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Peer Review File

Deconstruction of a spino-brain-spinal cord circuit drives chronic pain

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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)
This is a highly sophisticated manuscript the probes the role of the RVM mu-opioid receptor expressing neurons in modulating mechanical hypersensitivity in chronic inflammatory and neuropathic pain states. The authors created a new OPRM1-cre mouse line and engineered a more effective retrogradely transported AAV8 to selectively manipulated RVM opioid receptor expressing neurons that project to the spinal cord, and then manipulated this pathway in various ways. They then engaged in projection mapping to identify multiple connected upstream regions that form part of a circuit that regulates mechanical hypersensitivity. Overall, this is a well (albeit a bit densely) written paper that identifies key components of a long range neuronal circuit that regulates mechanical pain. I have a few minor comments, but I believe that I identified one rather major shortcoming that should be addressed with some additional experiments (see below).

Major concerns:
Fig 4 – the idea of tracing upstream inputs into the lateral superior colliculus is interesting. One major limitation here is that in contrast with the data in Fig 3 where the authors were able to selectively go after inputs that target the RVM OPRM1 neurons, in Fig 4 all inputs from upstream regions into the lateral superior colliculus are captured/manipulated, and not just those that then selectively send projections to the RVM OPRM1 cells (i.e., the specificity is lost). Indeed, any manipulation of the somatosensory cortex – superior colliculus axis may affect more than one downstream region, not just the opioid receptor expressing cells in the RVM. Ideally, the authors could design an experiment where they silence this connection as they did in Fig 4 h and I (or maybe ablate it altogether), and then they could in the same animals selectively manipulate the RVM-SC pathway to show that this overrides the effects of silencing the upstream pathway. This would not be too onerous to do.

Exactly the same consideration applies to the data in Fig 5 – again, the authors have no way of showing that this thalamus to SS projection ultimately acts by altering the RVM ORPM1 neurons. Clearly, the pathway is important, but RVM cell populations other than the opioid receptor expressing ones may be involved. The authors state in the abstract: “Here we report a closed-loop circuit extending from spinal cord to ventral posterolateral thalamus and posterior complex of the thalamus, proceeding to primary somatosensory cortex; then returning to the spinal cord via lateral superior colliculus, ultimately connecting to u-opioid receptor expressing RVMSC neurons.” I am not sure that I can agree with the latter part of the statement for the reasons articulated above. Yes, the RVM opioid receptor expressing neurons are important, and yes, upstream regions are important, but the current data set does not prove that the thalamus -SS connection acts exclusively (or at all) via the RVM opioid receptor neurons. Again, the way to show this is by concomitantly manipulating the RVM-SC projection and the specific upstream pathway to show that they are causally linked using pain behavioral assessments. Another way to potentially address this (rather than using mechanical thresholds) would be to use the calcium imaging approach used in Fig 1h and then show that that this signal is modulated when the various upstream parts of the circuit are activated or inhibited.

Along these lines, I am not sure that the authors have any evidence for a “closed loop circuit” as stated in the Discussion – all you have is evidence that there are individual connections in a pathway, but a closed loop would imply that there cannot be any other projections to other regions that leave the loop, and no other inputs that may modulate this part of the circuit.

Indeed, the data in Fig 3e and 4b suggest that there are other inputs even if they are less pronounced. So please reword this accordingly to something like a “long range circuit”.

Minor comments:

Another limitation that could be discussed and elaborated on is the nature of the projection that is being silenced or activated between the various relay stations – are these various projections excitatory, inhibitory or a mix of both? The way I read the manuscript, it seems that the hsyn promoter was being used which would hit all neuronal projections. This should be discussed.

Data in Fig 1e should be quantified

Bar graph in Fig 1f -the color coding of DAMGO vs DAMGO +CTAP is too similar – please change one of them

Fig 1 j – are the data at various time points statistically different from each other?

Fig 2 – why is mechanical threshold shown on a log scale?

Fig 2 – it may be useful to have the thermal data included ion the main figure to draw a clear contrast to the alterations in mechanical hypersensitivity in

Fig 3 – the authors find no effect upon silencing vlPAG excitatory inputs into the RVM OPRm1 neurons – this is interesting, Here the author may want to discuss differences to results reported by Huang et al Nat Neuroscience 2019 where a broader ablation of vlPAG-RVM connections did seem to ablate descending inhibitor modulation of mechanical hypersensitivity.

Methods:
Mice do not have a “gender” - they have a biological sex – please revise “genders” to “sexes”

Referee #3

(Remarks to the Author)
Summary of the Key Results:
The manuscript by Chen and colleagues investigates a spino-brain-spinal circuit loop that mediates mechanical hypersensitivity in mouse models of chronic inflammatory and neuropathic pain. This closed-loop circuit includes an ascending pathway from the spinal cord to the primary somatosensory cortex via the ventral posterolateral (VPL) thalamus and posterior complex of the thalamus (Po), and a descending pathway from the somatosensory cortex to the lateral superior colliculus (lSuC), then to the rostral ventromedial medulla (RVM), and back to the spinal cord. The study identifies a specific population of μ-opioid receptor-expressing RVM neurons, primarily GABAergic, that project to the spinal cord. Silencing any node in this multisynaptic pathway did not alter nociception in healthy mice but effectively eliminated mechanical hypersensitivity in chronic pain models. Notably, repetitive—but not acute—activation of these nodes was sufficient to induce chronic mechanical hypersensitivity in healthy mice.

Overall Assessment
This study offers novel insights that challenge established perspectives in the field. By employing a variety of state-of-the-art techniques, the authors meticulously dissected each connection within the spino-brain-spinal circuit and identified key cell types at critical nodes, including the RVM and lSuC. Through the manipulation of OPRM1+ spinal cord-projecting RVM neurons in chronic pain models, the study highlights the pivotal role of these neurons in the process of pain chronification. However, several significant concerns must be addressed before the study can be considered for publication:

(1) The number of animals used in key experiments is insufficient, reducing the statistical power and robustness of the conclusions. Behavioral and calcium imaging experiments are particularly affected by inadequate sample sizes.
(2) The claim that the circuit mediates mechanical but not thermal hypersensitivity is inadequately supported by the presented data. Additional experiments are needed to clarify this specificity.
(3) Missing control experiments undermine the interpretability of the results and leave alternative explanations unexplored.

Major Concerns:
1. The study concludes that silencing the spino-brain-spinal circuit loop eliminates mechanical hypersensitivity, but the specificity of this loop to mechanical pain remains questionable. While Oprm1+ RVM neurons responded to mechanical and cold stimuli, no response to thermal stimuli (Hargreaves test) was observed in Figure 1i. However, the sample sizes (4 for normal mice and 5 for SNI mice) are too small to draw firm conclusions. A larger sample size is required.
2. Figure 1j: Time-dependent changes in calcium responses of spinal cord-projecting Oprm1+ RVM (N=3) should be presented with individual lines connecting the data points for each mouse. Furthermore, the significance of the Day 28 response relative to pre-SNI should be clarified, especially given that chronic pain with mechanical hypersensitivity could persist for 28 days. The N=3 value is insufficient for statistical reliability in this context.
3. Given the significant results of plantar cold response in Figure 1i, chemogenetic manipulation experiments in Figure 2b should include a plantar cold test.
4. Figure 1f: The DAMGO experiments (baseline, DAMGO, DAMGO + CTAP) are from two independent sets of neurons.

Ideally, these conditions should be tested in the same neurons for consistency.
5. Signals in Figure 1e (Oprm1, vGAT, Tph2, Penk) resemble immunohistochemistry (IHC) rather than RNA scope imaging. Higher magnification and additional validation are required to confirm the specificity of RNA labeling.
6. Chronic pain has sensory and emotional components. In this study, chemogenetic inhibition of Oprm1+ spinal cord-projecting RVM neurons blocked mechanical stimuli induced conditioned place aversion in the SNI mice, suggesting the reduction of both sensory and affect components of pain. To strength this point, the authors should evaluate anxiety- and depression-like behaviors. Also, does repetitive activation of the spino-brain-spinal circuit specifically induce chronic mechanical pain without affecting the emotional (affective) component? This should be addressed experimentally and discussed.
7. Saline is not an appropriate control for CLZ in chemogenetic experiments. The authors should include a control with AAV encoding a fluorescent reporter without hM4D or hM3D to exclude hM4D- or hM3D-independent effects of CLZ.
8. Optogenetic approaches offer greater temporal precision in assessing pain circuits and nociceptive responses. Incorporating optogenetics alongside fiber photometry would strengthen the key findings.=
9. The study reports that pharmacogenetic terminal silencing of the PAG-RVM pathway has no effect on alleviating mechanical hypersensitivity in the SNI model. However, this negative result is not convincing without verification that terminal inactivation of PAG axons by hM4D successfully suppressed PAG-to-RVM transmission.

Minor Concerns:
1. Line 30 in Abstract is missing "of" in “activation each node”; revise to “activation of each node.”
2. The experimental timeline should be clarified. Neonatal spinal cord injections (P1.5–P2.5) and stereotaxic injections (5–7 weeks old) suggest a 4-week gap, which should be explicitly noted.
3. Figure 1c: Yellow (Clover3 and Ruby) signals represent Oprm1+ neurons and green (Clover3 but not Ruby) signals indicate Oprm1– neurons. It seems that green signal represents all Colver3-expressing RVM cells. Please clarity.
4. Figure 1f: Mention CTAP usage explicitly in the results section or figure legend.
5. Figure 2b: Clarify whether these data are from SNI/CFA models or healthy mice in the legend.
6. Figure 2g, it should be “fiber track” (not cannula track) for fiber photometry
7. Unlike other spinal cord injection at neonatal stage (figure 2), justify why injections were performed in adult stages particularly for Figure 5c.
8. Simultaneous silencing of VPL and Po pathways alleviated chronic pain, whereas individual silencing did not. This is still clear to me. If repetitive activation of SC to Po or SC to VPL can induce chronic pain, why silencing either of them has not effect on pain relief. Does it mean that chronic mechanical hypersensitivity induced by repetitive activation of SC to Po or SC to VPL differs from SNI-induced central sensitization.
9. Some critical discussions (e.g., paw licking/biting in Figure 3i and dorsal column nucleus in Figure 5) belong in the Results section.
10. Figure 1f Units: Correct the DAMGO unit in lines 705–706 from "μm" to "μM."
11. Methods: line 346, 200 μM, unit should be “μm”.
12. According to Methods, lines 484-485, amplitudes of EPSCs form baseline and the last 5 min of clozapine-N-oxide was bath applied. Obviously, the data is missing in Figure 5b.

Referee #4

(Remarks to the Author)
Wang, et al. present a study that further our understanding of circuits involved in mechanical sensitivity and neuropathic/inflammatory pain. Through development of novel genetic and viral tools, they specifically show a spinal cord->thalamus (VPL + Po)->SS->lSuC->RVM->spinal cord circuit and demonstrate that silencing nodes along this pathway disrupts injury-induced mechanical hypersensitivity and that driving activation of nodes along this pathway is sufficient to induce mechanical hypersensitivity. Generation of the AAV8retro viral tools should have broad applicability to many other areas and we appreciate additional Oprm1-Cre mice that can help with identifying the role of Oprm1+ neurons throughout the nervous system. The Oprm1+ RVM-SC fiber photometry results in the awake animal further our understanding of the dynamics of these cells and their prolonged activation. Critically, highlighting the role of VPL/Po in mechanical hypersensitivity should increase focus on the spinothalamic tract that will complement recent work focused on the spinoparabrachial tract. The authors effort and systematic analysis of this pathway through a series of well-defined experiments is much appreciated and these studies should further our understanding of sensory/pain pathways.

However, to make the work easier to read for a general audience and further clarify the advantages of the approaches taken, see below for questions and requests for updating the manuscript text along with a few necessary and optional experiments that will demonstrate that the circuit manipulations match neural activity changes observed. I hope these questions and suggestions, which indicate the interest and question the manuscript raises, help further improve this manuscript, whose approach and results hold great promise for further dissecting the nuances of the ascending and descending pain pathways.

If due to space limitations authors are not able to include all additional material in the main text, we would recommend adding a series of supplemental notes clarifying how their anatomical and neural activity manipulation results align with prior preclinical work or match any clinical observations that heretofore been unexplained.

Questions:

1. Why do the authors state on lines 22-23 that “the neuronal pathway that transmitting information about peripheral injury to

these descending RVM SC neurons is unknown"? As has been reviewed recently (for example, https://doi.org/10.1016/j.tins.2024.04.006, https://doi.org/10.3389/fpain.2022.932476), inputs from PB and other structures are known to synapse in the RVM and relay sensory/nociceptive information to the RVM. Further, Francois, 2017 also conducted cTRIO on GABAergic RVM-SC neurons. Please rephrase this statement as it is more appropriate to state that "little is known", "a more detailed study is needed" or a similar phrasing. Further, in light of Francois, 2017 using the same method in the same structure on a similar subset of neurons, the authors need to reference that paper in lines 165-166 (https://doi.org/10.1016/j.neuron.2017.01.008) and comment on the overlap they see with that paper, as they should have overlapping populations

2. Given ON-cells (and OFF-cells) are active before trail flick in many prior anesthetized and awake RVM studies (see Hellman, 2012 for awake RVM electrophysiology, please reference as it is not currently in the manuscript, https://doi.org/10.1523/JNEUROSCI.1551-12.2012), can the authors comment in the manuscript text and here about whether they observed a statistically significant difference in activation of Oprm1+ RVM-SC cells between withdrawal and non-withdrawal trials? For example, Hellman, 2012 observed that ON/OFF-cells were not active in trials that did not elicit a withdrawal. Further, the authors should discuss in the text further how their work compares to recent results in the field (for example, https://doi.org/10.1097/j.pain.0000000000003360) that have identified roles for RVM-MOR populations in nociception/pain.

3. Have other studies identified superior colliculus (SuC)->RVM pathway? If so, the authors need reference in the text when cTRIO experiments are described. For example, SuC->RVM was mentioned in Hermann, 1997 (https://doi.org/10.1016/S0891-0618(97)00019-7).

4. In the case of injuries that have a defined recovery period (for example, incisional), does this circuit normalize its activity?

5. Did the authors collect any manual or automated affective or escape behaviors for any of the CFA or SNI studies in this manuscript? While the manuscript focuses on affective and sensory brain circuits yet only nociceptive stimulus-induced post-injury behaviors are quantified. The manuscript's conclusions would be strengthened by showing whether manipulating nodes, especially in chronic pain states, in this pathway only modulate nociceptive behaviors (in contrast to ACC, BLA, etc.).

6. Does chemogenetic sensitization increases guarding/escape behaviors to innocuous stimuli, change paw pressure, or drive spontaneous pain behavior? Indicating ongoing pain or hypersensitivity. This is distinct from CPA and related assays in the manuscript, which test aversion and in certain cases may not be specific to mechanical sensitivity/pain.

7. If, as the authors claim, this is a closed loop circuit then stimulating STT terminals in VPL + Po should cause transients in RVM-Oprm1+ cells and those same SST neuron cell bodies (excluding antidromic activity). Do the authors have evidence that this is the case or other direct experimental evidence of a true closed-loop circuit? If that is not the case, it seems appropriate for the authors to replace all "closed-loop circuit" with "spino-brain-spinal circuit" throughout the text.

8. Is it possible to enhance Fig. 5j? This is a critical figure panel and is currently too small. Suggest moving Fig. 5c to extended data and giving more room for Fig. 5j and update the red/blue color scheme for excitatory inhibitory to the common -> and -| for each, respectively, along with thicker lines for visualization. The diagram also doesn't distinguish if it is RVM-SC or Oprm1+ RVM-SC neurons that are critical. Further, given the number of brain regions and experiments, it would help readers to include a panel-table (either in main or extended data figs) with columns for brain region, chemogenetic silence/activation, and direction of mechanical hypersensitivity change along with which experimental results were expected/unexpected given prior work.

9. Did the authors conduct anterograde tracing in Oprm1+ RVM-SC neurons to show which cells they synapse on in the spinal cord? Given the specificity and focus on mechanical hypersensitivity (for example, lines 148-149), additional discussion of this (beyond DCN) would help clarify potential cause for this. For example, building on lines 295-297.

10. The manuscript at times switches from anatomical work that specifies specific cell types and pathway to more general dissections of broader cell types in the pathway. For example, lSuc->RVM-Oprm1+ pathway is characterized in Fig. 3c-f but the silencing of inputs is switched to broader excitatory inputs (lines 180-186). Did the authors find that manipulating the complete lSuc->RVM-Oprm1+ pathway did not alter mechanical thresholds, or can they comment on why they assume the smaller inhibitory input might not have an outsized effect on the downstream RVM-Oprm1+ neuron response?

11. The authors generate an Oprm1-Cre mouse and reference a prior paper that did so (Bailly, 2020). However, the authors fail to mention why they generated a new knock-in mouse line and if there are differences from Bailly, 2020 or other lines created. A supplemental note discussing this would help clarify the advantages of the new mouse line compared to prior ones: https://doi.org/10.1002/dvg.23341, https://doi.org/10.1016/j.celrep.2019.11.096, https://doi.org/10.1016/j.neuron.2021.11.029, https://doi.org/10.1038/s41467-023-41407-2, etc.

12. Authors state on lines 217-218: "The VPL and Po show similar functional connectivity to the retrogradely labeled lSuC projecting neurons in the layer 5 of SS (Fig. 5b)." Fig. 5b show electrophysiological characterization of those pathways and yet no electrophysiological is done to show similar SS->lSuc connectivity in the manuscript. This should either be done or this sentence rephrased. If these experiments exist, they should be added to the manuscript and the figures referenced in both sections.

13. The repetitive activation of RVM-Oprm1+, lSuC, SS, and SC->(Po or VPL) show mechanical hypersensitivity. However, in all cases it takes several days for the effect to become persistent. Did the authors conduct single activation experiments (only on CLZ dose given) to show that is not sufficient to drive long-term sensitization? For example, follow mice in Fig. 2b. Does the sensitization stay locked at the mechanical sensitivity that mice are at when CLZ infusions are ceased or recover? For example, we know from Cai, 2014 (not reference, but would prefer if it was, https://doi.org/10.1186/1744-8069-10-70) that single optogenetic activation of RVM-TPH2+ cells can drive prolonged hypersensitivity. Discussion of this point in the manuscript would help with interpreting these experiments.

14. Do the authors expect to see prolonged state changes in Oprm1+ RVM-SC activity on chemogenetic activation of STT->(VPL + Po) pathway? Given there is no longer a prolonged peripheral input to drive Oprm1+ RVM-SC activity changes as is the case with SNI. Discussion of prior work looking at (noci)plastic changes that persist centrally in the absence of peripheral input should be briefly mentioned.

15. Do mice show any changes in grimace during any of the chemogenetic activation experiments? If the videos are available, this should be quantified and included as it would indicate if activating this pathway is causing a prolonged pain-

related percept leading to mechanical hypersensitivity.
16. Are spontaneous pain behaviors reduced during chemogenetic inhibition of Oprm1+ RVM-SC neurons? In line with Fig. 1h fiber photometry results showing spontaneous pain behaviors in SNI mice.
17. Given CLZ's pharmacokinetics and the dose used (0.1 mg/kg), these pathways will only be maximally active for 1-2 hours and overall active for several hours. Can the authors comment on whether this is in line with any studies showing repeated mechanical stimulation for similar durations leads to prolonged sensitization?
18. The floor texture preference studies are intriguing but do not appear complete, as the animal is asked to make two decisions during the texture change: preference for a novel texture and for the specific rougher texture. The authors do not have an additional control (for example, a texture smoother than the Baseline textures) that would help differentiate this. Please elaborate on why this was not done or perform these experiments to demonstrate that any novel texture would not be affected by these manipulations. This would mean that lines 234-235 ("suggesting that mice innately prefer the rough surface over the smooth surface") needs to be updated, as this is not demonstrated by the authors' experiments.
19. Do the CFA or SNI mice have an altered preference for the rough texture in preference assay?
20. Either in the main text or a supplemental note, can the authors please elaborate on where prior views on STT (line 230-231, "traditional view emphasize STT's role in somatosensory discrimination") are wrong or where their results disagree with prior studies?
21. Does identifying RVM-SC neurons via neonatal injections lead to missing certain RVM-SC neurons that would be identified in the adult or label RVM neurons that are not RVM-SC in the adult (for example, post-natal pruning)? If so, please provide quantification of an experiment with later intraspinal injections of AAV8retro-GFP combined with fluorogold, CTB, or similar retrograde tracer.
22. Do the authors observe any anterograde transport with their new AAV8retro? Please indicate this in the text or provide experimental evidence this is not the case.
23. Is the activity of the Oprm1+ RVM-SC neurons recorded in Fig. 1g-j correlated with "pain"-related movement or just to any general locomotion or startle-like movement? If the authors recorded synchronized videos, they should plot the dF/F activity as a function of locomotor speed before and after SNI in the setting of stimuli being delivered and during non-stimulation periods (when the animal is and is not locomoting).
24. In Fig. 5j, RVM-SC neurons are shown to synapses on superficial inhibitory dorsal horn neurons that then synapse on excitatory deep dorsal horn projection neurons. Where is the evidence of this in the manuscript? The model should clearly delineate what was experimentally shown in the manuscript vs. speculation or results from other studies.
25. Does manipulating this circuit affect general aversion or anxiety? For example, is behavior in the elevated plus maze affected by VPL+Po manipulation? Discussion of this would help indicate the specificity of manipulating this pathway.
26. Given Fig. 3f shows that PAG->(Oprm1+ RVM-SC) is mostly inhibitory, why did the authors choose to silence excitatory PAG->RVM (not Oprm1+ RVM-SC) cells (Fig. 3a-b)? If the authors had instead silenced the inhibitory PAG->RVM or PAG->(Oprm1+ RVM-SC) pathway, then this would lead to disinhibition of the inhibitory Oprm1+ RVM-SC, which might drive analgesia (depending on animal state) and be more consistent with prior results as mentioned on line 161. Please state in the text (for example, line 156-158) why excitatory but not inhibitory PAG->RVM cells were silenced.
27. Did the authors distinguish between LGPi and NRM? For example, recent work (https://doi.org/10.7554/eLife.78689) indicates that they can have differential effects on behavior. The focus appears to be on the NRM from images, but it is not clear if the LGPi is also being manipulated.
28. Why did the authors not conduct a sham experiment for CPA? Pairing the side with just the experimenter present and approaching the animal with their hands can be sufficient to induce neural responses and conditioning independent of mechanical drive.
29. Regarding blinding, this was not done as stated on line 582. Was this lack of blinding only to the injury the animal received, or did this extend to what compounds were being given (for example, CLZ, saline, etc.) or circuit pathway being test? Please state this explicitly in the text and explain why this was not done (the lack of blinding to injury is understandable).
30. Regarding Fig. 3g and 3i, does silencing lSuC(excitatory)->RVM affect CFA related behaviors or reproduce the formalin-like behaviors?
31. Can the authors speculate on why there is no partial restoration of sensory threshold when silencing only SC->VPL or SC->Po pathways (Fig. 5c)? Is the effect in Fig. 5d synergistic? For example, if you halve the dose of CLZ, is there any effect on mechanical thresholds?
32. As the frontal cortex and other structures that project to the RVM (directly or indirectly) have been shown to be involved, can the authors comment on whether they would expect the sensitization to disappear upon removal of those structures? For example, if this circuit maintains the sensitized state independent of those other pathways, that would greatly add to the importance of the identified pathway.

Experiments and data analysis:
33. What is the overlap between fluorogold or other chemical retrograde tracers and AAV8retro? That would appear to be a more comprehensive comparison than to AAV2retro. This should be shown as a Venn diagram overlap.
34. Neural activity is only recorded in the RVM using fiber photometry. Recording from at least one or two other brain areas in the pathway characterized would help strengthen the case that the inferred changes from the activation and silencing experiments match the changes that the circuit actually undergoes after injury. For example, if record from lSuC->RVM neurons before and after SNI, are changes observed?
35. Additional characterization of the new Oprm1-Cre mouse is needed. This includes showing expected expression in other areas of the CNS/PNS (for example, striasomes, sensory neurons, etc.) when crossed with a reporter line and any unexpected expression patterns.
36. There is no validation that the chemogenetic silencing is producing the downstream effects claimed. The manuscript would be strengthened if the authors performed fiber photometry in downstream regions while conducting chemogenetic

manipulation. For example, record from SS while silencing SST->Po and/or SST->VPL to show change in SS activity is specific for silencing both or recording from ISuC->RVM to show that activating it drives Oprm1+ RVM-SC activity.
37. Fig. 5i is missing a control for no silencing of either pathway, for example, it is not clear if the discrimination index with SC->(VPL+Po) silencing is lower than no brain silencing, even if it is higher than DCN->VPL. Lines 237-238 ("also had no effect on mice's preference for the sandpaper floor or to objects with novel textures") needs to be updated to indicate this was not directly shown or the experiment performed to indicate this.
38. Given CLZ can affect behavior, to assure readers about locomotion dynamics, the authors should show a temporal trace of the behavior (if the data is available) from the time of CLZ injection during chemogenetic activation experiments and at intervals to at least 1-2 hrs post-injection.
39. The authors need to show with a startle-based assay or by giving other sensory stimuli (for example, air puff, etc.) if the mouse is specifically sensitized to hind or forepaws stimulation or generally sensitized to all sensory stimuli. This will better show the specificity of activating this circuit to mechanical/pain-related hypersensitivity and build on Extended Data Fig. 4.
40. For Oprm1+ RVM-SC fiber photometry: make a plot similar to Fig 1h-j but separating out summarized plot by those that evoked a withdrawal and those that didn't along with spontaneous vs. non-spontaneous.

Requests:
41. "Data and materials availability": Please deposit images from the new Oprm1-Cre mouse crossed with a reporter line on a Zenodo or other public repository.
42. "Data and materials availability": Please state whether the new AAV8retro or other viruses/plasmids will be deposited on Addgene or otherwise made available to the community.
43. "Data and materials availability": Please deposit some of the code used for analysis (for example, for locomotion) into a GitHub/Zenodo associated with this paper.
44. Given the general audience that will read this paper, the final paragraph could be strengthened to reference the wider pain literature or how these results might influence next steps in preclinical research, relate to observations in the clinic, or might lead to novel types of analgesics or pain management.
45. Please provide a supplemental table of the statistical analysis with columns for figure, statistical test, number of animals, and significance value. That will be easier for readers than the current list in the Methods section.
46. For "Chemogenetic Manipulation" Method section, please specify for each of the different experiments where exactly the chemogenetic manipulation was done. Based on the current description, Fig. 5d is not clear if CLZ was infused into the VPL and Po or at some earlier point in the STT pathway. If the former, there should be two "X"s over each pathway in Fig. 5d, to clearly indicate this.
47. Extended Data Fig. 2d-f, please provide a Venn diagram of the AAV8retro and AAV2retro labeled RVM neurons, so readers can identify how many neurons labeled by AAV2retro are missed by AAV8retro.
48. In "von Frey Test" methods, please state the exact behaviors used to indicate withdrawal responses and those not considered withdrawal behaviors or not included in the analysis. This will affect interpretation of what mechanical thresholds mean throughout the study and whether non-nociceptive behaviors are being included.
49. Please provide a supplementary video of a mouse with RVM fiber photometry, for example, include the behavior synchronized with a trace of RVM GCaMP activity measured.
50. Building on Fig. 5h, please plot summary dF/F (for example, in Extended Data or Supplemental figures) stimulus induced vs. spontaneous or no locomotion.
51. The introduction refers to the well-defined ON-cells (line 46-49) but does not explicitly name them. Please do so.
52. Line 553-555, please clarify why a different stimulus amount or timing was giving for SNI vs. non-injured mice? Further, were non-injured mice actually stimulated every 6.5 s for 10 mins? And why was a different stimulus given to SNI and sham mice for cFos? Or if the same stimulus was given for Extended Data Fig. 5e-f, please clarify that in the methods.
53. Please provide additional details on the tracking software and algorithm used for the CPA, locomotion, and other related test (for example, expand on line 422-423) or make the code available on a GitHub.
54. Please comment in the Results or Discussion whether the fiber photometry RVM results align with other fiber photometry recordings from other papers (for example, https://doi.org/10.1016/j.ibror.2019.05.005 and https://doi.org/10.1101/2024.11.29.626042).
55. On line 431-432, it appears that different recording times were used for CLZ compared to morphine and the CLZ duration of recording is relatively short given CLZ's kinetics. Please explain this discrepancy.

Figures:
56. Fig. 1e and Extended Data Fig. 3e, please plot the overlap as Venn diagrams to readers can know % retrograde that are X marker and % of X marker that are retrograde.
57. Fig. 1g, make this and other diagrams include a descending axon or other indication to make it clearer what is being done. This extends to making the arrows having a line going to text of each injected substance.
58. Fig. 1g, please improve this timeline to indicate when optical fiber is placed and when experiments in the normal state are done.
59. Fig. 2a, please improve this timeline to indicate when cannula is placed and exact times for behavioral studies.
60. Fig. 2c, overlay RVM landmarks to indicate looking at NRM or other structures.
61. Fig. 2d, overlay outline of spinal cord, currently hard to visualize. Improve legend or panel to indicate what is being visualized with each color.
62. Fig. 3g, "Silencing ISuC *excitatory* input", please make clear in this and other figures when stimulating a specific cell type within a pathway, for example, this experiment is "excitatory" cells.
63. Fig. 4 needs a diagram of the experiments leading to Fig. 4a-b.

64. Fig. 4a, please directly label Mo and SS in the dashed green circles on the 3D model and change the color of the dashed green circles to one with greater contrast, it is currently hard to see.
65. Extended Data Fig. 6, please provide a diagram of the experiments performed and clearly state which brain areas are being manipulated in the figure panels and/or legends.
66. Extended Data Fig. 9. Please provide supplemental figure(s) showing example histology images paralleling the summary diagrams.
67. For CPA studies, it is often hard to see that the quantified effect matches the traces of animal locomotion/location. Is it possible to display these as heat maps or include the heat maps in a supplemental figure for all CFA experiments?

Text:
68. Please check the text throughout for spelling and grammar. For example (with text inside stars indicating suggested changes) line 130 “mechanical withdrawal threshold*s*”, line 252 “lays *the* foundation”, line 253 “circuit interact*s* with”, line 255 “*The* current model” or “Current model*s*”, line 382 “480 *nm* LED”, line 495 “2 ms *long pulse of* 470 nm”, etc.
69. Please use “significantly” if the result is statistically significant instead of “dramatically” or similar adjectives to describe results in the results section.
70. Line 228-230: Fig. 5c,d do not demonstrate that those pathways do not play a role in nociception in healthy mice. At most they show that the authors measure of mechanical sensitivity is not affected or that the experiment did not work since no other validation (for example, electrophysiology) of chemogenetic inhibition was done.
71. Please state the method employed in the text when mentioning experiments, for example, line “Silencing the SS->ISuC, but” should indicate chemogenetic.
72. Line 229-230 “indicating that neither pathway is involved in nociception in healthy mice (Fig. 5c,d)”. This is not shown by these experiments, only that mechanical hypersensitivity is not changed.
73. Line 238-240 needs to be revised. No recording of individual SS neurons was done, so these results merely show that there is not a behavioral manifestation after manipulating (VPL + Po)->SS rather than they are not transmitting tactile information to SS, which would require inhibiting these pathways then showing this does not alter SS neural codes via calcium imaging or electrophysiology.
74. Line 269-270 needs rewording. The experiments done do not show that ISuC->RVM pathway is not involved in orienting toward the wound, this would require additional experiments such as inhibiting and activating that pathway while giving von Frey or other stimulation at the wound site and then demonstrating animals maintain behavior. Further, the authors need to be specific and indicate ISuC(vglut2)->RVM for all cases in which they are looking at just VGLUT2+ excitatory neurons rather than the entire RVM pathway (which includes excitatory inputs).
75. In Methods, all instances of “previously described” (for example, line 351, line 362, etc.) or similar should cite the relevant study.
76. In the Methods, please include manufacturer information in all cases, for example, line 406-407 does not state von Frey filament manufacturer, clozapine source not stated, etc.
77. The entire sentence from line 530-538 is confusing and appears to contain hanging parentheses. Please re-write.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
I would like to commend the authors for their hard work in responding to the comments of the three referees. Thank you also for your detailed response document which made the re-review much easier. This study is important and of high technical quality and I have nothing further to critique. Congratulations on a beautiful piece of work!

Referee #3

(Remarks to the Author)
The study investigates a spino–brain–spinal circuit loop mediating mechanical hypersensitivity in chronic inflammatory and neuropathic pain. Through the combined use of anatomical tracing, chemogenetic and optogenetic manipulations, calcium imaging, and behavioral assays, the authors provide compelling evidence for a closed-loop pain circuit linking the spinal cord, thalamus, somatosensory cortex, superior colliculus, and rostral ventromedial medulla (RVM). The identification of μ-opioid receptor-expressing ($OPRM1^+$) GABAergic neurons in the RVM as a key node mediating pain chronification is a particularly noteworthy contribution to the field of pain research.

The authors have successfully addressed all my major and minor concerns in this revision. The manuscript is now technically sound, clearly written, and scientifically convincing. It provides novel insights into the mechanisms underlying pain chronification via a newly identified spino–brain–spinal circuit. I recommend the acceptance of this revised manuscript for publication.

Referee #1 (Remarks to the Author):

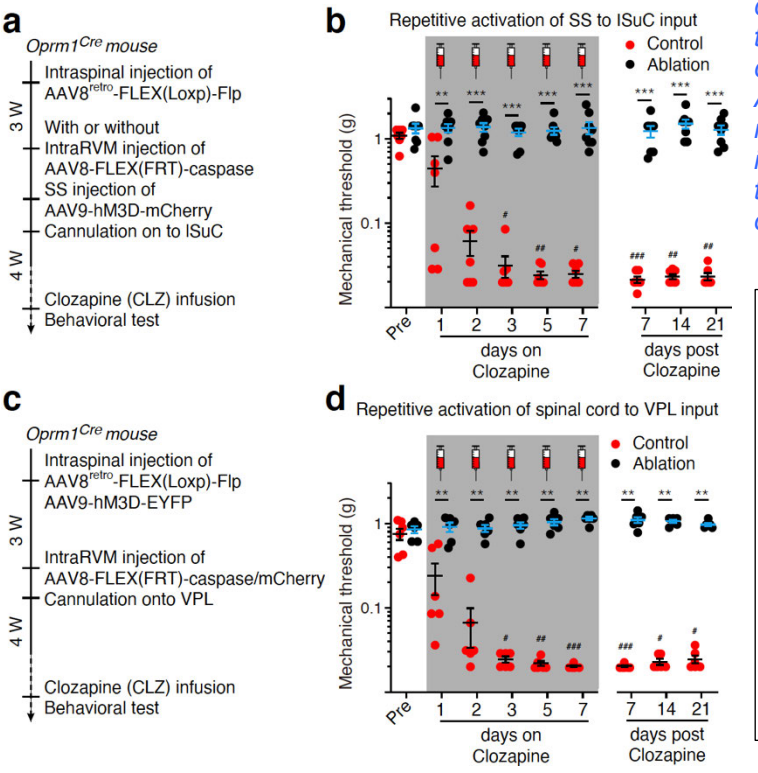
This is a highly sophisticated manuscript the probes the role of the RVM mu-opioid receptor expressing neurons in modulating mechanical hypersensitivity in chronic inflammatory and neuropathic pain states. The authors created a new OPRM1-cre mouse line and engineered a more effective retrogradely transported AAV8 to selectively manipulated RVM opioid receptor expressing neurons that project to the spinal cord, and then manipulated this pathway in various ways. They then engaged in projection mapping to identify multiple connected upstream regions that form part of a circuit that regulates mechanical hypersensitivity. Overall, this is a well (albeit a bit densely) written paper that identifies key components of a long range neuronal circuit that regulates mechanical pain. I have a few minor comments, but I believe that I identified one rather major shortcoming that should be addressed with some additional experiments (see below).

We sincerely appreciate the reviewer's enthusiastic for our work and thank the reviewer for raising the important questions. Addressing these points with new results and analysis has allowed us to further strengthening our conclusions.

Major concerns:

Fig 4 – the idea of tracing upstream inputs into the lateral superior colliculus is interesting. One major limitation here is that in contrast with the data in Fig 3 where the authors were able to selectively go after inputs that target the RVM OPRM1 neurons, in Fig 4 all inputs from upstream regions into the lateral superior colliculus are captured/manipulated, and not just those that then selectively send projections to the RVM OPRM1 cells (i.e., the specificity is lost). Indeed, any manipulation of the somatosensory cortex – superior colliculus axis may affect more than one downstream region, not just the opioid receptor expressing cells in the RVM. Ideally, the authors could design an experiment where they silence this connection as they did in Fig 4 h and I (or maybe ablate it altogether), and then they could in the same animals selectively manipulate the RVM-SC pathway to show that this overrides the effects of silencing the upstream pathway. This would not be too onerous to do.

Exactly the same consideration applies to the data in Fig 5 – again, the authors have no way of showing that this thalamus to SS projection ultimately acts by altering the RVM ORPM1 neurons. Clearly, the pathway is important, but RVM cell populations other than the opioid receptor expressing ones may be involved. The authors state in the abstract: "Here we report a closed-loop circuit extending from spinal cord to ventral posterolateral thalamus and posterior complex of the thalamus, proceeding to primary somatosensory cortex; then returning to the spinal cord via lateral superior colliculus, ultimately connecting to u-opioid receptor expressing RVMSC neurons." I am not sure that I can agree with the latter part of the statement for the reasons articulated above. Yes, the RVM opioid receptor expressing neurons are important, and yes, upstream regions are important, but the current data set does not prove that the thalamus -SS connection acts exclusively (or at all) via the RVM opioid receptor neurons. Again, the way to show this is by concomitantly manipulating the RVM-SC projection and the specific upstream pathway to show that they are causally linked using pain behavioral assessments. Another way to potentially address this (rather than using mechanical thresholds) would be to use the calcium imaging approach used in Fig 1h and then show that that this signal is modulated when the various upstream parts of the circuit are activated or inhibited.



Rebuttal Fig.1 Ablation of OPRM1^{+} RVM^{SC} neurons prevents mechanical sensitization caused by repetitive activation of upstream nodes in the long range circuit. a, Experimental timeline for (**b**). W, week. **b**, Quantification of mechanical thresholds after repetitive chemogenetic activation of the SS→ISuC pathway in mice with (ablation, black, n = 8) or without (control, red, n = 7) caspase expression in OPRM1^{+} RVM^{SC} neurons. **c**, Experimental timeline for (**d**). W, week. **d**, Quantification of mechanical thresholds after repetitive chemogenetic activation of the spinal cord→VPL pathway in mice with (ablation, black, n = 6) or without (control, red, n = 6) caspase expression in OPRM1^{+} RVM^{SC} neurons. CLZ was infused 23 hours before each von Frey test for 7 consecutive days (shade area). Control vs. ablation: Mann-Whitney test, ** $P < 0.01$, *** $P < 0.001$; Before vs. after CLZ: Dunn's multiple comparison test, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$.

The reviewer raised important points. We agree that the original data in Fig.4f and Fig.5f did not conclusively demonstrated that chronic mechanical pain sensitization, induced by repetitive activation of the somatosensory cortex (SS)→lateral superior colliculus (lSuC) pathway or the spinal cord (SC)→thalamus→SS pathway, depends on OPRM1^{+} RVM^{SC} neurons. Following the reviewer's suggestion, we ablated the OPRM1^{+} RVM^{SC} neurons before repetitively activate these upstream pathways. In consistent with our circuitry model, this ablation prevented the development of mechanical sensitization, providing direct evidence that OPRM1^{+} RVM^{SC} neurons are essential for chronic mechanical pain induced by upstream pathway activation (Rebuttal Fig.1). Again, we sincerely thank the reviewer for raising this important question, as addressing it has significantly strengthened our conclusions.

We added the ablation data in Rebuttal Fig.1b to Fig.4f and added Rebuttal Fig.1 c,d to Extended Data Fig. 10a,b in the revised manuscript.

Along these lines, I am not sure that the authors have any evidence for a "closed loop circuit" as stated in the Discussion – all you have is evidence that there are individual connections in a pathway, but a closed loop would imply that there cannot be any other projections to other regions that leave the loop, and no other inputs that may modulate this part of the circuit. Indeed, the data in Fig 3e and 4b suggest that there are other inputs even if they are less pronounced. So please reword this accordingly to something like a "long range circuit".

We apologize for any confusion caused by the term "closed-loop circuit" and have revised the manuscript to use "long range circuit loop" instead. We did not intend to imply that this long-range circuit operates in isolation from the broader brain network. However, we believe the term "loop" remains useful to emphasize the key anatomical feature of this pathway—projections originating in the spinal cord, traversing four intermediate relays, and ultimately returning to the spinal cord.

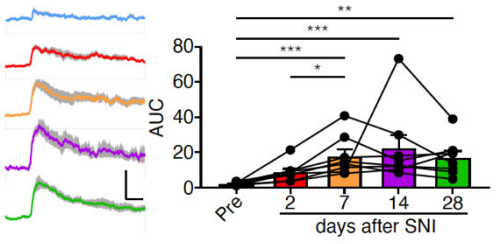
Minor comments:

Another limitation that could be discussed and elaborated on is the nature of the projection that is being silenced or activated between the various relay stations – are these various projections excitatory, inhibitory or a mix of both? The way I read the manuscript, it seems that the hsyn promoter was being used which would hit all neuronal projections. This should be discussed.

Thanks for the suggestion. As shown in Fig.3f, approximately 95% mCherry^{+} neurons in the lSuC are vGlut2^{+} excitatory neurons. While we have not specifically characterized the retrogradely labeled lSuC projecting neurons in the SS, these neurons are located in Layer 5, which predominantly contains long-range projection neurons that are almost entirely excitatory pyramidal cells (Harris and Shepherd, 2015). Similarly, the Po and VPL are thalamic regions composed also almost entirely of excitatory neurons. We have used different colors (red for excitatory, blue for inhibitory) to clarify the nature of these projections in Fig.6a of the revised manuscript.

Data in Fig 1e should be quantified

The quantification of Fig.1e has been presented in Extended data Fig.3d of the original manuscript because of the space limitation. Now the quantification is presented in Venn Diagram as requested by reviewer#4. Sorry for the confusion.



Rebuttal Fig.2 Representative traces (left) and quantification of calcium responses (right) in OPRM1^{+} RVM^{SC} neurons to mechanical stimuli before SNI (Pre) and 2,7,14,28 days after SNI (n = 8). Scale bar: 2s, 2% ΔF/F. Data from same mice are connected with lines. Non-parametric ANOVA test,, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Bar graph in Fig 1f -the color coding of DAMGO vs DAMGO +CTAP is too similar – please change one of them

We changed the color coding of DAMGO + CTAP to purple.

Fig 1 j – are the data at various time points statistically different from each other?

Reviewer#3 also raised similar question in his/her major concern #2. To answer this question, we increased N to 8 and performed statistical analysis to compare data at each time points (Rebuttal Fig.2). We found that mechanical responses in OPRM1^{+} RVM^{SC} neurons were significantly

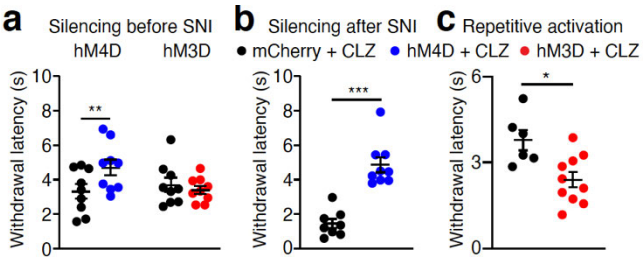
elevated at 7, 14, and 28 days post-SNI compared to baseline (Pre-SNI). The new result is now showed in the updated Fig.1i of the revised manuscript.

Fig 2 – why is mechanical threshold shown on a log scale?

Both linear and log scales are used to report mechanical thresholds. However, in models of neuropathic pain like SNI, successful surgery induces a strong hypersensitivity, driving thresholds to very low values (e.g., below 0.05 g) with significant variability. We use a log scale to better represent this variation of sensitization. On a linear scale, these low values would be compressed, making differences between groups difficult to discern. The log scale expands this critical low-value range, allowing for clearer data visualization.

Fig 2 – it may be useful to have the thermal data included in the main figure to draw a clear contrast to the alterations in mechanical hypersensitivity

Thank you for this suggestion. We agree that including thermal data is valuable for a complete comparison. In our initial submission, the Hargreaves test (heat) showed that the circuit we identified is not involved in heat hypersensitivity following inflammation. However, as rightly noted by Reviewer #3, role of the OPRM1^{+} RVM^{SC} neurons in cold allodynia remained a crucial question. To address this, we performed the plantar cold test. We found that silencing OPRM1^{+} RVM^{SC} neurons eliminated nerve injury-induced cold allodynia, while repetitive activating these neurons induced cold allodynia in healthy mice (Rebuttal Fig. 3).



Rebuttal Fig.3 Withdrawal latency of plantar cold test. a, Quantification of saline (black) or CLZ on withdrawal latency of hM4D (blue, n = 9) or hM3D (red, n = 9) expressing healthy mice. **b,** Quantification of withdrawal latency after CLZ treatment in mCherry (black, n = 8) or hM4D (blue, n = 9) expressing mice with SNI. **c,** Quantification of withdrawal latency of mCherry (black, n = 6) or hM3D (red, n = 10) expressing healthy mice with repetitive CLZ treatment. CLZ: clozapine. Wilcoxon matched-pairs signed rant test or Mann-Whitney test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Due to space constraints in the main figures, we have replaced the original Hargreaves test data (Fig. 2b) with these new plantar cold test results. The original Hargreaves data has now been moved to Extended Data Fig. 6a. Furthermore, we have added the new cold allodynia data from chemogenetic inhibition and activation experiments to panels Fig. 2g and Extended Data Fig. 6f, respectively.

Fig 3 – the authors find no effect upon silencing vlPAG excitatory inputs into the RVM OPRm1 neurons – this is interesting, Here the author may want to discuss differences to results reported by Huang et al Nat Neuroscience 2019 where a broader ablation of vlPAG-RVM connections did seem to ablate descending inhibitor modulation of mechanical hypersensitivity.

We thank the reviewer for bringing the Huang et al. (2019) study to our attention. The reviewer is likely referring to the data in their Fig. 5d (as showed on the right), which supports their conclusion that ablating vlPAG→RVM projections prevents analgesia induced by optogenetic inhibition of BLA→mPFC inputs.

However, regarding our findings, the data in Huang et al.'s Fig. 5d appear largely consistent with, rather than contradictory to, our results. Focusing on the light-off condition in their study, the paw withdrawal (PW) threshold in sham-operated rats (~7–8 g) reflects baseline sensitivity, while SNI reduces this threshold to ~4 g in the control group (dtA-only, no ablation, light off). Notably, when both excitatory and inhibitory vlPAG→RVM projections were ablated, SNI surgery also bring the PW threshold down to ~4 g after—indistinguishable from the dtA-only control with SNI (PAG-RVM, light off). This suggests that neither excitatory nor inhibitory vlPAG→RVM neurons significantly contribute to SNI-induced mechanical hypersensitivity, aligning with our observation that silencing excitatory vlPAG→RVM inputs alone does not alter mechanical sensitivity. We have now cited Huang et al. (2019) and incorporated this discussion into the revised manuscript (line 335-338).

Methods:
Mice do not have a "gender" - they have a biological sex – please revise "genders" to "sexes"
We changed 'genders' to 'sexes' in the revised manuscript. Thanks.

Referee #3 (Remarks to the Author):

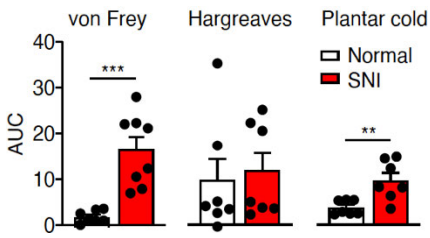
Summary of the Key Results:
The manuscript by Chen and colleagues investigates a spino-brain-spinal circuit loop that mediates mechanical hypersensitivity in mouse models of chronic inflammatory and neuropathic pain. This closed-loop circuit includes an ascending pathway from the spinal cord to the primary somatosensory cortex via the ventral posterolateral (VPL) thalamus and posterior complex of the thalamus (Po), and a descending pathway from the somatosensory cortex to the lateral superior colliculus (lSuC), then to the rostral ventromedial medulla (RVM), and back to the spinal cord. The study identifies a specific population of μ-opioid receptor-expressing RVM neurons, primarily GABAergic, that project to the spinal cord. Silencing any node in this multisynaptic pathway did not alter nociception in healthy mice but effectively eliminated mechanical hypersensitivity in chronic pain models. Notably, repetitive—but not acute—activation of these nodes was sufficient to induce chronic mechanical hypersensitivity in healthy mice.

Overall Assessment
This study offers novel insights that challenge established perspectives in the field. By employing a variety of state-of-the-art techniques, the authors meticulously dissected each connection within the spino-brain-spinal circuit and identified key cell types at critical nodes, including the RVM and lSuC. Through the manipulation of OPRM1+ spinal cord-projecting RVM neurons in chronic pain models, the study highlights the pivotal role of these neurons in the process of pain chronification. However, several significant concerns must be addressed before the study can be considered for publication:

(1) The number of animals used in key experiments is insufficient, reducing the statistical power and robustness of the conclusions. Behavioral and calcium imaging experiments are particularly affected by inadequate sample sizes.
(2) The claim that the circuit mediates mechanical but not thermal hypersensitivity is inadequately supported by the presented data. Additional experiments are needed to clarify this specificity.
(3) Missing control experiments undermine the interpretability of the results and leave alternative explanations unexplored.

We sincerely appreciate the reviewer's positive overall assessment on our work and their insightful feedback. Following the suggestions, we increased number of animals and added new experiments that should successfully addressed all concerns raised by the reviewer.

Major Concerns:
1. The study concludes that silencing the spino-brain-spinal circuit loop eliminates mechanical hypersensitivity, but the specificity of this loop to mechanical pain remains questionable. While Oprm1+ RVM neurons responded to mechanical and cold stimuli, no response to thermal stimuli (Hargreaves test) was observed in Figure 1i. However, the sample sizes (4 for normal mice and 5 for SNI mice) are too small to draw firm conclusions. A larger sample size is required.



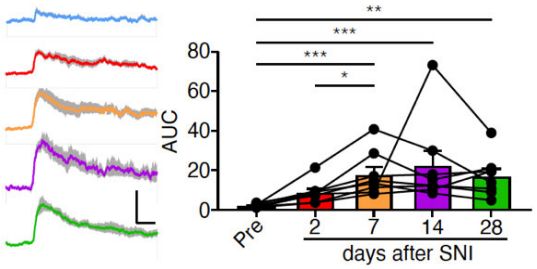
Rebuttal Fig.4 Histogram of area under curve (AUC) of fiber photometry recording. AUC of OPRM1^{+} RVM^{SC} neurons' response to three types of noxious stimuli at baseline (white) and after SNI (red). n=7-8. Mann-Whitney test, ** $P < 0.01$, *** $P < 0.001$.

Sorry for the confusion. As showed in our original Fig.1i,h. OPRM1^{+} RVM^{SC} neurons respond to all three types of noxious stimuli, including mechanical, cold and thermal stimuli (Hargreaves test). In original Fig.1i (now in Extended Data Fig.4c with n increased to 7-8), we compared calcium signals in OPRM1^{+} RVM^{SC} neurons before and after SNI surgery. We observed that SNI significantly enhanced neuronal responses to mechanical and cold, but not to heat, stimuli. These findings align with the well-established phenotype of SNI-induced mechanical and cold allodynia, but lacks a pronounced thermal hypersensitivity. We rephrase the sentences to '*In healthy mice, these neurons exhibited calcium transients time-locked to paw withdrawal in response to all three types of noxious stimuli (Fig.1h, Extended Data Fig. 4a, b). Following spared nerve injury (SNI) to induce a persistent neuropathic pain state, these neurons displayed*

heightened responses to mechanical and cold, but not heat, stimuli (Fig.1h, Extended Data Fig. 4c).' in line 111-115 of the revised manuscript.

Following the reviewer's suggestion, we have increased the sample size to n=7-8 for both normal and SNI mice in the fiber photometry experiments (Rebuttal Fig.4). Due to space limitation, these data have now been moved to Extended Data Fig.4c to accommodate the new results, which highlight the positive correlation between calcium responses in the OPRM1^{+} RVM^{SC} neurons with the speed of animal's escape response after painful stimuli, but not during voluntary locomotion (Rebuttal Fig. 19).

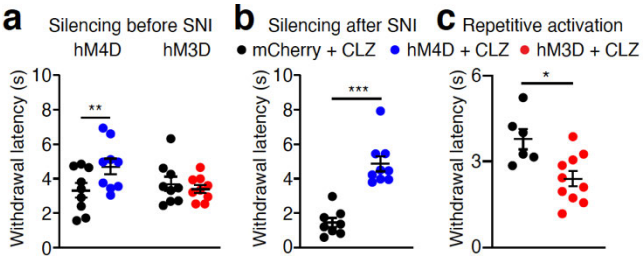
2. Figure 1j: Time-dependent changes in calcium responses of spinal cord-projecting Oprm1+ RVM (N=3) should be presented with individual lines connecting the data points for each mouse. Furthermore, the significance of the Day 28 response relative to pre-SNI should be clarified, especially given that chronic pain with mechanical hypersensitivity could persist for 28 days. The N=3 value is insufficient for statistical reliability in this context.



Rebuttal Fig.2 Example traces (left) and quantification (right) of fiber photometry recording of OPRM1^{+} RVM^{SC} neurons' response to Von Frey stimuli before (Pre) and 2,7,14, and 28 days after SNI. n=8.Lines connect same animal at different recording time. Scale bar: 2s, 2% ΔF/F. Data from same mice are connected with lines. Non-parametric ANOVA test,, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

As suggested, we have increased the sample size to n=8 in this figure and now presented the time-dependent calcium responses with individual trajectories for each mouse, connecting the data points across time points (Rebuttal Fig.2). Now this data is presented as Fig.1i in the revised manuscript. Statistical analysis confirmed that mechanical responses in OPRM1^{+} RVM^{SC} neurons were significantly elevated at 7, 14, and 28 days post-SNI compared to baseline (Pre-SNI). However, no statistic differences were observed among post-SNI days 7, 14, and 28, indicating a sustained hypersensitivity throughout the chronic phase. These results align with the persistent mechanical allodynia typically observed in the SNI model up to 28 days. Moreover, as suggested by reviewer#4, we also recorded mechanical responses in OPRM1^{+} RVM^{SC} neurons across different days before and after CFA injection (Rebuttal Fig. 10, Extended Data Fig.4d,e). We added *'Calcium responses in the OPRM1^{+} RVM^{SC} neuron increased from day 2 to day 7 post-SNI and remained elevated (Fig.1i). In contrast, in a Complete Freund's Adjuvant (CFA)-induced inflammatory pain model, calcium responses peaked at day 2 before gradually returning to baseline over 21 days (Extended Data Fig. 4d, e).'* to line 115-118 to describe these results in the revised manuscript.

3. Given the significant results of plantar cold response in Figure 1i, chemogenetic manipulation experiments in Figure 2b should include a plantar cold test.



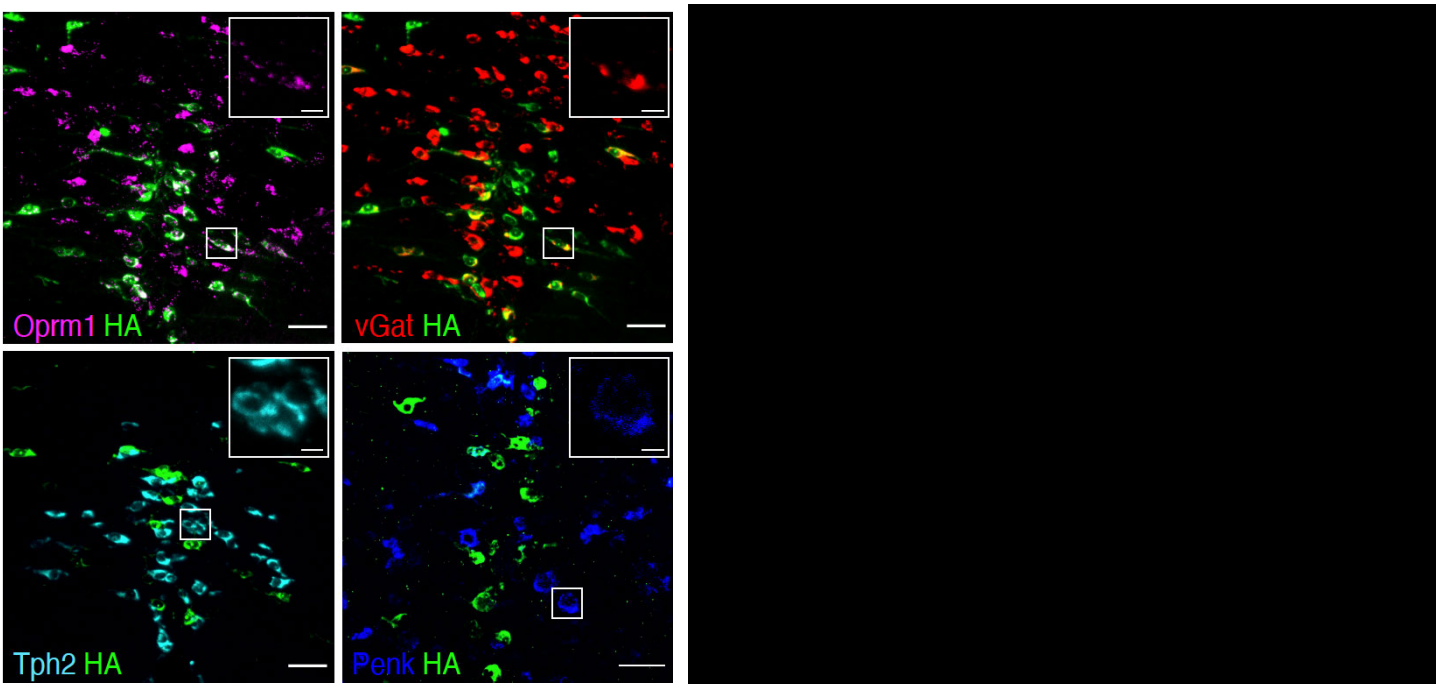
Rebuttal Fig.3 Withdrawal latency of plantar cold test. a, Quantification of saline (black) or CLZ on withdrawal latency of hM4D (blue, n = 9) or hM3D (red, n = 9) expressing healthy mice. **b,** Quantification of withdrawal latency after CLZ treatment in mCherry (black, n = 8) or hM4D (blue, n = 9) expressing mice with SNI. **c,** Quantification of withdrawal latency of mCherry (black, n = 6) or hM3D (red, n = 10) expressing healthy mice with repetitive CLZ treatment. CLZ: clozapine. Wilcoxon matched-pairs signed rant test or Mann-Whitney test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

We thank the reviewer for this insightful suggestion. As recommended, we performed the plantar cold test following our chemogenetic manipulations. We found that silencing OPRM1^{+} RVM^{SC} neurons reversed injury-induced cold allodynia, whereas activating these neurons was sufficient to induce a cold allodynia phenotype in naive mice (Rebuttal Fig. 3). The plantar cold test results have now been included in Fig.2b,g and Extended Data Fig. 6f in the revised manuscript. We described these results in line 157-159 and line 178-179.

4. Figure 1f: The DAMGO experiments (baseline, DAMGO, DAMGO + CTAP) are from two independent sets of neurons. Ideally, these conditions should be tested in the same neurons for consistency.

We fully agree that recording all conditions (baseline, DAMGO, DAMGO + CTAP) from the same neurons would enhance consistency of our findings. Over the past several months, we have diligently attempted to achieve this; however, obtaining stable, long-duration patch-clamp recordings from OPRM1^{+} RVM^{SC} neurons in adult mice has proven exceptionally challenging, due to the inherent challenges of brainstem electrophysiology in old mice. Despite extensive efforts, we have not yet succeeded in maintaining recordings of sufficient duration and quality to reliably assess all conditions within the same cell. That said, the primary goal of this experiment was to determine whether OPRM1^{+} RVM^{SC} neurons express functional μ-opioid receptors. Our data robustly demonstrate that these neurons indeed exhibit DAMGO-sensitive responses that are blocked by CTAP, confirming the presence of functional μ-opioid receptor signaling.

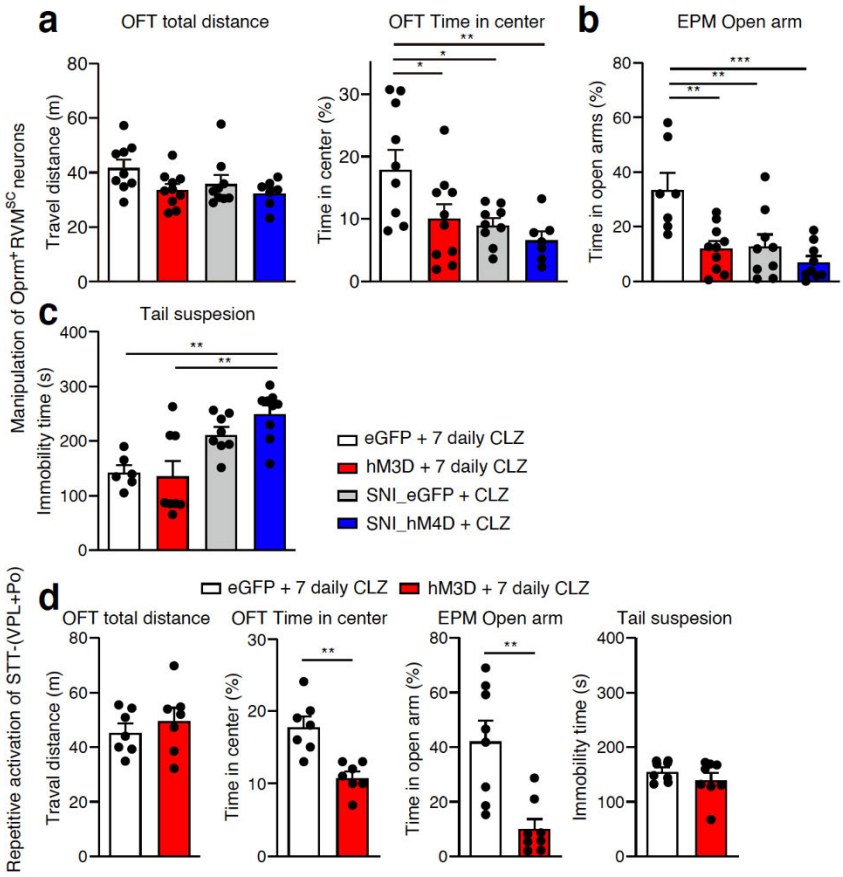
5. Signals in Figure 1e (Oprm1, vGAT, Tph2, Penk) resemble immunohistochemistry (IHC) rather than RNA scope imaging. Higher magnification and additional validation are required to confirm the specificity of RNA labeling.



Rebuttal Fig.5 High magnification RNAscope images visualize the expression of Oprm1, vGat, Tph2, and Penk expression in the retrogradely labeled OPRM1^{+} RVM^{SC} neurons. Left, Same images as Fig.1e but added a high magnification image for one representative cells as insert. Scale bar, 50μm for the large image, 10 μm for insert. Right, Example images from allen brain atlas in situ data for each of the same probe in the similar RVM region.

Thank you for this comment. We agree that the lower-magnification images in Fig. 1e could resemble IHC due to the high signal intensity. To unequivocally confirm the specificity of our RNA labeling and provide validation at the necessary resolution, we have added high-magnification images of single neurons for each gene to Fig.1e (Rebuttal Fig. 5,left) in the revised manuscript.

Furthermore, to benchmark our results against an established standard, we include reference images from the Allen Brain Atlas acquired using traditional *in situ* hybridization for the same genes in a comparable RVM region (Rebuttal Fig. 5, right). The expression patterns are highly concordant, confirming the specificity of our probes. The dense, confluent staining for vGAT, Tph2, and Penk is not an artifact but rather an expected



Rebuttal Fig.6 Anxiety and depression-like behaviors.
Six weeks after repetitive CLZ treatment to manipulate the OPRM1⁺ RVM^{SC} neurons **a,** reduces time in the center of the open field box (right) in hM3D- (red, n = 10) but not in eGFP- (white, n = 9) expressing mice but has no effect on total travel distance (left); **b,** reduces time in open arms in elevated plus maze (EPM) test in hM3D- (red, n = 10) but not in eGFP- (white, n = 7) expressing mice; **c**, but has no effect on immobility time in tail suspension test in hM3D- (red, n = 8) vs. eGFP- (white, n = 6) expressing mice.

Six weeks after SNI **a,** CLZ treatment showed similar level of total travel distance (left) and reduced time in the center of the open field box (right) in both hM4D- (blue, n = 7) or eGFP- (gray, n = 9) expressing mice; **b,** CLZ treatment showed similar level of reduced time in open arms in EPM test in both hM4D- (blue, n = 9) or eGFP- (gray, n = 9) expressing mice; **c,** CLZ treatment showed similar level of increased immobility in tail suspension test in both hM4D- (blue, n = 9) or eGFP- (gray, n = 8) expressing mice.

d, Repetitive CLZ treatment of hM3D-(red, n = 7-8) but not eGFP- (white, n = 7-8) expressing neurons in the VPL+Po thalamus reduces time in the center of the open field box and reduced time in open arms in EPM test, but not total travel distance and immobility time in tail suspension test. OFT: Open Field Test; EPM: elevate plus maze, CLZ: clozapine. Wilcoxon matched-pairs signed rank test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

outcome for genes with high expression levels in this cell population, where individual fluorescent dots are too numerous to resolve. The signal for Oprm1, a gene with lower expression, clearly demonstrates the superior sensitivity and resolution of the RNAscope platform, as individual transcript dots are readily visible.

6. Chronic pain has sensory and emotional components. In this study, chemogenetic inhibition of Oprm1+ spinal cord-projecting RVM neurons blocked mechanical stimuli induced conditioned place aversion in the SNI mice, suggesting the reduction of both sensory and affect components of pain. To strength this point, the authors should evaluate anxiety- and depression-like behaviors. Also, does repetitive activation of the spino-brain-spinal circuit specifically induce chronic mechanical pain without affecting the emotional (affective) component? This should be addressed experimentally and discussed.

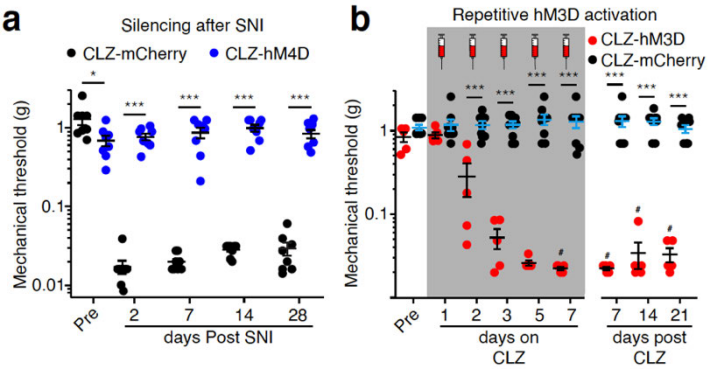
We thank the reviewer for raising this important question. Following the suggestion, we manipulated the OPRM1^{+} RVM^{SC} neurons and examined its impact on SNI-induced anxiety and depression-like behaviors, using open field, elevated plus maze, and tail suspension tests. Our results showed that six weeks after repetitive activation of the OPRM1^{+} RVM^{SC} neurons not only drives chronic mechanical sensitization but also reduces time spent in the center of the open field and in the open arm of the elevated plus maze, indicating an heightened anxiety-like states, similar to those observed in mice six weeks after SNI. However, chemogenetic inhibition of these neurons did not alter the anxiety level in SNI mice (Rebuttal Fig. 6a-c). Together, these results indicate that activity in OPRM1^{+} RVM^{SC} neurons is sufficient, but not necessary, to drive anxious state following neuropathic injury.

To address the reviewer's second point, we also evaluated anxiety-like behaviors six weeks after repetitive activation of the VPL+Po neurons that received spinal cord inputs. We similarly observed a heightened anxious-like state, characterized by reduced center time in the open field test and less time in the open arm of the elevated plus maze (Rebuttal Fig. 6d). These results closely mirror those obtained from direct activation of OPRM1^{+} RVM^{SC} neurons, strongly suggesting that both manipulations engage the same spino-brain-spinal circuit to promote affective pain-related behaviors.

We also attempted to assess depression-like behavior using the sucrose preference test. However, in our preliminary experiments with SNI mice, we did not observe a significant decrease in sucrose preference compared to non-injured controls across various conditions (e.g., 1% or 2% sucrose solutions, 1-2 hour testing periods). Consequently, we were unable to utilize this test to evaluate the chemogenetically manipulated mice.

We added these new results to Extended Data Fig. 8,10 and discussed these results in line 185-196.

7. Saline is not an appropriate control for CLZ in chemogenetic experiments. The authors should include a control with AAV encoding a fluorescent reporter without hM4D or hM3D to exclude hM4D- or hM3D-independent effects of CLZ.



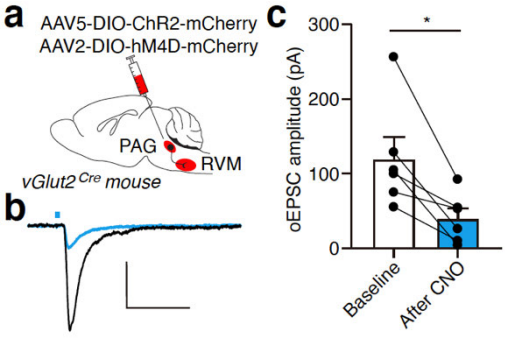
Rebuttal Fig. 7 Clozapine (CLZ) control. a, CLZ injection eliminated SNI-induced mechanical hypersensitization in mice expressing hM4D (blue, n = 8) but not mCherry (black, n = 8) in Oprm1^{+} RVM^{SC} neurons. **b,** Repetitive CLZ injection drives mechanical hypersensitization in mice expressing hM3D (red, n = 5) but not mCherry (black, n = 9) in OPRM1^{+} RVM^{SC} neurons. Comparison between mCherry vs. hM3D/hM4D groups, Mann-Whitney test, * $P < 0.05$, *** $P < 0.001$; Comparison before and after repetitive CLZ treatment, nonparametric ANOVA test, # $P < 0.05$.

Following the suggestion, we expressed mCherry in the OPRM1^{+} RVM^{SC} neurons and found CLZ injection has no effect on pain thresholds in both SNI or healthy mice (Rebuttal Fig.7). We now use these results to replace saline controls in the updated Fig.2f, i of the revised manuscript.

8. Optogenetic approaches offer greater temporal precision in assessing pain circuits and nociceptive responses. Incorporating optogenetics alongside fiber photometry would strengthen the key findings.

We fully agree that the optogenetic approach can offer 'greater temporal precision'. We initially pursued this method in our study. However, during preliminary experiments, we observed significant alterations in pain thresholds in mice tethered with optical fibers, which introduced confounding variables. To ensure the robustness of our findings, we ultimately adopted chemogenetic manipulation as our primary approach.

9. The study reports that pharmacogenetic terminal silencing of the PAG-RVM pathway has no effect on alleviating mechanical hypersensitivity in the SNI model. However, this negative result is not convincing without verification that terminal inactivation of PAG axons by hM4D successfully suppressed PAG-to-RVM transmission.



Rebuttal Fig.8 Validation of chemogenetic inhibition. a. Experimental diagram. Cre-dependent AAVs expressing ChR2 and hM4D are co-injected in the PAG of the vGlut2-Cre mice. **b, c**, Example traces (**b**) and histogram (**c**) of Light-evoked oEPSCs before (black) and after (blue) perfusion of 5μM CNO into the slice recording chamber. Scale bar: 50 pA, 25 ms. 6 cells from 3 mice. Wilcoxon matched-pairs signed rank test, * $P < 0.05$.

We apologize for not including this important data in the original manuscript. We actually have indeed confirmed the effectiveness of our chemogenetic terminal silencing approach in the PAG-RVM pathway. In these validation experiments, we co-injected AAV5-DIO-ChR2 and AAV2-DIO-hM4Di into the PAG of vGlut2-Cre mice. Four to six weeks after virus injection, we prepared acute RVM slices containing ChR2/hM4Di expressing terminals from the PAG. Electrophysiological recording demonstrated that CNO (5 μm) application effectively suppressed light-evoked EPSCs in RVM neurons, validating the functional silencing of PAG-RVM pathway (Rebuttal Fig.8). Given that we have previously used similar approach to validate chemogenetic silencing efficiency in Zhu et al. (2016), we initially only cited this work rather than presenting the data. We have now added them as Extended Data Fig. 5d-f and described the result in line 206-207 of the revised manuscript.

Minor Concerns:

1. Line 30 in Abstract is missing "of" in "activation each node"; revise to "activation of each node."

We added the missing ‘of’ to line 30, thanks.

2. The experimental timeline should be clarified. Neonatal spinal cord injections (P1.5–P2.5) and stereotaxic injections (5–7 weeks old) suggest a 4-week gap, which should be explicitly noted.
We have 4W (W=week) on the x-axis of the timeline to indicate the time gap between each injection. Sorry for the confusion.

3. Figure 1c: Yellow (Clover3 and Ruby) signals represent Oprm1+ neurons and green (Clover3 but not Ruby) signals indicate Oprm1− neurons. It seems that green signal represents all Colver3-expressing RVM cells. Please clarity.
We appreciate the reviewer's question regarding the labeling strategy. Our approach, as outlined in Fig. 1b, was designed to distinguish $OPRM1^{+}$ from $OPRM1^{-}$ neurons within the retrogradely labeled population. The AAV construct used (AAV8retro-H2B-Clover3-FLEX-H2B-Ruby3) results in constitutive expression of the green marker H2B-Clover3 in all transduced neurons. The red marker H2B-Ruby3 is only expressed following Cre-mediated recombination. Thus:

Yellow signal (Clover3+ & Ruby3+): Identifies retrogradely labeled neurons that are Oprm1+.
Green signal (Clover3+ only): Identifies retrogradely labeled neurons that are Oprm1-.

4. Figure 1f: Mention CTAP usage explicitly in the results section or figure legend.
CTAP concentration is 1 μM. We added this information to the figure legend, thanks.

5. Figure 2b: Clarify whether these data are from SNI/CFA models or healthy mice in the legend.
These data are from healthy mice. We added ‘healthy’ in the legend.

6. Figure 2g, it should be “fiber track” (not cannula track) for fiber photometry
We changed ‘cannula track’ to ‘fiber track’ in Fig.1g. Thanks

7. Unlike other spinal cord injection at neonatal stage (figure 2), justify why injections were performed in adult stages particularly for Figure 5c.
High titer AAV1-hSyn-Cre is needed to achieve anterograde transsynaptic labeling. In Fig.5, we injected high titer AAV1-hSyn-Cre (1.0 E13 gc ml^{-1}) into the spinal cord and AAV8-hSyn-DIO-hM4D or hM3D into the Po or VPL to specifically express chemogenetic tools in these thalamic neurons that received spinal cord input.

The justification for using adult injections, unlike the neonatal injections in other figures, is based on our observations of cell toxicity. In other projects, we have observed toxicity at the injection site after long incubation times (greater than two months) with high titer AAV1-hSyn-Cre. For neonatal injections, the incubation time is necessarily long (more than two months) as we wait for the mice to fully grow up before behavioral testing. In contrast, for the experiments in Fig. 5, we only waited 4-5 weeks after the adult injections before performing behavioral tests. This shorter incubation period allowed us to use the necessary high titer virus without observing cell toxicity at the spinal cord injection sites.

8. Simultaneous silencing of VPL and Po pathways alleviated chronic pain, whereas individual silencing did not. This is still unclear to me. If repetitive activation of SC to Po or SC to VPL can induce chronic pain, why silencing either of them has not effect on pain relief. Does it mean that chronic mechanical hypersensitivity induced by repetitive activation of SC to Po or SC to VPL differs from SNI-induced central sensitization.
Our current hypothesis is that SNI induces sensitization in DRG neurons, which in turn provide sustained input to both the spinal cord→Po and spinal cord→VPL pathways. Consequently, SNI leads to the repetitive co-activation of both of these thalamic pathways.

Since our data in Fig.5f show that the repetitive activation of either pathway alone is sufficient to induce mechanical hypersensitivity, we propose that in the SNI model, these pathways act in a redundant or parallel manner. Therefore, silencing only one pathway is ineffective because the other remains active and continues to drive the pain state. We added the following discussion in line 353-355 ‘*These observations lead us to*

hypothesize that SNI sensitizes both STT→SS pathways, which operate in a redundant or parallel manner to initiate and maintain injury-induced pain sensitization.'

To test this hypothesis, future electrophysiological studies are needed to investigate the nature of the interaction between VPL and Po inputs in the somatosensory cortex and to characterize the specific synaptic plasticity that occurs within these two pathways following SNI.

9. Some critical discussions (e.g., paw licking/biting in Figure 3i and dorsal column nucleus in Figure 5) belong in the Results section.
Thanks for the suggestion. We now moved our discussion on Fig. 3i to the results section in line 236-242, and the discussion on Fig. 5h,I to line 310-318 in the revised manuscript.

10. Figure 1f Units: Correct the DAMGO unit in lines 705–706 from "μm" to "μM."
This error has been corrected. Thanks.

11. Methods: line 346, 200 μM, unit should be "μm".
This error has been corrected. Thanks

12. According to Methods, lines 484-485, amplitudes of EPSCs form baseline and the last 5 min of clozapine-N-oxide was bath applied. Obviously, the data is missing in Figure 5b.
We apologize for this oversight. Experiments in Fig.5b were described in line 281-283. Instead, the methods described in line 484-485 belongs to the validation experiment to confirm the efficacy of CNO-induced synaptic silencing via hM4Di (Rebuttal Fig. 8). These specific validation data were not included in the original manuscript by mistake. As the answer to your major point #9, we have now included this dataset in Extended Data Fig. 5d-f of the revised version of the manuscript.

Referee #4 (Remarks to the Author):

Wang, et al. present a study that further our understanding of circuits involved in mechanical sensitivity and neuropathic/inflammatory pain. Through development of novel genetic and viral tools, they specifically show a spinal cord->thalamus (VPL + Po)->SS->ISuC->RVM->spinal cord circuit and demonstrate that silencing nodes along this pathway disrupts injury-induced mechanical hypersensitivity and that driving activation of nodes along this pathway is sufficient to induce mechanical hypersensitivity. Generation of the AAV8retro viral tools should have broad applicability to many other areas and we appreciate additional Oprm1-Cre mice that can help with identifying the role of Oprm1+ neurons throughout the nervous system. The Oprm1+ RVM-SC fiber photometry results in the awake animal further our understanding of the dynamics of these cells and their prolonged activation. Critically, highlighting the role of VPL/Po in mechanical hypersensitivity should increase focus on the spinothalamic tract that will complement recent work focused on the spinoparabrachial tract. The authors effort and systematic analysis of this pathway through a series of well-defined experiments is much appreciated and these studies should further our understanding of sensory/pain pathways.

We sincerely appreciate the reviewer's enthusiasm for the potential impact of our work on advancing the understanding of somatosensory and pain pathways.

However, to make the work easier to read for a general audience and further clarify the advantages of the approaches taken, see below for questions and requests for updating the manuscript text along with a few necessary and optional experiments that will demonstrate that the circuit manipulations match neural activity changes observed. I hope these questions and suggestions, which indicate the interest and question the manuscript raises, help further improve this manuscript, whose approach and results hold great promise for further dissecting the nuances of the ascending and descending pain pathways.

If due to space limitations authors are not able to include all additional material in the main text, we would recommend adding a series of supplemental notes clarifying how their anatomical and neural activity manipulation results align with prior preclinical work or match any clinical observations that heretofore been unexplained.

We are grateful to the reviewer for their comprehensive and insightful feedback. In response, we have conducted all the recommended experiments over the past seven months. The revised manuscript now incorporates these new data and the reviewer's valuable suggestions. We have addressed all major concerns raised, and we believe these revisions have substantially improved the quality and impact of the manuscript.

Questions:

1. Why do the authors state on lines 22-23 that "the neuronal pathway that transmitting information about peripheral injury to these descending RVM SC neurons is unknown"? As has been reviewed recently (for example, https://doi.org/10.1016/j.tins.2024.04.006, https://doi.org/10.3389/fpain.2022.932476), inputs from PB and other structures are known to synapse in the RVM and relay sensory/nociceptive information to the RVM. Further, Francois, 2017 also conducted cTRIO on GABAergic RVM-SC neurons. Please rephrase this statement as it is more appropriate to state that "little is known", "a more detailed study is needed" or a similar phrasing. Further, in light of Francois, 2017 using the same method in the same structure on a similar subset of neurons, the authors need to reference that paper in lines 165-166 (https://doi.org/10.1016/j.neuron.2017.01.008) and comment on the overlap they see with that paper, as they should have overlapping populations.

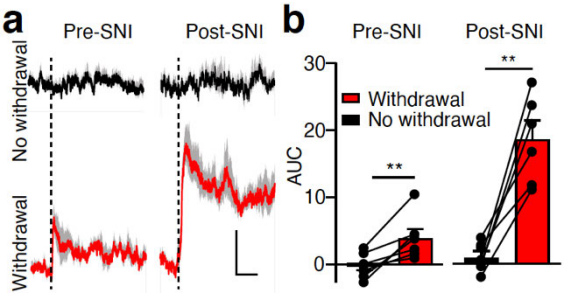
We thank the reviewer for bring these important review papers to our attention. Following the suggestion, we rephrase lines 20-23 to '*Although the spinal cord-projecting neurons in the rostral ventromedial medulla (RVM^{SC} neurons) are known can promote pain chronification, the pathway by which peripheral injury signals drive these neurons is poorly understood.*'. This change highlights the specific knowledge gap in functional connection that drives chronic pain. In line 23 and 43 of the revised manuscript, we also cited these two additional review papers you mentioned (Chen and Heinricher, 2022; De Preter and Heinricher, 2024). We also rephrase lines 43-44 to '*However, little is known how information about peripheral injury is transmitted to these descending RVM^{SC} neurons.*'.

We are very familiar with series works from Mary Heinricher's group on the PBN→RVM pathway and have discussed the discrepancy between our cTRIO tracing results with their works in lines 219-224 of the original manuscript and have cited the original paper from her group (Chen Q et al., 2017). However, the electrophysiological recording in this paper was targeted on the broader RVM neurons but not selectively on the descending RVM^{SC} neurons .

We have also cited Francois' 2017 paper in line 88 of the original manuscript, where we first revealed the OPRM1^{+} RVM^{SC} neurons is part of the GABAergic neurons in the RVM. Following the suggestion, we now cited Francois' paper again in line 218, and also revised the text to '*In contrast, cTRIO tracing from the entire population of GABAergic RVM^{SC} neurons revealed input from PBN. This discrepancy could reflect the tropism of the rabies virus, or because lPBN neurons prefer to innervate local ON-cells or OPRM1^{-} RVM^{SC} neurons but not to OPRM1^{+} RVM^{SC} neurons.*' in the revised manuscript.

2. Given ON-cells (and OFF-cells) are active before trail flick in many prior anesthetized and awake RVM studies (see Hellman, 2012 for awake RVM electrophysiology, please reference as it is not currently in the manuscript, https://doi.org/10.1523/JNEUROSCI.1551-12.2012), can the authors comment in the manuscript text and here about whether they observed a statistically significant difference in activation of Oprm1+ RVM-SC cells between withdrawal and non-withdrawal trials? For example, Hellman, 2012 observed that ON/OFF-cells were not active in trials that did not elicit a withdrawal. Further, the authors should discuss in the text further how their work compares to recent results in the field (for example, https://doi.org/10.1097/j.pain.0000000000003360) that have identified roles for RVM-MOR populations in nociception/pain.

Following the suggestion, we compared fiber photometry data in withdrawal and non-withdrawal trials from mice before and after SNI (Rebuttal Fig. 9). In both conditions, we found significant larger responses in the withdrawal trials compare to that of non-withdrawal trials, which is consistent with the observation by Hellman,



Rebuttal Fig.9 OPRM1⁺ RVM^{SC} neurons activity in withdrawal vs. non-withdrawal trials. a,b. Example traces (**a**) and histogram (**b**) showed significant larger response in withdrawal trials in both Pre- (left) and Post-SNI (right). Scale Bar: 1% ΔF/F, 3s. n = 6. Paired t test, ** *P* < 0.01.

2012. We added this data to Extended Data Fig. 4a,b and cited Hellman 2012 in line 120 of the revised manuscript.

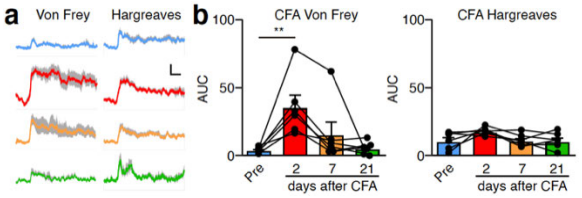
We appreciate the reviewer for bring the recent paper by Dogrul et al. to our attention which we have cited in line 142 and 149 of our original manuscript (Dogrul et al., 2025). We have also cited another classic paper by Porreca et al., 2001 in line 123 of our original manuscript (Porreca et al., 2001), which have shown that ablation of a broader population of MOR-expressing neurons in the RVM had no effect on paw withdrawal thresholds and latency in non-injured rat but prevented spinal nerve ligation-induced neuropathic pain.

Dogrul et al. injected CNO into the spinal cord to silence the hM4D-expressing terminals from the MOR-expressing neurons in the RVM, and also observed significant reduction of mechanical hypersensitization caused by partial sciatic nerve ligation. Their main conclusion 'Descending facilitation from rostral ventromedial medulla mu opioid receptor-expressing neurons is necessary for maintenance of sensory and affective dimensions of chronic neuropathic pain' is largely consistent with our data in Fig. 2f,g. We now cite this paper again in line 22 in the abstract of the revised manuscript.

However, our study moves beyond testing necessity to establish sufficiency: we show that repeated chemogenetic activation of OPRM1^{+} RVM^{SC} neurons alone can drive chronic mechanical sensitization in healthy mice. We have acknowledged their important contributions by citing both their papers multiple times. Collectively, their work and ours provide a solid foundation for the central aim of our manuscript: to map the circuit connecting the ascending pain pathway to these key descending modulatory neurons.

3. Have other studies identified superior colliculus (SuC)->RVM pathway? If so, the authors need reference in the text when cTRIO experiments are described. For example, SuC->RVM was mentioned in Hermann, 1997 (https://doi.org/10.1016/S0891-0618(97)00019-7).

We are sorry for missing this reference. We now cited this paper in line 219 of the revised manuscript.



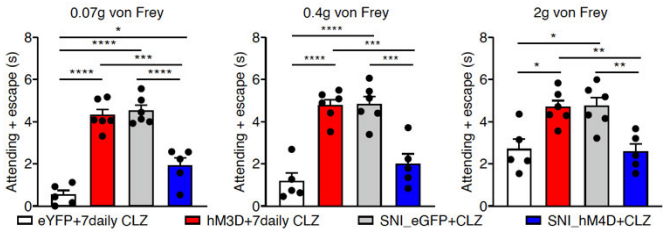
Rebuttal Fig.10 Fiber photometry recording of Oprm1⁺ RVM^{SC} neurons in CFA mice. a,b. Example traces (**a,** left Von Frey; right Hargreaves) and AUC histogram (**b**, left Von Frey; right Hargreaves) of calcium transient pre-CFA injection and 2,7,21 days after CFA injection. Scale Bar: 1% ΔF/F, 2s. n = 6. Friedman test, ** *P* < 0.01

4. In the case of injuries that have a defined recovery period (for example, incisional), does this circuit normalize its activity?

Thanks for the suggestion. Because CFA-induced mechanical pain sensitization can recover within three weeks and we have established the essential role of the OPRM1^{+} RVM^{SC} neurons in the initiation of CFA-induced inflammation pain (Fig. 2e), we thus performed fiber photometry to record mechanical responses in OPRM1^{+} RVM^{SC} neurons at different time points after CFA injection (Rebuttal Fig. 10). Indeed, mechanical responses in OPRM1^{+} RVM^{SC} neurons peaks at 2 days after CFA injection, then gradually recovered to normal over three weeks. We added this data to Extended Data Fig. 4d,e and added '*in a Complete Freund's Adjuvant (CFA)-induced inflammatory pain model, calcium responses peaked at day 2 before gradually returning to baseline over 21 days (Extended Data Fig. 4d, e).*' to line 116-118 in the revised manuscript.

5. Did the authors collect any manual or automated affective or escape behaviors for any of the CFA or SNI studies in this manuscript? While the manuscript focuses on affective and sensory brain circuits yet only nociceptive stimulus-induced post-injury behaviors are quantified. The manuscript's conclusions would be strengthened by showing whether manipulating nodes, especially in chronic pain states, in this pathway only modulate nociceptive behaviors (in contrast to ACC, BLA, etc.).

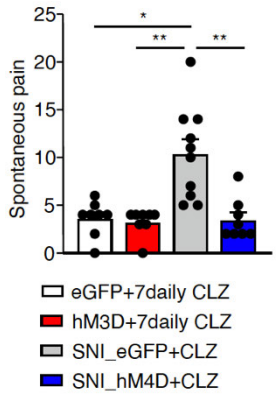
Thank you for the suggestion. We agree that assessing affective and escape behaviors can substantiate our conclusions regarding the role of this pathway in the chronic pain state. In direct response to this comment, we performed new experiments to quantify attending and escape behavior as readouts of the affective component of pain (Corder et al., 2019). We found that silencing OPRM1^{+} RVM^{SC} neurons in SNI mice significantly suppressed the attending and escape response (Rebuttal Fig. 11). These new data strengthen our manuscript by demonstrating that manipulating this specific node modulates not only sensory nociceptive behaviors but also the affective dimensions of chronic pain. We added this data to Extended Data Fig. 8a and discussed this result in line 160 of the revised manuscript.



Rebuttal Fig.11 Attending and escape behaviors after manipulation of the OPRM1^{+} RVM^{SC} neurons. Repetitive activation of OPRM1^{+} RVM^{SC} neurons in healthy mice increase stimuli-evoked attending and escape behaviors to the level similar to SNI mice, whereas silencing of these neurons significantly reduce these behaviors in SNI mice. Attending and escape behaviors were tested three time using different von Frey fibers. Groups: eGFP +7 daily CLZ, white, n = 5; hM3D +7 daily CLZ, red, n = 6; SNI_eGFP+CLZ: grey, n = 6; SNI_hM4D+CLZ, blue, n = 5. One way ANOVA test, * $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

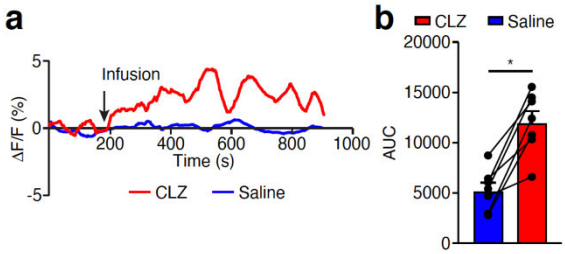
6. Does chemogenetic sensitization increases guarding/escape behaviors to innocuous stimuli, change paw pressure, or drive spontaneous pain behavior? Indicating ongoing pain or hypersensitivity. This is distinct from CPA and related assays in the manuscript, which test aversion and in certain cases may not be specific to mechanical sensitivity/pain.

As showed in above Rebuttal Fig. 11, repetitive chemogenetic activation of OPRM1^{+} RVM^{SC} neurons in healthy mice (eGFP vs. hM3D) significantly increased escape behaviors to innocuous stimuli. We also assessed spontaneous pain by measuring paw licking and flinching in the absence of von Frey stimulation. While we observed a significant increase in spontaneous pain in SNI mice—which was reversed to normal levels by silencing OPRM1^{+} RVM^{SC} neurons—repetitive activation of these same neurons in healthy mice did not alter spontaneous pain behaviors (Rebuttal Fig. 12). This dissociation suggests that chemogenetic sensitization of this central pathway is sufficient to drive hypersensitivity to evoked stimuli but is insufficient to generate spontaneous pain. We hypothesize that spontaneous pain likely requires episodic, injury-driven action potential firing in primary sensory (DRG) neurons, a condition not recapitulated by our central sensitization manipulation alone. We added this data to Extended Data Fig. 8c and discussed this result in lines 162-166 of the revised manuscript.



Rebuttal Fig.12 Spontaneous pain. Spontaneous paw licking and flinching in the absence of von Frey stimulation indicates spontaneous pain, which is significantly increase in the SNI group but not in other groups. Groups: eGFP +7 daily CLZ, white, n = 8; hM3D +7 daily CLZ, red, n = 8; SNI_eGFP+CLZ: grey, n = 10; SNI_hM4D+CLZ, blue, n = 8. One way ANOVA test, * $P < 0.05$, ** $P < 0.01$.

7. If, as the authors claim, this is a closed loop circuit then stimulating STT terminals in VPL + Po should cause transients in RVM-Oprm1+ cells and those same SST neuron cell bodies (excluding antidromic activity). Do the authors have evidence that this is the case or other direct experimental evidence of a true closed-loop circuit? If that is not the case, it seems appropriate for the authors to replace all "closed-loop circuit" with "spino-brain-spinal circuit" throughout the text.



Rebuttal Fig.13 Activation of STT to VPL pathway drives calcium increase in OPRM1^{+} RVM^{SC} neurons. Injection of AAV9-hM3D into the spinal cord, followed by VPL cannulation and fiber photometry recording of calcium response in OPRM1^{+} RVM^{SC} neurons. Example traces (**a**) and quantification (**b**) of calcium responses in OPRM1^{+} RVM^{SC} neurons after infusion of CLZ (red) or saline (blue) in the VPL. Wilcoxon matched-pairs signed-rank test * $P < 0.05$.

Thanks for raising this important question, which aligns with one of the major concern from reviewer#1. To further support our 'circuit loop' claim, we have added two lines of evidence. First, as the reviewer suggested, we performed chemogenetic activation of STT terminals in the VPL and indeed observed increased calcium signals in OPRM1^{+} RVM^{SC} neurons (Rebuttal Fig.13); Second, we ablated the OPRM1^{+} RVM^{SC} neuron, and found it can eliminate chronic mechanical sensitization induced by repetitive activation of upstream circuit

nodes (Rebuttal Fig.1). Together, these new results significantly strengthen our argument for a functional circuit loop. However, reviewer#1 is concerning the potential confusion caused by the term 'closed-loop'. We agree that it could be misleading and replaced it with 'long range circuit loop' for great clarity.

8. Is it possible to enhance Fig. 5j? This is a critical figure panel and is currently too small. Suggest moving Fig. 5c to extended data and giving more room for Fig. 5j and update the red/blue color scheme for excitatory inhibitory to the common -> and -| for each, respectively, along with thicker lines for visualization. The diagram also doesn't distinguish if it is RVM-SC or Oprm1+ RVM-SC neurons that are critical. Further, given the number of brain regions and experiments, it would help readers to include a panel-table (either in main or extended data figs) with columns for brain region, chemogenetic silence/activation, and direction of mechanical hypersensitivity change along with which experimental results were expected/unexpected given prior work.

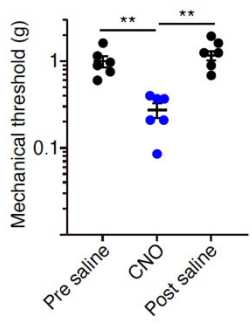
Thanks for the suggestion. Although Fig.5c is a negative result but this result is quite unexpected and promote us to manipulate both pathways in Fig.5d, we thus think it is important to include this data in the main figure. Following the suggestion, we enhanced Fig,5j to a separate Fig.6a and incorporated all the suggestions to the new figure. Moreover, we added another diagram to describe the separated ascending pathways for nociception (based on Chen et al., 2024) and touch/tactile (Fig. 6b), respectively.

9. Did the authors conduct anterograde tracing in Oprm1+ RVM-SC neurons to show which cells they synapse on in the spinal cord? Given the specificity and focus on mechanical hypersensitivity (for example, lines 148-149), additional discussion of this (beyond DCN) would help clarify potential cause for this. For example, building on lines 295-297.

We agree with the reviewer that figuring out the identity of the OPRM1^{+} RVM^{SC} neurons' postsynaptic partner will be very helpful to better understand how this pathway contribute to mechanical sensitization. To identify the postsynaptic targets of these neurons in the spinal cord, we attempted anterograde transsynaptic tracing by injecting H129ΔTK-TT virus (Lo and Anderson, 2011) or newly developed AAV-DIO-mWGA-mCherry (Tsai et al., 2022) into the RVM of the Oprm1-Cre mice. While these tools work effectively for brain circuitry, we were unable to detect any tdT expressing neurons in the spinal cord. Future studies will require new anterograde transsynaptic tools that works for the RVM-spinal cord pathway to fully resolve this synaptic connectivity.

10. The manuscript at times switches from anatomical work that specifies specific cell types and pathway to more general dissections of broader cell types in the pathway. For example, lSuc->RVM-Oprm1+ pathway is characterized in Fig. 3c-f but the silencing of inputs is switched to broader excitatory inputs (lines 180-186). Did the authors find that manipulating the complete lSuc->RVM-Oprm1+ pathway did not alter mechanical thresholds, or can they comment on why they assume the smaller inhibitory input might not have an outsized effect on the downstream RVM-Oprm1+ neuron response?

Our fiber photometry recordings demonstrated robust activation of OPRM1^{+} RVM^{SC} neurons in response to mechanical stimuli (Fig. 1h), which led us to focus our study on the excitatory inputs to these neurons. To clarify this rationale, we have added the following statement to lines 200-202 in the revised manuscript *'Since peripheral stimuli strongly activate OPRM1^{+} RVM^{SC} neurons (Fig.1h), we thus focused on identifying the excitatory monosynaptic inputs onto these neurons that drives chronic pain.'*. Using cTRIO tracing and RNAscope, we identified the lSuC as a major source of monosynaptic excitatory input onto OPRM1^{+} RVM^{SC} neurons (Fig. 3c–f). We thus performed chemogenetic silencing of lSuC excitatory terminals in the RVM, rather than the entire lSuC→RVM pathway, and found that this manipulation eliminated mechanical sensitization (Fig. 3g).



Rebuttal Fig.14 Chemogenetic silencing of the inhibitory lSuC→RVM pathway. Pre-saline, CNO, Post-saline are 24 hours apart. Saline infusion is used as control for infusion, n=6. One-Way ANOVA, ** $P < 0.01$.

In preliminary experiments, chemogenetic silencing of inhibitory lSuC terminals in the RVM induced a robust pain sensitization in uninjured healthy mice (Rebuttal Fig. 14). Given that manipulation of OPRM1^{+} RVM^{SC} neurons has minimal impact on nociception in healthy mice, we hypothesize that inhibitory lSuC inputs may modulate pain through other RVM neuronal populations. A full characterization of how the inhibitory lSuC→RVM pathway modulates pain will require further study and falls outside the scope of the present work.

11. The authors generate an Oprm1-Cre mouse and reference a prior paper that did so (Bailly, 2020). However, the authors fail to mention why they generated a new knock-in mouse line and if there are differences from Bailly, 2020 or other lines created. A supplemental note discussing this would help clarify the advantages of the new mouse line compared to prior ones: https://doi.org/10.1002/dvg.23341, https://doi.org/10.1016/j.celrep.2019.11.096, https://doi.org/10.1016/j.neuron.2021.11.029, https://doi.org/10.1038/s41467-023-41407-2, etc.

Sorry for the confusion. Our Oprm1Cre knock-in/knock-out mouse line was generated in 2014, prior to our awareness of other labs developing similar Oprm1-Cre lines. Our targeting strategy closely resembles that of the mouse line created by the Palmiter lab (Liu et al., 2021) and we now cite this paper instead in the revised manuscript. Both lines express Cre (our lab) or Cre:GFP fusion protein (Palmiter lab) under the control of endogenous Oprm1 promotor while disrupting the expression of endogenous Oprm1 gene. Heterozygous mice exhibit normal phenotype, whereas homozygotes lack functional μ-opioid receptor expression and do not respond to exogenous μ-opioid receptor ligands (Extended Data Fig.1). These mouse lines enable precise targeting Oprm1 expressing cells using heterozygous mice. Moreover, by injecting Cre-dependent AAVs expressing the μ-opioid receptor into specific brain regions of homozygous mice, we can investigate the role of individual brain areas in varies morphine-induced behaviors, such as analgesia, reward, and tolerance (ongoing study).

Here is the supplemental note for discussing different mouse and viral approaches to targeting OPRM1 expressing cells.

Strategies for Genetic Targeting of Oprm1-Expressing Cells
Several approaches have been developed to achieve precise genetic manipulation of μ-opioid receptor (MOR/Oprm1)-expressing cells.

1. The Chen lab's Oprm1Cre knock-in/knock-out line (current manuscript).
The Cre recombinase cDNA, followed by the rabbit β-globin poly-A signal, was introduced via homologous recombination immediately after the start codon in exon 1 of the mouse Oprm1 gene. This line allows Cre expression under control of endogenous Oprm1 promotor. Heterozygous mice exhibit normal phenotype, whereas homozygotes lack functional μ-opioid receptor expression and do not respond to endogenous or exogenous μ-opioid receptor ligands.

2. The Palmiter lab's Oprm1Cre knock-in/knock-out line (Liu et al., 2021)
The cDNA of Cre recombinase and GFP fusion protein, followed by the rabbit β-globin poly-A signal, was introduced via homologous recombination immediately after the start codon in exon 1 of the mouse Oprm1 gene. This line allows expression of Cre:GFP fusion protein under control of endogenous Oprm1 promotor. Heterozygous mice exhibit normal phenotype, whereas homozygotes lack functional μ-opioid receptor expression and do not respond to endogenous or exogenous μ-opioid receptor ligands.

3. The Meletis lab's Oprm1-2A-Cre mice (Martin et al., 2019)
A T2A-Cre cassette was inserted into the fifth exon of the Oprm1 gene and replacing a TAA stop codon by homologous recombination. This line allows expression of Cre recombinase under control of endogenous Oprm1 promotor. The endogenous μ-opioid receptor expression has not been disrupted.

4. The Kieffer lab's Oprm1-2A-Cre:eGFP mice (Bailly et al., 2020)
A T2A-Cre:eGFP fusion protein cassette was inserted into the fourth exon of the Oprm1 gene and replacing a TAA stop codon by homologous recombination. This line allows expression of Cre:eGFP fusion protein under control of endogenous Oprm1 promotor. The endogenous μ-opioid receptor expression has not been disrupted.

5. The Watanabe lab's Oprm1-2A-CreER mice (Okunomiya et al., 2020)

A T2A-CreER cassette was inserted into the fourth exon of the Oprm1 gene and replacing a TAA stop codon by homologous recombination. This line allows expression of a tamoxifen-inducible Cre recombinase under control of endogenous Oprm1 promotor. The endogenous μ-opioid receptor expression has not been disrupted.

6. The Corder lab's Oprm1 targeting virus (Salimando et al., 2023)

The researcher developed a new promotor system for viral-assisted, species-specific, selective targeting of $OPRM1^+$ cells. They cloned genomic sequence spanning -1797 to -265bp upstream of the ATG site of the mouse *Oprm1* gene into plasmids encoding various reporter, then packaged these constructs into AAV1 vectors (AAV1-mMORp1-reporter). The resulting mMORp construct exhibit robust and specific expression in endogenous MOR+ neurons across the mouse central and peripheral nervous systems. This specificity was confirmed through co-localization with MOR protein, *Oprm1* mRNA, and Cre-recombinase in transgenic lines, with minimal off-target glial expression. Remarkably, the mMORp system also functioned effectively in rats, shrews, and human iPSC-derived nociceptors, but not in non-neuronal cells (like cardiomyocytes) with low receptor expression. Similarly, the human construct, hMORp1 (-1841 to -257bp upstream of the human *OPRM1*ATG site), enabled efficient and selective transduction of $OPRM1^+$ neurons in rhesus macaques. This versatile toolkit facilitates precise targeting and manipulation of opioidergic cells across multiple species. It supports diverse transgenes (reporters, sensors, actuators) and is compatible with various AAV serotypes optimized for different delivery routes and tissues.

12. Authors state on lines 217-218: "The VPL and Po show similar functional connectivity to the retrogradely labeled lSuC projecting neurons in the layer 5 of SS (Fig. 5b)." Fig. 5b show electrophysiological characterization of those pathways and yet no electrophysiological is done to show similar SS->lSuc connectivity in the manuscript. This should either be done or this sentence rephrased. If these experiments exist, they should be added to the manuscript and the figures referenced in both sections.

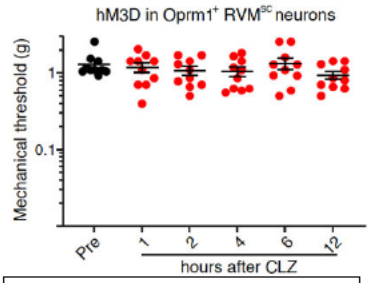
We apologize for the confusion and rephrase the sentence to *'Electrophysiological recording of retrogradely labeled lSuC projecting neurons in the layer 5 of SS (SS_{L5}) reveal robust functional connectivity in both VPL→SS_{L5} and Po→SS_{L5} pathways (Fig. 5b).'* in lines 281-283 of the revised manuscript.

We injected AAV8-retro-mCherry in the lSuC and AAV8-ChR2 into Po or VPL. We then performed recordings from retrogradely labeled lSuC-projecting neurons in layer 5 of the SS. We observed comparable connectivity rates (i.e., the proportion of neurons with light-evoked EPSCs) when stimulating Po or VPL terminals. Our conclusion regarding similar functional connectivity between 'Po to SS_{L5}' and 'VPL to SS_{L5}' is based on this observation (Fig. 5b). Our analysis focused solely on functional connectivity (presence/absence of light-evoked EPSCs), not other electrophysiological properties (e.g., amplitude, kinetics).

We highlighted this L5 connectivity in the main figure because it contrasts with the classical view that thalamocortical inputs primarily target L4. The thalamic innervation of L5 has been reported (Constantinople and Bruno, 2013) but understudied, thus merits further investigation.

13. The repetitive activation of RVM-Oprm1+, lSuC, SS, and SC->(Po or VPL) show mechanical hypersensitivity. However, in all cases it takes several days for the effect to become persistent. Did the authors conduct single activation experiments (only one CLZ dose given) to show that is not sufficient to drive long-term sensitization? For example, follow mice in Fig. 2b. Does the sensitization stay locked at the mechanical sensitivity that mice are at when CLZ infusions are ceased or recover? For example, we know from Cai, 2014 (not reference, but would prefer if it was, https://doi.org/10.1186/1744-8069-10-70) that single optogenetic activation of RVM-TPH2+ cells can drive prolonged hypersensitivity. Discussion of this point in the manuscript would help with interpreting these experiments.

In Fig.2b, the mechanical thresholds were measured only at 30 mins after a single CLZ injection and we did not assess later time points in those experiments.



Rebuttal Fig.15 Single injection of CLZ in mice expressing hM3D in the Oprm1^{+} RVM^{SC} neurons did not show mechanical sensitization measured 1,2,4,6,and 12 hours after CLZ injection. n=10.

To address your question directly, we performed new experiments in which we monitored mechanical thresholds continuously for 12 hours after a single CLZ injection in mice expressing hM3D in the Oprm1^{+} RVM^{SC} neurons. Consistent with our original conclusions, we observed no persistent hypersensitization following a single activation (Rebuttal Fig. 15). This data is now added to Extended Data Fig. 6b in the revised manuscript.

This contrasts with the effects of activating descending serotonergic RVM neurons, where even a single stimulation can induce prolonged hypersensitivity (Cai et al., 2014). We have now cited this work and added the following clarification to the revised manuscript (lines 135-136): *This is different from the activation of descending serotoninergic RVM neurons, where single stimulation suffices to elicit prolonged hypersensitivity (Cai et al, 2014).*

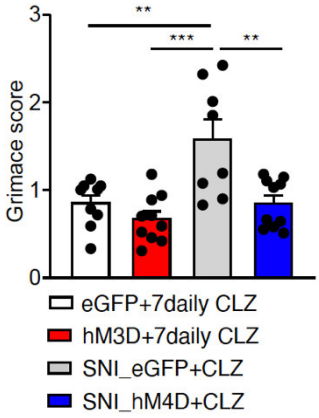
14. Do the authors expect to see prolonged state changes in Oprm1+ RVM-SC activity on chemogenetic activation of STT->(VPL + Po) pathway? Given there is no longer a prolonged peripheral input to drive Oprm1+ RVM-SC activity changes as is the case with SNI. Discussion of prior work looking at (noci)plastic changes that persist centrally in the absence of peripheral input should be briefly mentioned.

[redacted]

As shown in our response to your point #7, chemogenetic activation of the STT terminals in the VPL can evoke a prolonged calcium elevation in Oprm1^{+} RVM^{SC} neurons (Rebuttal Fig.13).

We thank the reviewer for raising the insightful point regarding nociplastic pain, which occurs in the absence of identifiable injury or nerve damage. Two recent studies on nociplastic pain have demonstrated that repetitive activation of excitatory neurons (Sun *et al.,* 2020) or *Calca*-expressing neurons (Condon *et al.,* 2024) in the parabrachial nucleus (PBN) can induce long lasting-mechanical sensitization. This mirrors the persistent effects from repetitive activation of each node in the long-range circuit loop reported in our manuscript. [redacted]

15. Do mice show any changes in grimace during any of the chemogenetic activation experiments? If the videos are available, this should be quantified and included as it would indicate if activating this pathway is causing a prolonged pain-related percept leading to mechanical hypersensitivity.



Rebuttal Fig. 17 Facial grimace score. Histogram shows facial grimace score in control mice or mice with manipulation of Oprm1^{+} RVM^{SC} neurons. Groups: eGFP +7 daily CLZ, white, n = 9; hM3D +7 daily CLZ, red, n = 10; SNI_eGFP+CLZ: grey, n = 8; SNI_hM4D+CLZ, blue, n = 10. One way ANOVA test, ** P < 0.01, *** P < 0.001.

Thanks for the suggestion. Our original video recording did not have clear focus on mice's face, therefore was not able to perform grimace analysis. To address this question, we recorded video from a new sets of mice and analyzed change of facial grimace online at http://painface.net (Langford et al., 2010; McCoy et al., 2024). We found that SNI has heightened grimace score indicating the ongoing pain, whereas silencing of the Oprm1^{+} RVM^{SC} neurons can restore normal grimace score in SNI mice (Rebuttal Fig. 17). Repetitive activation of the Oprm1^{+} RVM^{SC} neurons had no effect on the grimace score. This grimace score results is similar to the results from measurement of spontaneous pain (Rebuttal Fig. 12), suggesting that peripheral damage is required for promoting the ongoing pain, which is not recapitulated in our model. We included this results in Extended Data Fig. 8b and discussed in lines 162-166 of the revised manuscript.

16. Are spontaneous pain behaviors reduced during chemogenetic inhibition of Oprm1+ RVM-SC neurons? In line with Fig. 1h fiber photometry results showing spontaneous pain behaviors in SNI mice.

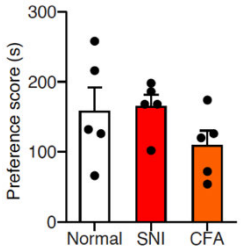
Thanks for the suggestion. We analyzed spontaneous pain behaviors in SNI mice before and after chemogenetic silencing of Oprm1^{+} RVM^{SC} neurons. We found significant reduction of licking and flinching when these neurons been silenced, suggesting that ongoing activity of these neurons is required to support the chronic pain state after SNI (Rebuttal Fig.12). We added this new result to Extended Data Fig. 8c and discussed in lines 162-166 of the revised manuscript.

17. Given CLZ's pharmacokinetics and the dose used (0.1 mg/kg), these pathways will only be maximally active for 1-2 hours and overall active for several hours. Can the authors comment on whether this is in line with any studies showing repeated mechanical stimulation for similar durations leads to prolonged sensitization?

This is an interesting point. We are not aware of any studies that have administered repeated mechanical stimulation over a period of several hours. Such a prolonged protocol might induce acute stress in the mice, leading to stress-induced analgesia. Alternatively, it might cause local tissue injury and swelling, which could itself lead to pain sensitization. Both of these potential outcomes could confound the measurement of pain thresholds.

18. The floor texture preference studies are intriguing but do not appear complete, as the animal is asked to make two decisions during the texture change: preference for a novel texture and for the specific rougher texture. The authors do not have an additional control (for example, a texture smoother than the Baseline textures) that would help differentiate this. Please elaborate on why this was not done or perform these experiments to demonstrate that any novel texture would not be affected by these manipulations. This would mean that lines 234-235 ("suggesting that mice innately prefer the rough surface over the smooth surface") needs to be updated, as this is not demonstrated by the authors' experiments.

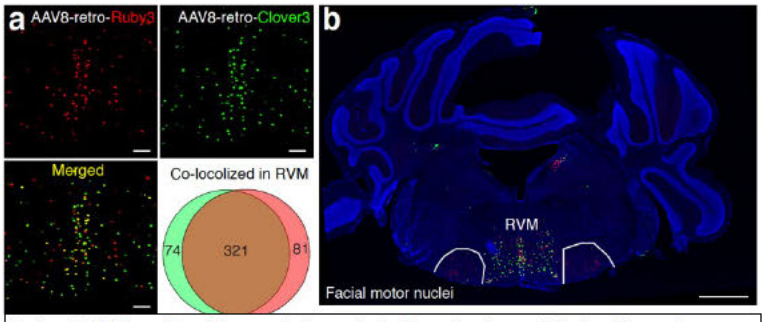
We used plexiglass floor in baseline test then switch one side of the chamber to fine grit sand paper floor. We will not able to find a smoother texture than the baseline condition. We removed the sentence 'suggesting that mice innately prefer the rough surface over the smooth surface' in the revised manuscript, as we agree with the reviewer that mice's preference toward the textured floor could be the result of novelty. However, the mice still need detect the texture difference in this behavioral test. We really appreciate the reviewer for pointing this out.



Rebuttal Fig. 18. Mice with nerve injury (SNI, red) or inflammation (CFA, orange) have similar preference score as non-injured control (Normal, white) toward the chamber with rough texture. n=5. One-way ANOVA, P > 0.05

19. Do the CFA or SNI mice have an altered preference for the rough texture in preference assay?

Both CFA and SNI mice showed similar preference for the rough texture as control mice (Rebuttal Fig. 18). The procedure of floor texture preference test is described in details in lines 572-580 of the revised manuscript.



Rebuttal Fig. 19. Neonatal vs. Adult spinal cord injection of retrograde AAV. (**a**) Representative RVM section following neonatal spinal cord injection of AAV8^{retro} H2B Ruby3 and adult spinal cord injection of AAV8^{retro} H2B Clover 3, and Venn diagram illustrated the number of Ruby3-labeled, Clover3-labeled, and colocalized neurons in the RVM. Scale Bar: 100μm. (**b**) Representative section following neonatal spinal cord injection of AAV8^{retro}-H2B Ruby3 and adult spinal cord injection of AAV8^{retro}-H2B Clover3 showing RVM and facial motor nucleus (FMN). Scale Bar: 1mm.

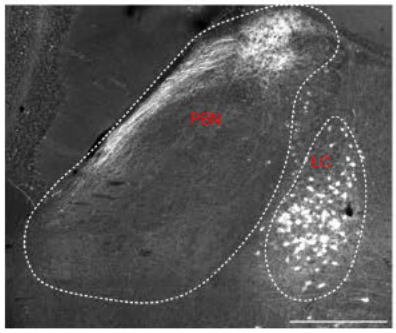
20. Either in the main text or a supplemental note, can the authors please elaborate on where prior views on STT (line 230-231, "traditional view emphasize STT's role in somatosensory discrimination") are wrong or where their results disagree with prior studies?

We discussed our views on STT's function in lines 270-276 and 344-355 of the revised manuscript.

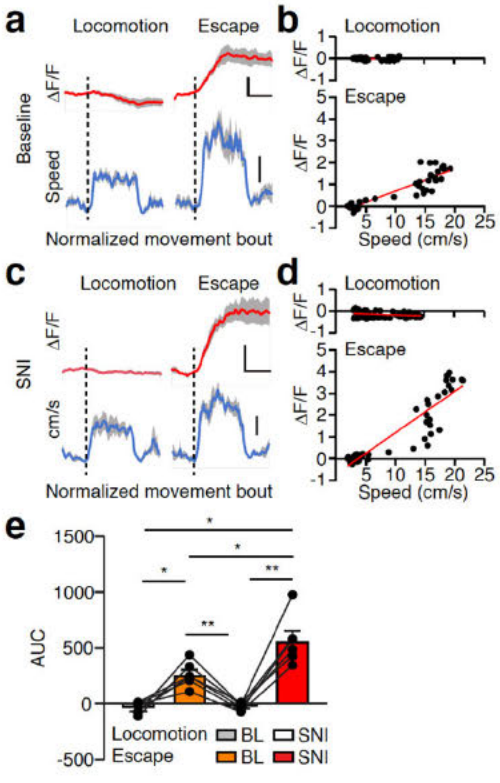
21. Does identifying RVM-SC neurons via neonatal injections lead to missing certain RVM-SC neurons that would be identified in the adult or label RVM neurons that are not RVM-SC in the adult (for example, post-natal pruning)? If so, please provide quantification of an experiment with later intraspinal injections of AAV8retro-GFP combined with fluorogold, CTB, or similar retrograde tracer.

Thanks for the suggestion. We injected same amount of AAV8retro-Ruby3 and AAV8retro-Clover3 in the spinal cord of the same mice at P1 and P28, respectively. We found ~70% overlap between two retrogradely labeled neurons on the RVM(Rebuttal Fig. 19a). However, the nearby facial motor nucleus was only labeled by neonatal injection suggesting the post-natal pruning in this nucleus that need further experiment to confirm (Rebuttal Fig. 19b). We added this data to Extended Data Fig. 2b,c. and commented this feature in lines 443-4.

22. Do the authors observe any anterograde transport with their new AAV8retro? Please indicate this in the text or provide experimental evidence this is not the case.



We did not observe any anterograde transsynaptic transport with AAV8retro. As showed on the right, following injection of AAV8retro-mCherry into the spinal cord, we observed robust retrograde labeling of locus coeruleus (LC) neuron cell bodies, consistent with known descending projections (Arvidsson *et al.*, 1995). However, in the parabrachial nucleus (PBN)—a region that receives anterograde projections from the spinal cord—we detected only mCherry-labeled axon terminals. The absence of labeled cell bodies in the PBN indicates that AAV8retro-mCherry is not transported anterogradely across synapses. We added this data to Extended Data Fig.2i and commented this feature in lines 78-79.



23. Is the activity of the Oprm1+ RVM-SC neurons recorded in Fig. 1g-j correlated with "pain"-related movement or just to any general locomotion or startle-like movement? If the authors recorded synchronized videos, they should plot the dF/F activity as a function of locomotor speed before and after SNI in the setting of stimuli being delivered and during non-stimulation periods (when the animal is and is not locomoting).

Rebuttal Fig. 20. Locomotion or pain-evoked escape. a, Representative traces of calcium response (upper panel) and movement speed (lower panel) in uninjured healthy mice. Dash line indicates the time of movement start. **b,** correlation of calcium response vs. movement speed in uninjured healthy mice. Dot indicates the average speed calcium signal during each movement event. Red line: linear fitting of dots. R^2= 0.021 or 0.842 for locomotion and escape, respectively. Scale bar: 50 % of normalized movement bout, 1 % ΔF/F, 5cm/s. **c,d.** Representative traces of calcium response (c upper panel) and movement speed (c, lower panel), and correlation of calcium response vs. movement speed in SNI mice. Dash line indicates the time of movement start. Dot indicates the average speed calcium signal during each movement event. Red line: linear fitting of dots. R^2= 0.087 or 0.845 for locomotion and escape, respective. Scale bar: 50 % of normalized movement bout, 2 % ΔF/F, 5cm/s. **e,** Quantification of AUC. n=6, One-way ANOVA, * P <0.05, ** P <0.01.

Thanks for the suggestion. We recorded synchronized videos and fiber photometry traces, then plot the ΔF/F vs. locomotor speed before and after SNI as suggested. These results showed that general locomotion without von Frey stimulus before or after SNI would not activate the OPRM1^{+} RVM^{SC} neurons (Rebuttal Fig. 20, locomotion). However, activity of these neurons correlate with the speed of von Frey stimuli causes 'pain'-related escape response (Rebuttal Fig. 20, escape). We added this data to Fig. 1j, Extended Data Fig. 4f,g and discussed in lines 118-122 of the revised manuscript.

24. In Fig. 5j, RVM-SC neurons are shown to synapses on superficial inhibitory dorsal horn neurons that then synapse on excitatory deep dorsal horn projection neurons. Where is the evidence of this in the manuscript? The model should clearly delineate what was experimentally shown in the manuscript vs. speculation or results from other studies.

We are sorry for the mistake of not clearly delineating the actual results vs. speculation. We used dash line for the speculated working model, whereas using solid lines for the actual data in Fig.6a of the revised manuscript. We discussed our speculated model in line 357-367 in the revised manuscript.

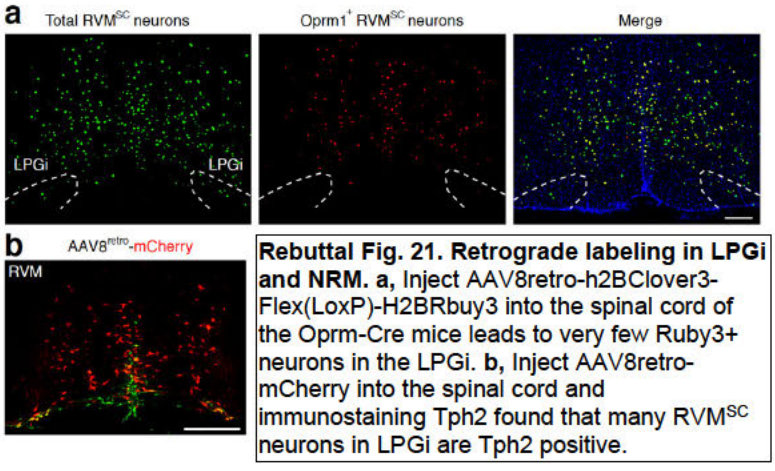
25. Does manipulating this circuit affect general aversion or anxiety? For example, is behavior in the elevated plus maze affected by VPL+Po manipulation? Discussion of this would help indicate the specificity of manipulating this pathway.

Thank you for this important question, which is also raised by reviewer #3's point 6. In Rebuttal Fig. 6d, we showed heightened anxious states, characterized by reduced center time in the open field test and less time in the open arm of the elevated plus maze, six weeks after repetitively activating the VPL+Po thalamus that received input from the spinal cord. These results closely mirror those obtained from direct activation of OPRM1^{+} RVM^{SC} neurons, strongly suggesting that both manipulations engage the same spino-brain-spinal circuit to promote affective pain-related behaviors. These results were added to Extended Data Fig, 10c,d and were discussed in line 291-293 of the revised manuscript.

26. Given Fig. 3f shows that PAG->(Oprm1+ RVM-SC) is mostly inhibitory, why did the authors choose to silence excitatory PAG->RVM (not Oprm1+ RVM-SC) cells (Fig. 3a-b)? If the authors had instead silenced the inhibitory PAG->RVM or PAG->(Oprm1+ RVM-SC) pathway, then this would lead to disinhibition of the inhibitory Oprm1+ RVM-SC, which might drive analgesia (depending on animal state) and be more consistent with prior results as mentioned on line 161. Please state in the text (for example, line 156-158) why excitatory but not inhibitory PAG->RVM cells were silenced.

We are sorry for the confusion. We explained our rational of focusing on excitatory input in lines 200-202 as follows: '*Since mechanical stimulus strongly activate Oprm1^{+} RVM^{SC} neurons (Fig. 1h), we thus focusing on mapping the excitatory monosynaptic inputs onto these neurons that drives chronic pain.*' Please also see our discussion on the inhibitory PAG→RVM pathway in lines 335-338 of the revised manuscript.

27. Did the authors distinguish between LGPi and NRM? For example, recent work (https://doi.org/10.7554/eLife.78689) indicates that they can have differential effects on behavior. The focus appears to be on the NRM from images, but it is not clear if the LGPi is also being manipulated.

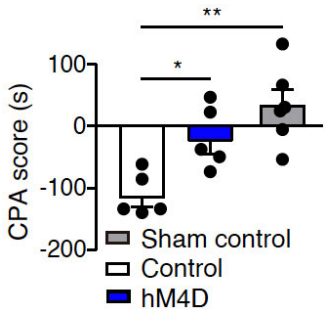


Rebuttal Fig. 21. Retrograde labeling in LPGi and NRM. a, Inject AAV8retro-h2BClover3-Flex(LoxP)-H2BRbuy3 into the spinal cord of the Oprm-Cre mice leads to very few Ruby3+ neurons in the LPGi. **b,** Inject AAV8retro-mCherry into the spinal cord and immunostaining Tph2 found that many RVM^{SC} neurons in LPGi are Tph2 positive.

Thanks for the question. We have not distinguished between LPGi and NRM in our study. A detailed examination of our retrograde tracing results (Fig.1 b-d) revealed that very few Oprm1^{+} RVM^{SC} neurons were located within the LGPi (Rebuttal Fig. 21a). Conversely, we found many RVM^{SC} neurons in LPGi are Tph2 positive (Rebuttal Fig. 21b, from Extended Data Fig.3e of the revised manuscript). Since our chemogenetic manipulations specifically targeted OPRM1-expressing neurons, this indicates that our experimental effect was predominantly driven by

the modulation of Oprm1^{+} neurons in the NRM, rather than those in the LPGi. We added Rebuttal Fig. 21a to Extended Data Fig. 2j and highlighted in line 83-84 of the revised manuscript.

28. Why did the authors not conduct a sham experiment for CPA? Pairing the side with just the experimenter present and approaching the animal with their hands can be sufficient to induce neural responses and conditioning independent of mechanical drive.



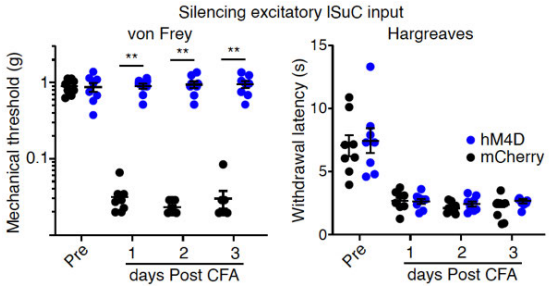
Rebuttal Fig. 22. Sham control for CPA. Approaching the SNI mice with hands holding a Von Frey fiber but not actually touching paw did not cause CPA (gray, n=6). Stimulation of the SNI mice with Von Frey fiber causes robust aversion (White, n=5) but not after chemogenetic silencing of the OPRM1^{+} RVM^{SC} neurons (blue, n=5). One-way ANOVA, *P <0.05, ** P< 0.01.

We performed a sham experiment for CPA by experimenter present and approaching the SNI mice with hands holding a von Frey fiber but not actually touching paw. Sham stimulation did not induce CPA (Rebuttal Fig. 22).

29. Regarding blinding, this was not done as stated on line 582. Was this lack of blinding only to the injury the animal received, or did this extend to what compounds were being given (for example, CLZ, saline, etc.) or circuit pathway being test? Please state this explicitly in the text and explain why this was not done 3(the lack of blinding to injury is understandable).

As expected by the reviewer, it was very easy to tell whether the animal is injured or not. Moreover, it was also easy to tell which circuit was been tested based on cannular location on mice's head. We did blinding for what compounds were being tested as much as we could. However, significant amount of experiments was done during COVID pandemic, when we sometime can have only one person per lab conducting the experiments, then blinding became very difficult.

30. Regarding Fig. 3g and 3i, does silencing lSuC(excitatory)->RVM affect CFA related behaviors or reproduce the formalin-like behaviors?



Rebuttal Fig. 23. Chemogenetic silencing of the excitatory lSuC→RVM pathway reduces inflammatory pain. Left, CLZ infusion reverse CFA injection caused mechanical sensitization in hM4D- (blue, n=8) but not mCherry- (black, n=8) expressing mice. Right, CLZ infusion has no effect on CFA injection induced thermal sensitization in both hM4D- (blue, n=8) and mCherry- (black, n=8) expressing mice. Mann-Whitney test, ** P < 0.01.

Following the suggestion, we examined the impact of silencing lSuC→RVM pathway on CFA related behavior. We injected AAV8-DIO-hM4D or AAV8-DIO-mCherry into the lSuC of the vGlut2-cre mice and cannulate onto the RVM. We found that local infusion of CLZ caused significant suppression of CFA-induced mechanical but not thermal sensitization in hM4D- but not in mCherry-expressing mice (Rebuttal Fig. 23). These results are similar to the effect of silencing of the OPRM1^{+} RVM^{SC} neurons. We added these new results to Extended Data Fig. 6h,i and discussed it in line 235-236 of the revised manuscript.

31. Can the authors speculate on why there is no partial restoration of sensory threshold when silencing only SC->VPL or SC->Po pathways (Fig. 5c)? Is the effect in Fig. 5d synergistic? For example, if you halve the dose of CLZ, is there any effect on mechanical thresholds?

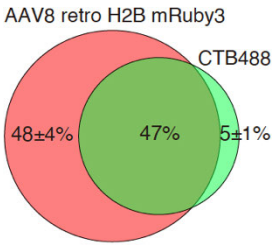
Thanks for the interesting question. As our answer to reviewer #3's minor point #8, we speculated that SNI causes sensitization in both the SC→VPL and SC→Po pathways. These two pathways act in a redundant or parallel manner. Therefore, silencing only one pathway is ineffective because the other remains active and continues to drive the pain state. Future electrophysiological studies are needed to validating this speculation. We discussed our hypothesis in line 351-355 in the revised manuscript.

32. As the frontal cortex and other structures that project to the RVM (directly or indirectly) have been shown to be involved, can the authors comment on whether they would expect the sensitization to disappear upon removal of those structures? For example, if this circuit maintains the sensitized state independent of those other pathways, that would greatly add to the importance of the identified pathway.

Our results in Extended Data Fig. 6d,e show that silencing of OPRM1^{+} RVM^{SC} neurons significantly reduces pain induced c-Fos expression in higher-order brain regions, including the anterior cingulate cortex (ACC). This suggests that the circuit loop we identified exerts a powerful influence on broad pain matrix. However, our transsynaptic cTRIO tracing indicate that very few cortical neurons provide direct descending innervation onto the OPRM1^{+} RVM^{SC} neurons (Fig. 3e). How ACC interact with other nodes in the circuit loop we reported is another very important question for future research.

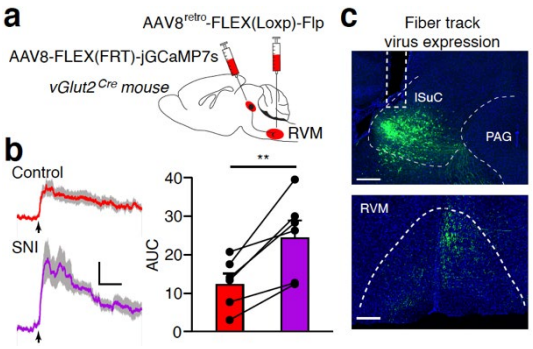
Experiments and data analysis:

33. What is the overlap between fluorogold or other chemical retrograde tracers and AAV8retro? That would appear to be a more comprehensive comparison than to AAV2retro. This should be shown as a Venn diagram overlap.



As suggested, we co-injected mixture of AAV8retro-H2B-mRuby3 (red circle) with CTB-488 (green circle) in wild-type adult mice (n=3). We found that mCherry expressing cells includes most of CTB-traced cells in the RVM and AAV8retro labels near twice as many cells as CTB (Rebuttal Fig. 24). We described this result in Extended Data Fig. 2h and added *'AAV8^{retro} also labeled twice as many neurons as co-injected chemical tracer CTB (Extended Data Fig. 2h)'* line 78 of the revised manuscript.

34. Neural activity is only recorded in the RVM using fiber photometry. Recording from at least one or two other brain areas in the pathway characterized would help strengthen the case that the inferred changes from the activation and silencing experiments match the changes that the circuit actually undergoes after injury. For example, if record from lSuC->RVM neurons before and after SNI, are changes observed?



As suggested, we recorded activity of the lSuC^{RVM} neurons and found significant increase of paw withdrawal related activity in these neurons after SNI (Rebuttal Fig. 25). This new result was included in Extended Data Fig. 4j-k and discussed in lines 230-231 of the revised manuscript.

Rebuttal Fig. 25. Fiber photometry recording of the excitatory lSuC^{RVM} neurons. a, Experimental diagram. **b**, Example traces (left) and histogram (right) of paw withdrawal related activity before (red) and after (purple) SNI. n=6. Scale Bar: 1% ΔF/F, 2s. Paired t test, ** $P < 0.01$. **c,** Example images of fiber track and virus expression in the lSuC (upper panel) and their terminals in the RVM (lower panel). Scale Bar: 500μm for lSuC, 200μm for RVM.

35. Additional characterization of the new Oprm1-Cre mouse is needed. This includes showing expected expression in other areas of the CNS/PNS (for example, striasomes, sensory neurons, etc.) when crossed with a reporter line and any unexpected expression patterns.

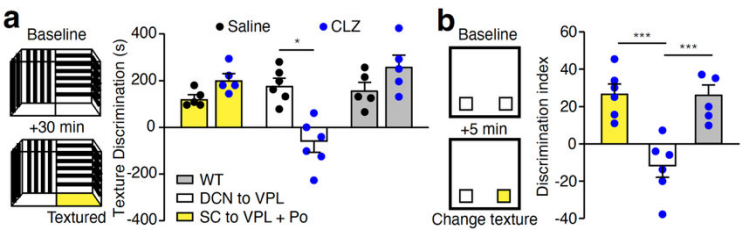
Following the suggestion, we have documented brain images of Oprm1-Cre X Ai14 mice and uploaded these images to Zenodo:
https://zenodo.org/uploads/17110834?token=eyJhbGciOiJIUzUxMiJ9.eyJpZCI6IjVjNGU4ZWQ1LTViNjAtNDliNC1hN2Y1LTJjZjg0YjMyOGFmNyIsImRhdGEiOnt9LCJyYW5kb20iOiI2MWJmY2U4YTA3OWQ3OTgzNWU4ZDE1YTAwMjE0MDZlOCJ9.X_wmoi8TXd-kNrBi5RdofGVZeTF66ofo7S6Gfjh575x_xb77-97Xn0tV-avdF2BYhVrM8yxArErC63yVZR0xIA

We did not see any unexpected expression patterns when compare to similar mouse lines generated by other labs.

36. There is no validation that the chemogenetic silencing is producing the downstream effects claimed. The manuscript would be strengthened if the authors performed fiber photometry in downstream regions while conducting chemogenetic manipulation. For example, record from SS while silencing SST->Po and/or SST->VPL to show change in SS activity is specific for silencing both or recording from lSuC->RVM to show that activating it drives Oprm1+ RVM-SC activity.

Thanks for this important question. As we showed in our answer to Reviewer#3's Point 9, we indeed have validated our chemogenetic silencing method using slice recording (Rebuttal Fig. 8). We now included this result in Extended data Fig. 5d-f and discussed it in lines 206-207 of the revised manuscript.

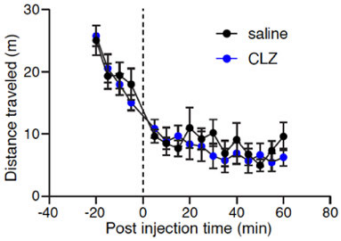
37. Fig. 5i is missing a control for no silencing of either pathway, for example, it is not clear if the discrimination index with SC->(VPL+Po) silencing is lower than no brain silencing, even if it is higher than DCN->VPL. Lines 237-238 ("also had no effect on mice's preference for the sandpaper floor or to objects with novel textures") needs to be updated to indicate this was not directly shown or the experiment performed to indicate this.



Rebuttal Fig. 26. a. Diagram (left) and quantification (right) of silencing the SC→VPL + SC→Po pathways together (yellow, n = 5) or the DCN-VPL pathway (white, n = 6) or wildtype mice without manipulation (WT, gray, n = 5) on discrimination score. Black, saline; blue, CLZ. **b.** Diagram (left) and quantification (right) of silencing the SC→VPL + SC→Po pathways together (yellow, n = 6) or the DCN→VPL pathway (white, n = 6) or wildtype mice without manipulation (WT, gray, n = 5) on novel object with novel texture. Wilcoxon matched-pairs signed rank test or one-way ANOVA, *** $P < 0.001$.

Following the suggestion, we now added a wild type (WT) control without silencing of either pathway for both Fig. 5h and 5i. Silencing SC to (VPL + Po) gives similar discrimination index compare to no-silencing WT group (Rebuttal Fig. 26). This new control data has been added to Fig. 5h,i (gray panel) in the revised manuscript. These results supported our clam in line 237-238, now lines 301-304 in the revised manuscript.

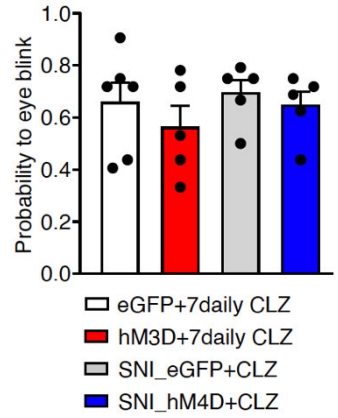
38. Given CLZ can affect behavior, to assure readers about locomotion dynamics, the authors should show a temporal trace of the behavior (if the data is available) from the time of CLZ injection during chemogenetic activation experiments and at intervals to at least 1-2 hrs post-injection.



Rebuttal Fig. 27. Locomotion dynamics after injection of CLZ or saline. Dash line indicate time of injection. n = 6. Two-way ANOVA, $P > 0.05$.

Following the suggestion, we recorded locomotion dynamics for one hour after CLZ or saline injection from the same group of mice. Our results show no difference in locomotion dynamics between these two conditions (Rebuttal Fig. 27). This data is included in Extended Data Fig.5c of the revised manuscript.

39. The authors need to show with a startle-based assay or by giving other sensory stimuli (for example, air puff, etc.) if the mouse is specifically sensitized to hind or forepaws stimulation or generally sensitized to all sensory stimuli. This will better show the specificity of activating this circuit to mechanical/pain-related hypersensitivity and build on Extended Data Fig. 4.

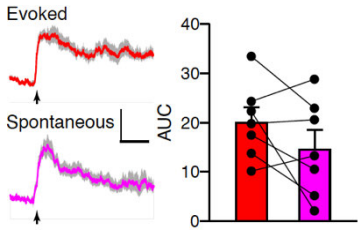


Rebuttal Fig. 28. Eye blink probability to air puff. Eye blink responses to 20PSI air puff directed at the cornea are no difference between each group. eGFP+CLZ control: white n = 6; hM3D+CLZ: red, n = 5;SNI_eGFP+CLZ, gray, n = 5; SNI_hM4D+CLZ: blue, n = 5.

Our lab does not have the equipment for a startle-based assay. Instead, we employed an air-puff eye-blink procedure, recently developed by our colleagues (Kauvar et al., 2025), to test for general sensory sensitization. We found that mice with SNI or repetitive activation of OPRM1^{+} RVM^{SC} neurons showed a blink probability similar to that of control and SNI+hM4D-silenced mice when exposed to a 20 psi air puff directed at the cornea (Rebuttal Fig. 28). This result demonstrates that activation of OPRM1^{+} RVM^{SC} neurons induces chronic mechanical pain without producing a generalized state of hyperarousal or non-specific sensory hypersensitivity. This result is shown in Extended Fig. 8d and discussed in lines 182-183 in the revised manuscript.

40. For Oprm1+ RVM-SC fiber photometry: make a plot similar to Fig 1h-j but separating out summarized plot by those that evoked a withdrawal and those that didn't along with spontaneous vs. non-spontaneous.

Following the suggestion, we plot average calcium transients associated with evoked paw withdrawal and spontaneous paw withdrawal, and found no difference between these two types of event (Rebuttal Fig. 29). This result is showed in Extended Fig. 4h in the revised manuscript. Please also see Rebuttal Fig.9 for activity of OPRM1^{+} RVM^{SC} neurons in withdrawal vs. non-withdrawal trials.



Rebuttal Fig. 29. Evoked vs. Spontaneous. Example traces (left) and histogram (right) of calcium transients associated with evoked- (red) and spontaneous- (magenta) paw withdrawal. Scale Bar: 1.5% ΔF/F, 3s. n = 7. Arrow head indicate time of paw withdrawal. Paired t test, $P > 0.05$.

Requests:
41. "Data and materials availability": Please deposit images from the new Oprm1-Cre mouse crossed with a reporter line on a Zenodo or other public repository.

We have deposited representative images of the Oprm1-cre X Ai14 mice on Zenodo:

https://zenodo.org/uploads/17110834?token=eyJhbGciOiJIUzUxMiJ9.eyJpZCI6IjVjNGU4ZWQ1LTViNjAtNDliNC1hN2Y1LTJjZjg0YjMyOGFmNyIsImRhdGEiOnt9LCJyYW5kb20iOiI2MWJmY2U4YTA3OWQ3OTgzNWU4ZDE1YTAwMjE0MDZlOCJ9.X_wmoi8TXd-kNrBi5RdofGVZeTF66ofo7S6Gfjh575x_xb77-97Xn0tV-avdF2BYhVrM8yxArErC63yVZR0xIA

42. "Data and materials availability": Please state whether the new AAV8retro or other viruses/plasmids will be deposited on Addgene or otherwise made available to the community.

We are happy to distribute the AAV8retro or other viruses/plasmids for research upon request.

43. "Data and materials availability": Please deposit some of the code used for analysis (for example, for locomotion) into a GitHub/Zenodo associated with this paper.

The tracking software was developed by Yueqing Peng's lab at Columbia University. It is available in GitHub using the link: https://github.com/thepenglab/posTracker.

44. Given the general audience that will read this paper, the final paragraph could be strengthened to reference the wider pain literature or how these results might influence next steps in preclinical research, relate to observations in the clinic, or might lead to novel types of analgesics or pain management.

We thank the reviewer for the suggestion. We have mentioned the potential clinical implication of targeting each node of this circuit for treating chronic pain in the discussion section, such as line 251 and 300 in the original manuscript. Instead, we'd like to take this opportunity to expand our discussion on the model we proposed to explain a potential spinal cord interneuron that is required for this circuit loop to driving chronic pain, and the future work needed to solve the missing pieces of the puzzle.

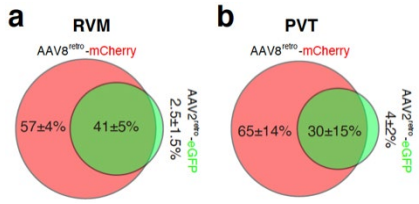
45. Please provide a supplemental table of the statistical analysis with columns for figure, statistical test, number of animals, and significance value. That will be easier for readers than the current list in the Methods section.

Following the suggestions, we added a supplemental table summarizing the statistical analysis and animal used for each figure.

46. For "Chemogenetic Manipulation" Method section, please specify for each of the different experiments where exactly the chemogenetic manipulation was done. Based on the current description, Fig. 5d is not clear if CLZ was infused into the VPL and Po or at some earlier point in the STT pathway. If the former, there should be two "X"s over each pathway in Fig. 5d, to clearly indicate this.

Sorry for the confusion. CLZ was infused into the VPL and Po together to silence both pathways in Fig.5d. As suggested, we used two 'X' on each pathway in the diagram in revised Fig.5d.

47. Extended Data Fig. 2d-f, please provide a Venn diagram of the AAV8retro and AAV2retro labeled RVM neurons, so readers can identify how many neurons labeled by AAV2retro are missed by AAV8retro.



As suggested, we replaced the original bar graph with the Venn diagrams in revised Extended Data Fig. 2f-g. Thanks.

48. In "von Frey Test" methods, please state the exact behaviors used to indicate withdrawal responses and those not considered withdrawal behaviors or not included in the analysis. This will affect interpretation of what mechanical thresholds mean throughout the study and whether non-nociceptive behaviors are being included.

We added *'A positive withdrawal response was defined as a brisk paw withdrawal, shaking, or licking directed at the stimulated paw. Movements related to general locomotion, weight shifting, grooming, or exploratory behavior were not considered withdrawal responses and were excluded from the analysis.'* to line 500-503 of the revised manuscript.

49. Please provide a supplementary video of a mouse with RVM fiber photometry, for example, include the behavior synchronized with a trace of RVM GCaMP activity measured.

Following the suggestion, we have provided a supplementary video showing behavioral recording synchronized with the RVM fiber photometry trace of GCaMP activity.

50. Building on Fig. 1h, please plot summary dF/F (for example, in Extended Data or Supplemental figures) stimulus induced vs. spontaneous or no locomotion.

As shown in our response to your previous points 23 and 40, there is no difference in response amplitude between spontaneous and evoked calcium response in Oprm1^{+} RVM^{SC} population (Rebuttal Fig. 29). Moreover, spontaneous locomotion in both control and SNI mice do not activate Oprm1^{+} RVM^{SC} population (Rebuttal Fig. 20).

51. The introduction refers to the well-defined ON-cells (line 46-49) but does not explicitly name them. Please do so.

Added 'which is known as ON-cell' to line 47 of the revised manuscript.

52. Line 553-555, please clarify why a different stimulus amount or timing was giving for SNI vs. non-injured mice? Further, were non-injured mice actually stimulated every 6.5 s for 10 mins? And why was a different stimulus given to SNI and sham mice for cFos? Or if the same stimulus was given for Extended Data Fig. 5e-f, please clarify that in the methods.

The stimulation protocol was optimized based on preliminary data to ensure robust c-Fos induction in both experimental groups. Although we began with an identical protocol for both SNI and non-injured mice (60 stimuli delivered every 10 s over 10 minutes), this frequency was insufficient to induce detectable c-Fos in some brain regions of non-injured animals. We thus increased the total number of stimuli for non-injured mice to 90, delivered over the same 10-minute duration. This resulted in an average inter-stimulus interval of 6.5-6.7 s, which successfully produced a reliable c-Fos signal for comparison.

53. Please provide additional details on the tracking software and algorithm used for the CPA, locomotion, and other related test (for example, expand on line 422-423) or make the code available on a GitHub.

The tracking software was developed by Yueqing Peng's lab at Columbia University. It is available in GitHub using the link: https://github.com/thepenglab/posTracker.

54. Please comment in the Results or Discussion whether the fiber photometry RVM results align with other fiber photometry recordings from other papers (for example, https://doi.org/10.1016/j.ibror.2019.05.005 and https://doi.org/10.1101/2024.11.29.626042).

We appreciate the reviewer's suggestion to consider the studies by Moriya et al. (2019) and Rani et al. (2024). These papers provide valuable evidence that fiber photometry can successfully capture nociception-related activity in other RVM neuron populations, such as serotoninergic neurons and those projecting to the lateral hypothalamus (LHA).

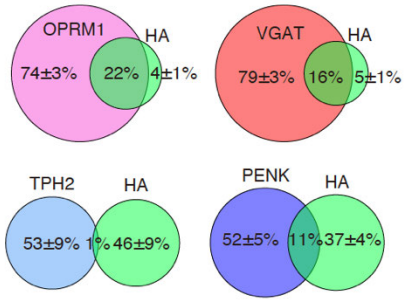
While this broadly aligns with our technical approach, the functional focus of these studies differs from our own. They primarily examine responses to acute nociceptive stimuli in naive animals. In contrast, our work specifically investigates the OPRM1^{+} RVM^{SC} population in the context of chronic pain, demonstrating its critical role in mediating mechanical hypersensitivity after injury or inflammation, rather than acute nociception. Given this fundamental difference in physiological context and neuronal function, we have chosen to focus the discussion on literature more directly related to the mechanisms of hyperalgesia.

55. On line 431-432, it appears that different recording times were used for CLZ compared to morphine and the CLZ duration of recording is relatively short given CLZ's kinetics. Please explain this discrepancy.

The locomotor activity recordings for morphine and CLZ were conducted in two separate experiments. The morphine data (Extended Data Fig. 1c) and the CLZ data (originally in Extended Data Fig. 4, now in Extended Data Fig. 5) were not intended for direct comparison, so the recording durations were established independently for each experiment.

As we showed in our response to your previous point 38, we have now performed a new experiment continuously recording locomotion for one hour after CLZ administration. This new data, presented in Rebuttal Fig. 27 and added to Extended Data Fig. 5c of the revised manuscript, confirms the absence of a locomotor effect.

Figures:



56. Fig. 1e and Extended Data Fig. 3e, please plot the overlap as Venn diagrams to readers can know % retrograde that are X marker and % of X marker that are retrograde.

As suggested, we replaced the original bar graph with the Venn diagrams and updated in Extended Data Fig. 3d of the revised manuscript. Thanks.

57. Fig. 1g, make this and other diagrams include a descending axon or other indication to make it clearer what is being done. This extends to making the arrows having a line going to text of each injected substance.

As suggested, we added descending axon to the diagram in Fig.1g, Fig.2a, and Extended Data Fig. 3a. And changed the arrow head to arrow with line link to the text of each injected substance. Thanks.

58. Fig. 1g, please improve this timeline to indicate when optical fiber is placed and when experiments in the normal state are done.

we added optic fiber implantation, pre-SNI and after-SNI FP recording to the time line.

59. Fig. 2a, please improve this timeline to indicate when cannula is placed and exact times for behavioral studies.

We added clozapine injection and behavioral test in normal state (for Fig.2b) in the timeline. For experiments in Fig.2, clozapine injection is through I.P., no cannula is implanted.

60. Fig. 2c, overlay RVM landmarks to indicate looking at NRM or other structures.

We added RVM landmarks to the images and explained the abbreviations in figure legend. Thanks.

61. Fig. 2d, overlay outline of spinal cord, currently hard to visualize. Improve legend or panel to indicate what is being visualized with each color.

We added outline of the spinal cord dorsal horn to the images and added more details in figure legend.

*62. Fig. 3g, "Silencing lSuC *excitatory* input", please make clear in this and other figures when stimulating a specific cell type within a pathway, for example, this experiment is "excitatory" cells.*

We added 'excitatory' to Fig.3b and 3g. In other places, we explained explicitly which cell type has been manipulated in figure legend.

63. Fig. 4 needs a diagram of the experiments leading to Fig. 4a-b.

Thanks for the suggestion but there is no space for adding the experimental diagram to Fig.4. We have described this approach in details in Fig.3c. In text, we mentioned "*We again employed the cTRIO approach to map brain-wide monosynaptic inputs onto the RVM-projecting excitatory lSuC neurons.*" in line 251-253 of the revised manuscript. Moreover, detailed procedure for rabies tracing was also described in line 628-643 in 'cTRIO tracing and analysis' in method section of the revised manuscript.

64. Fig. 4a, please directly label Mo and SS in the dashed green circles on the 3D model and change the color of the dashed green circles to one with greater contrast, it is currently hard to see.

We now changed the color of the dashed circles to black and labeled MOa and SS right next the each circle.

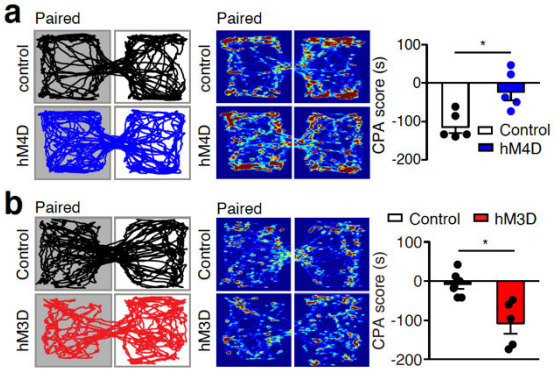
65. Extended Data Fig. 6, please provide a diagram of the experiments performed and clearly state which brain areas are being manipulated in the figure panels and/or legends.

We added timeline to this figure. Now this figure becomes Extended Data Fig. 7 in the revised manuscript.

66. Extended Data Fig. 9. Please provide supplemental figure(s) showing example histology images paralleling the summary diagrams.

Following the suggestion, we added example histology images paralleling the summary diagrams, now presented as Extended Data Fig. 11 in the revised manuscript.

67. For CPA studies, it is often hard to see that the quantified effect matches the traces of animal locomotion/location. Is it possible to display these as heat maps or include the heat maps in a supplemental figure for all CFA experiments?



Thanks for the suggestion, we have then plotted the traces of animal locomotion as heat map. As you can see from the example from the Fig.2g and Extended Data Fig. 7b of the original manuscript, the heat map does not help to illustrate the difference between two treatments in these two experiments. Moreover, heat map will not allow us using different color too indicate different treatment. We thus did not use heat map in our manuscript.

Text:

*68. Please check the text throughout for spelling and grammar. For example (with text inside stars indicating suggested changes) line 130 "mechanical withdrawal threshold*s*", line 252 "lays *the* foundation", line 253 "circuit interact*s* with",*

*line 255 "*The* current model" or "Current model*s*", line 382 "480 *nm* LED", line 495 "2 ms *long pulse of* 470 nm", etc.*

Thanks. All the errors have been changed as suggested.

69. Please use "significantly" if the result is statistically significant instead of "dramatically" or similar adjectives to describe results in the results section.

We removed the word 'dramatically' in line 113.

70. Line 228-230: Fig. 5c,d do not demonstrate that those pathways do not play a role in nociception in healthy mice. At most they show that the authors measure of mechanical sensitivity is not affected or that the experiment did not work since no other validation (for example, electrophysiology) of chemogenetic inhibition was done.

This statement was based on our findings that silencing these pathways in healthy mice (Pre-SNI and Pre-SNI + CLZ data in Fig.5c,d) did not alter their baseline mechanical nociceptive thresholds.

We apologize for missing the datasets for validation of chemogenetic silencing. As our answer to reviewer #3 point #9, we have performed slice recording and validated the efficiency of chemogenetic silencing. We added this data to Extended Data Fig. 5d-f in the revised manuscript.

71. Please state the method employed in the text when mentioning experiments, for example, line "Silencing the SS->ISuC, but" should indicate chemogenetic.

We added 'chemogenetic' to the test when mentioned chemogenetic manipulation.

72. Line 229-230 "indicating that neither pathway is involved in nociception in healthy mice (Fig. 5c,d)". This is not shown by these experiments, only that mechanical hypersensitivity is not changed.

We apologize for the lack of clarity. Line 228-230 of the original manuscript said 'Silencing either the STT→VPL→SS or STT→Po→SS pathway along or both pathways together also showed no effect on nociceptive thresholds before SNI, indicating that neither pathway is involved in nociception in healthy mice (Fig. 5c,d).' This statement was based on our findings that silencing these pathways in healthy mice (Pre-SNI and Pre-SNI + CLZ data in Fig.5c,d) did not alter their baseline mechanical nociceptive thresholds.

We have revised the text to specify '(Fig. 5c,d, Pre, Pre + SNI) ; and have clearly defined 'Pre' as 'Pre-SNI' in figure legend to avoid confusion.

73. Line 238-240 needs to be revised. No recording of individual SS neurons was done, so these results merely show that there is not a behavioral manifestation after manipulating (VPL + Po)->SS rather than they are not transmitting tactile information to SS, which would require inhibiting these pathways then showing this does not alter SS neural codes via calcium imaging or electrophysiology.

Following the suggestion, we rewrote the sentence to '*These results reveal the limited contribution of the STT→thalamus→SS pathway in tactile discrimination'* in line 306-307of the revised manuscript.

74. Line 269-270 needs rewording. The experiments done do not show that ISuC->RVM pathway is not involved in orienting toward the wound, this would require additional experiments such as inhibiting and activating that pathway while giving von Frey or other stimulation at the wound site and then demonstrating animals maintain behavior. Further, the authors need to be specific and indicate ISuC(vglut2)->RVM for all cases in which they are looking at just VGLUT2+ excitatory neurons rather than the entire RVM pathway (which includes excitatory inputs).

Following the suggestion, we now removed the claim *'ISuC→RVM pathway is not involved in orienting toward the wound'* , and moved the entire section regarding the orienting behavior from discussion in the original manuscript to lines 236-243 of the revised manuscript.

75. In Methods, all instances of "previously described" (for example, line 351, line 362, etc.) or similar should cite the relevant study.

Reference are added to all instances of 'previously described'. Thanks.

76. In the Methods, please include manufacturer information in all cases, for example, line 406-407 does not state von Frey filament manufacturer, clozapine source not stated, etc.

von Frey filaments (Ugo Basile), clozapine (Cayman Chemical), morphine (Hikma) was added to methods. Thanks.

77. The entire sentence from line 530-538 is confusing and appears to contain hanging parentheses. Please re-write.

Thanks for the suggestion. We re-write the section as follows: '*For immunostaining, 50 μm sections were washed three times for 5 min each with 1× PBS. They were then incubated in a blocking solutions (0.3% PBST containing 10% normal donkey serum (LAMPIRE Biological Products 7332100)) for 2 h at room temperature. Subsequently, the section were incubated with primary antibodies diluted in 0.3% PBST containing 3% normal donkey serum overnight at 4°C. After three 10 min washes with PBS, the section were incubated with secondary antibodies diluted in 0.3% PBST containing 5% normal donkey serum for 2 h at room temperature. Following this, the sections were washed three times for 10 min each with PBS; Hoechst 33342 solution (ThermoFisher) was included in the second wash at 1:10,000 dilution. Finally, the sections were mounted with Fluoromount-G (Southern Biotech).*' in lines 660-668 of the revised manuscript.

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