It looks more robust than previous pain-behavior software.

AUTHOR RESPONSE: We greatly appreciate you taking the time to download, install, and test LUPE. Your feedback that the platform runs smoothly and surpasses earlier pain-behavior software is very encouraging and affirms our focus on robustness and usability. Thank you for this positive evaluation.

Re: Response to previous review – Manuscript # 2024-08-17007C

October 1, 2025
Dear Editor and Reviewers,

We genuinely appreciate the continued thorough review of our manuscript and are excited to present this submission to the reviewers that integrates their insightful comments.

Specifically, we have

- Changed all mentions of 'gene therapy' to 'chemogenetic gene therapy' in line with reviewer suggestions and editor instructions.
- Added clarifying text about the training datasets of the AMPS model using all acute and chronic pain injury models for training.
- Addressed the editorial requirements provided, especially overall word length.

In this revised submission we have addressed all reviewer comments in the following point-by-point response, as well as in the manuscript text. These changes will improve the understanding of our manuscript, and we appreciate the reviewers' ongoing efforts that have helped strengthen the content and impact of this work. Please find **all reviewer comments in Black Text**, and **our responses in Blue Text**.

With kind regards from all Senior Authors,

Greg Corder
Eric Yttri
Karl Deisseroth
Ben Reiner

Referee's comments:

Referee #3 (Remarks to the Author):

We appreciate the authors' thoughtful and extensive efforts in revising the manuscript and addressing the concerns raised in the previous round of review. The inclusion of additional analyses, clarifications, and discussion points—particularly regarding the behavioral classifier, ACC circuit function, and translational framing—reflects a genuine attempt to engage with reviewer feedback and improve the scientific clarity and rigor of the manuscript.

AUTHORS: We appreciate the reviewer's effort in evaluating our manuscript and continual engagement with the review process.

Referee 3, Comment 1. While we understand that the regulatory framework has broadened its definition of gene therapy, in the context of a scientific journal article rather than commercial or marketing material, this still seems to be an expansion of the term, for what otherwise is normally referred to in scientific journals as chemogenetic inhibition.

We appreciate that the authors have provided numerous examples in commercial contexts where chemogenetic-based therapies are referred to as "gene therapy". However, we note that even in these commercial contexts, the term "gene therapy" typically includes conditional qualifiers such as "Chemogenetic gene therapy" or "Optogenetic gene therapy", presumably to differentiate it from gene therapy that corrects diseases due to genetic defects. In the one academic document cited: Kang, H. J., Krumm, B. E., Tassou, A., Geron, M., DiBerto, J. F., Kapolka, N. J., ... & Roth, B. L. (2024). Structure-guided design of a peripherally restricted chemogenetic system. Cell, 187(26), 7433-7449., nowhere in the paper is the term "gene therapy" used and their technology is referred to in the manuscript as a "peripherally restricted chemogenetic system/platform". We believe terminological drift in a scientific journal article risks compromising mechanistic

accuracy in scientific communication.

The authors' proposed solution to clarify their use of "gene therapy" in the discussion section represents a well-intentioned compromise. However, we believe that incorporating the conditional qualifier "chemogenetic gene therapy" THROUGHOUT the manuscript would better serve communication to a basic science audience, while also helping place emphasis on the potential translational relevance of the intervention, particularly given that this is a publication in a scientific journal rather than a commercial document.

We suggest revising the proposed clarification as: "Throughout this study we use the term chemogenetic gene therapy in the regulatory sense—AAV delivery of a therapeutic transgene—implemented here with chemogenetic effectors.", or any alternative phrasings that achieve the same terminological precision, with the same qualifying term.

AUTHORS: We appreciate the additional explanation of the reviewer's perspective and understand their concern with the use of 'gene therapy' in this situation. **We have updated the text to reflect this by using 'chemogenetic gene therapy' throughout the manuscript.**

Referee 3, Comment 4. The authors assert that their classifier generalizes well to chronic pain behavior. However, this claim remains only partially substantiated. The model was trained using acute pain conditions, and licking was used as the behavioral ground truth—limiting the potential to fully capture chronic pain states, which often involve reduced motivation, altered emotional tone, and passive coping.

The authors acknowledge that decoding accuracy from ACC signals is degraded in chronic pain (Figure S13), and that morphine partially restores this ability. However, this could reflect restoration of acute-like pain processing rather than true affective relief. Thus, it remains unclear whether the classifier captures chronic-specific affective signatures or merely reflects a shift in behavioral visibility.

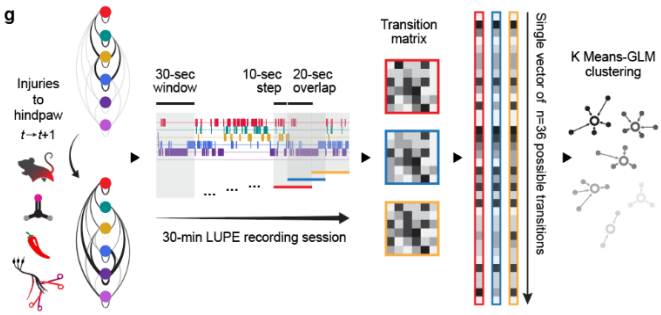
To improve interpretability, the authors should more clearly discuss the limitations of using an acute pain-trained model to classify chronic pain behaviors. If feasible, retraining the model on chronic pain data or validating it against chronic-specific behavioral features would better support the generalizability of the approach (see below)

AUTHORS: The reviewer is mistaken. Our model was in fact trained on all types of pain models in the paper, including spared nerve injury mice (**see *Results* section on page 4 line 157-9, as well as *Methods* section on page 24 line187-190)**. Therefore, we avoid the potential pitfall of misclassifying chronic pain behaviors to which the referee refers. There is no issue here, and our classifier remain the first method to capture acute pain and chronic pain affective signatures.

Discovery of latent affective-motivational pain states sensitive to morphine.

Inferring internal states, such as pain, from sparse spontaneous behavior is a central challenge in ethological neuroscience. An injury to the left hindpaw may or may not elicit licking at a given moment, yet the animal may still be experiencing pain. We therefore tested whether latent cognitive–affective pain states could be determined from LUPE-scored behavior. From 58 mice (formalin n=19, capsaicin n=20, SNI n=19; **Fig. 1g, S7**), we modeled behavioral transitions as Markov processes using 30-sec sliding windows to produce per-animal transition matrices (**Fig. 1g, S7a**). Matrices were clustered by k-means (k=6; 100-fold cross-validation; **Fig. 1g, S7b**). Classification of animals to these six clusters exceeded chance (Euclidean distance between real vs shuffled cluster centroids; **Fig. S7c–h, Supp. Table 1 Rows 66–72**). Importantly, no single behavior drove the clustering—systematic removal of individual behaviors disrupted classification less than expected by chance (**Fig. S7c**).

In addition to the main text and methods that clearly describe the data going into the model, to improve communication to the *Nature* readership on this point, **we have updated Figure 1, Panel g** to now include didactic illustrations of the acute and chronic pain injuries that went into the model: capsaicin (chili pepper), formalin (formaldehyde molecule), and SNI (scissors for nerve transection).



Referee #4 (Remarks to the Author): The authors identified a population of anterior cingulate cortex (ACC) neurons that encode spontaneous pain-related behaviors and are selectively modulated by morphine. Using TRAP2 reporter mice, Fos-driven Cre recombination, histological methods, in situ hybridization, and single-cell sequencing in acute and chronic pain models, they found that Oprm1 expression remains stable across cell types, conditions, and time points—supporting its feasibility as a MOR-promoter target for cell-type–specific therapies regardless of chronic pain–induced transcriptomic changes. While morphine-induced Oprm1 reduction did not affect mechanical withdrawal thresholds, it impaired morphine's relief of affective-motivational pain behaviors. To improve behavioral assessment, they developed LUPE, an open-source hardware–software system using deep learning to classify six behavioral states distinguishing pain from non-pain conditions, validated through reproducibility tests. Calcium imaging via GRIN lens implants revealed paw-licking as a key activity, and LUPE analyses enabled inference of latent cognitive-affective pain states that were morphine-sensitive, culminating in ARMS—a continuous index of pain-related states responsive to injury and analgesia. Finally, inhibitory DREADDs driven by the Oprm1 promoter selectively suppressed mu-opioidergic ACC neurons, reducing pain behaviors as effectively as or better than morphine, but without tolerance or addiction risk.

I think the author's revisions have continued to improve this extremely dense manuscript. The supplementary notes are a wonderful addition that address many of the open-ended discussion issues. The statistics are more uniform now. I think this is a spectacular ambitious body of work.

AUTHORS: We appreciate the reviewer's recognition of the quantity and quality of work presented in this manuscript, as well as their thorough and thoughtful review from the previous round of revision.

Referee #4 (Remarks on code availability): I accessed the code in the last round of review and it was functional.

AUTHORS: We thank the reviewer for taking the time to test the code.