nature portfolio

Peer Review File

Deconstruction the feedforward inhibition changes in the layer III of anterior cingulate cortex after peripheral nerve injury

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The mediodorsal thalamus (MD) is intricately linked with the anterior cingulate cortex (ACC) and plays a pivotal role in the emotional aspect of pain processing. Under conditions of peripheral neuropathy, this connection undergoes dysregulation. While MD neurons regulate the activity of both L3 and L5 cingulate neurons through feedforward inhibition, how this inhibition is altered under peripheral neuropathic conditions remains unclear. In their manuscript, Lian et al. demonstrated that peripheral nerve injury exacerbates excitatory synaptic transmission to L3 cingulate neurons while concurrently impairing feedforward inhibition, as corroborated by pathclamp recordings. This stands in contrast to prior research indicating a reduction in MD inputs to L5 cingulate neurons post-nerve injury, a difference attributed to cortical layer variances, as discussed in the manuscript.
Another noteworthy discovery in this study was the impact of chemogenetic manipulation targeting the MD-to-ACC projection. Activation of this pathway heightened mechanical sensitivity and induced place aversion, while its inhibition alleviated mechanical hyperalgesia and induced place preference in nerve-injured mice. However, the manuscript's impact is somewhat tempered by the absence of a direct correlation between the patch clamp recording results and the behavioral outcomes of chemogenetic modulation.
Major
1. It is imperative to elucidate how chemogenetic interventions influence feedforward inhibition through patch clamp techniques.
2. Given the potential diffusion of DREADD agonists within the ACC and the widespread expression of AAV-retro-cre-GFP, it remains unclear which layers of ACC neurons are affected. Fos experiments could illuminate the proportion of L3 and L5 cingulate neurons impacted by chemogenetics. Additionally, displaying mCherry expression in the ACC would provide further clarity.
3. Moreover, addressing the discrepancy between the current study and a previous finding reported by Wang et al., who noted an increase in silent synapses between MD and L3 cingulate neurons post-peripheral nerve injury, is crucial.
Minor
1. Rectifying inconsistencies in the figure depicting cannula placement and the method describing midline positioning.
2. Noting that the baseline PWT (g) appears unusually low (0.2-0.3), typically averaging around 1.
3. Highlighting that preference indexes are negative in control groups during the CPP test using the nerve injury model.

Reviewer #2 (Remarks to the Author):

Summary: In this paper, Lian et all investigate a circuit between the mediodorsal thalamus (MD) and the anterior cingulate cortex (ACC) in the context of nociception in mice. This study adds to an

existing literature regarding both structures in nociception and neuropathic models. First, the authors showed that ChR2 photostimulation could drive EPSC and IPSC's in the L3 of ACC and the latency of each was suggestive of a FFI circuit. Next, the circuit was evaluated 7 days after a nerve injury and there was evidence for enhanced inputs to ACC after injury. For intermediate firing (pyramidal) neurons in the ACC, nerve injury increased APs to current injection. There was no change in regular spiking pyramidal neurons nor in intermediate neurons (fast spiking). Next EPSCs and IPSCs onto ACC neurons were evaluated suggesting changes in the FFI after nerve injury. Next, chemogenetic activation of MD-to-ACC neurons induced CPA and caused mechanical hypersensitivity in the hindpaw although the specific paw(s) are not clear (ACC activation was bilateral so this is a minor issue). Finally, inactivating the MD-to-ACC chemogenetically reduced neuropathic injury mechanical hypersensitivity. Overall, the study used a diverse set of studies to further our specialty knowledge of one brain circuit involved in neuropathic aversion and mechanical hypersensitivity. The studies, though, largely expand on existing findings and literature rather than proposing new questions or hypotheses. The methods are generally solid (although see issues below) and conclusions well justified.

Major:

- Please justify the use of 7 days after nerve injury in this study. This is a time period that could very easily be in the transition phase between acute injury and chronic injury. Without specific markers showing one versus the other, it is impossible to interpret these data in any specific context.
- For physiology, please clearly describe side of brain recorded especially in the nerve injury versus control experiments since the injury was made to the left nerve but viral injections are described at being bilateral. This suggests that you may have done recordings on the left and right. If that is true, data should be presented as ipsilateral and bilateral to injury.
- Fast spiking neurons were noted as being parvalbumin neurons but no staining was done to justify this. This possibility should be moved to the discussion and out of the results.
- Please justify the use of male only study and include limitations section related to sex in the discussion.
- There is no mention of a power analysis or other method for sample size determination. Please indicate how samples were determined since they vary widely in the experiments.
- Blinding is mentioned at the end of the viral construct section. Please include more details on blinding. Was the experiment blinded to virus construct completely (e.g. DREADD vs Opto) or just within an experiment (e.g. ChR2 vs control)? Was the experimenter blinded to surgery type when relevant (e.g. nerve injury vs sham) for behavior? Was targeting of virus completed blinded to viral type and/or behavioral result? Was the experimenter blinded to surgery type when relevant (e.g. nerve injury vs sham) for slice physiology?
- Please include information on whether selection of mice for injury or virus was conducted in a randomized fashion. If so, what process was used for randomization.
- Suggest that raw data be included in publication as a supplemental data set and/or on GitHub or OSF.
- For statistics – Please indicate whether statistical tests were pre-determined (registered) before the start of data collection or where determined a posteriori?

Minor:

- For the figures you show a brain slice to indicate viral injection and then projection. These are helpful. However, I would suggest you flip the left and right to match the direction left to right in the B part of the figure. In other word, if MD is on the left in B then it should be on the left in A (or vice versa). Either direction is fine but make them the same for ease of interpretation.
- Intro lines 61-63 – The way FFI is described is a bit confusing as written and will likely confuse many readers. Please re-write and clarify. For example, the last part of the sentence refers to a "post synaptic neuron" but this could be either the inhibitory neuron OR another neuron in the circuit.
- Intro lines 65-72 – The organization of this paragraph is odd and doesn't flow well. Please restructure to carefully and intentionally layout the existing and unknown aspects of this circuitry.
- Suggest removing "MP" as an acronym for membrane potentials. Please just write out.
- Line 199 – Please clarify whether photostimulation was of the MD or the terminals in the ACC.
- Line 247-249 – Re-write – Sentence fragment

Reviewers' comments:

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The mediodorsal thalamus (MD) is intricately linked with the anterior cingulate cortex (ACC) and plays a pivotal role in the emotional aspect of pain processing. Under conditions of peripheral neuropathy, this connection undergoes dysregulation. While MD neurons regulate the activity of both L3 and L5 cingulate neurons through feedforward inhibition, how this inhibition is altered under peripheral neuropathic conditions remains unclear. In their manuscript, Lian et al. demonstrated that peripheral nerve injury exacerbates excitatory synaptic transmission to L3 cingulate neurons while concurrently impairing feedforward inhibition, as corroborated by patch-clamp recordings. This stands in contrast to prior research indicating a reduction in MD inputs to L5 cingulate neurons post-nerve injury, a difference attributed to cortical layer variances, as discussed in the manuscript.

Another noteworthy discovery in this study was the impact of chemogenetic manipulation targeting the MD-to-ACC projection. Activation of this pathway heightened mechanical sensitivity and induced place aversion, while its inhibition alleviated mechanical hyperalgesia and induced place preference in nerve-injured mice. However, the manuscript's impact is somewhat tempered by the absence of a direct correlation between the patch clamp recording results and the behavioral outcomes of chemogenetic modulation.

Major

1. It is imperative to elucidate how chemogenetic interventions influence feedforward inhibition through patch clamp techniques.

Response: Thanks for the reviewer’s suggestion! We have conducted corresponding experiments to elucidate the question, as illustrated in Figure 6.

The specific experiments and results are as follows:

We unilaterally injected AAV9-CaMKIIα-hM3Dq-mCherry virus into the right MD. Then, we conducted in vitro whole-cell recordings of ipsilateral ACC L3 pyramidal neurons to measure spontaneous excitatory postsynaptic currents (sEPSCs) and spontaneous inhibitory postsynaptic currents (sIPSCs) at least 21 days after the virus injection (Fig. 6A-C). Interestingly, we observed a significant difference in the frequency of both sEPSCs and sIPSCs after adding 30 μM CNO to the

ACSF for 10 min. Additionally, sEPSCs were significantly blocked by 20 μM CNQX (Fig. 6 D&E), while sIPSC frequency and amplitude were reduced following the addition of 20 μM CNQX to the ACSF for 10 min (Fig. 6 D&E). Hence, chemogenetic activation of MD-ACC pathway would affect the synaptic transmission of ACC neurons, thus influencing the FFI within the microcircuits.

2. Given the potential diffusion of DREADD agonists within the ACC and the widespread expression of AAV-retro-cre-GFP, it remains unclear which layers of ACC neurons are affected. Fos experiments could illuminate the proportion of L3 and L5 cingulate neurons impacted by chemogenetics. Additionally, displaying mCherry expression in the ACC would provide further clarity.

Response: Thanks for the reviewer's suggestion! Following with the reviewer's suggestion, we performed further experiments, as illustrated in Figure 7.

The specific experiments and results are as follows:

We bilaterally injected AAV2-retro-hSyn-cre-GFP into the ACC and AAV2-hSyn-DIO-hM3Dq-mCherry into the MD, the mice with the injection of AAV2-retro-hSyn-cre-GFP (ACC) and AAV2-hSyn-DIO-mCherry (MD) as control, to selectively activate the MD-to-ACC inputs. Compared with the control group, immunofluorescence staining revealed increased c-Fos expression in both L3 and L5 of the ACC, 90 min after intraperitoneal administration of CNO (4 mg/kg) to mice (Fig. 7D & E).

3. Moreover, addressing the discrepancy between the current study and a previous finding reported by Wang et al., who noted an increase in silent synapses between MD and L3 cingulate neurons post-peripheral nerve injury, is crucial.

Response: Thank you for the reviewer's insightful question. We have added corresponding analyse in the **Discussion** part:

Additionally, Wang et al., who noted an increase in silent synapses between MD and L3 cingulate neurons post-chronic constriction injury (CCI) [19] . These synapses gradually decreased in number over time following prolonged CCI. Moreover, the potential maturation of these synapses could lead to remodeling of the MD-to-ACC projection, creating new circuit connectivity and

strengthening neurotransmission, which might contribute to the development of allodynia induced by nerve injury. Therefore, following neural injury, even with an increase in silent synapses, enhanced AMPAR currents may still be observed due to the presence of inactive AMPARs.

Minor

1. Rectifying inconsistencies in the figure depicting cannula placement and the method describing midline positioning.

Response: Thank you for the reviewer's meticulous review. We have revised the description of cannula implantation and microinjection methods as follows:

The double-lumen cannula was positioned at coordinates 0.7 mm anterior to the bregma, 0.0 mm lateral to the midline, and 1.20 mm ventral to the surface of the skull. Each sleeve is located 0.25 mm on either side of the midline.

2. Noting that the baseline PWT (g) appears unusually low (0.2-0.3), typically averaging around 1.

Response: Thank you for noting the observed baseline PWT (g) in our study. The lower values (0.2-0.3) compared to the typical average (around 1) are indeed notable. We attribute this discrepancy to factors such as the specific strain of mice used, variability inherent in pain response measurements, or potential influences from recent experimental procedures like virus injection and cannula implantation. Despite these variations, we ensured that all animals were carefully monitored and maintained under consistent experimental conditions throughout the study.

3. Highlighting that preference indexes are negative in control groups during the CPP test using the nerve injury model.

Response: Thank you for highlighting the negative preference indexes observed in control groups during the CPP test using the nerve injury model. This finding is indeed noteworthy.

We believe several factors may contribute to this observation, including potential sensitization or conditioning effects unrelated to the intended experimental manipulation. Additionally, variations in baseline preferences or inherent differences in response to the CPP paradigm could also play a role. We ensured rigorous experimental controls and consistent procedures across all groups to minimize confounding factors and accurately interpret our results.

Reviewer #2 (Remarks to the Author):

Summary: In this paper, Lian et all investigate a circuit between the mediodorsal thalamus (MD) and the anterior cingulate cortex (ACC) in the context of nociception in mice. This study adds to an existing literature regarding both structures in nociception and neuropathic models. First, the authors showed that ChR2 photostimulation could drive EPSC and IPSC's in the L3 of ACC and the latency of each was suggestive of a FFI circuit. Next, the circuit was evaluated 7 days after a nerve injury and there was evidence for enhanced inputs to ACC after injury. For intermediate firing (pyramidal) neurons in the ACC, nerve injury increased APs to current injection. There was no change in regular spiking pyramidal neurons nor in intermediate neurons (fast spiking). Next EPSCs and IPSCs onto ACC neurons were evaluated suggesting changes in the FFI after nerve injury. Next, chemogenetic activation of MD-to-ACC neurons induced CPA and caused mechanical hypersensitivity in the hindpaw although the specific paw(s) are not clear (ACC activation was bilateral so this is a minor issue). Finally, inactivating the MD-to-ACC chemogenetically reduced neuropathic injury mechanical hypersensitivity. Overall, the study used a diverse set of studies to further our specialty knowledge of one brain circuit involved in neuropathic aversion and mechanical hypersensitivity. The studies, though, largely expand on existing findings and literature rather than proposing new questions or hypotheses. The methods are generally solid (although see issues below) and conclusions well justified.

Major:

1. Please justify the use of 7 days after nerve injury in this study. This is a time period that could very easily be in the transition phase between acute injury and chronic injury. Without specific markers showing one versus the other, it is impossible to interpret these data in any specific context.

Response: Thanks for the reviewer's question! It is a good question. We selected 7 days as a time point to study FFI with following reasons:

Firstly, nerve injury triggers a cascade of molecular and cellular events that evolve over time. Different phases of the injury response, such as neuronal hyper-excitability, and chronic remodeling, can have distinct impacts on neural circuits. Studying FFI specifically at the 7-day mark allows researchers to capture a phase where initial responses have occurred but before more long-term

adaptations fully manifest.

Secondly, 7 days post-injury is a period where chronic pain-like behaviors and neural changes start to emerge, making it a relevant time point for studying alterations in FFI.

Thirdly, it is consistent with previous research, prior studies have demonstrated that significant alterations in synaptic transmission, neuronal excitability, and circuit function can be observed around the 7-day mark following nerve injury. Therefore, choosing this time point allows for comparisons and continuity with existing literature.

Overall, the selection of "7 days after nerve injury" as a time point in the study is grounded in the temporal dynamics of neural injury responses, relevance to chronic pain models, consistency with previous research findings, and practical considerations in experimental design.

2. For physiology, please clearly describe side of brain recorded especially in the nerve injury versus control experiments since the injury was made to the left nerve but viral injections are described at being bilateral. This suggests that you may have done recordings on the left and right. If that is true, data should be presented as ipsilateral and bilateral to injury.

Response: Thanks for the reviewer's suggestion! In our study, we made nerve injury to the left side of mice. According to information transmission pathway, we made ChR2 virus injection into the right MD and conducted electrophysiological recording of the right brain side, contralateral to the nerve injury.

In the **Whole-cell Patch-clamp Recording** part of method, we described the recording sites as follows:

All recordings were made from L3 neurons located in the ACC contralateral to the nerve ligation surgery.

3. Fast spiking neurons were noted as being parvalbumin neurons but no staining was done to justify this. This possibility should be moved to the discussion and out of the results.

Response: Thanks for the reviewer's suggestion! We further conducted immunostaining experiments to verify that fast spiking neurons express parvalbumin, as shown in **Figure 4**.

The specific experiments and results are as follows:

Initially, we conducted whole-cell patch-clamp recording on the GFP-positive cells (GFP^+) in the ACC of GAD67-GFP mice. Among the recorded GFP^+ cells, over 70% of cells fired action potentials (APs) at a very high frequency, indicating these are fast-spiking (FS) neurons. Biocytin was injected into the recorded neurons showing high-frequency AP firing, followed by immunostaining to label the neurons. Subsequently, immunostaining using the PV antibody was conducted on the same brain slices, confirming that the FS neurons are indeed PV-positive.

4. Please justify the use of male only study and include limitations section related to sex in the discussion.

Response: Thanks for the reviewer's suggestion! We acknowledge the importance of addressing sex as a variable in our study. We added the reason of use of male animals in the parts of **Animals and CPN model** and **Discussion**, as follows:

Animals and CPN model:

To rule out the effects of estrogen on the estrous cycle, experiments were performed on adult male C57BL/6J mice and male GAD67-GFP mice (8-12 weeks old).

Discussion:

Notably, much of the existing research has focused on male subjects, further studies should emphasize the importance of sex differences in thalamic-cortical communication and their impacts on cognitive function.

5. There is no mention of a power analysis or other method for sample size determination. Please indicate how samples were determined since they vary widely in the experiments.

Response: Thank you for the reviewer's question! We have addressed the determination of sample sizes in the '**Data Analysis**' section:

Before starting experiments, based on our previous behavioral studies, we estimated the sample size by using G*Power software.

6. Blinding is mentioned at the end of the viral construct section. Please include more details on blinding. Was the experiment blinded to virus construct completely (e.g. DREADD vs Opto) or just within an experiment (e.g. ChR2 vs control)? Was the experimenter blinded to surgery type when

relevant (e.g. nerve injury vs sham) for behavior? Was targeting of virus completed blinded to viral type and/or behavioral result? Was the experimenter blinded to surgery type when relevant (e.g. nerve injury vs sham) for slice physiology?

Response: Thanks for the reviewer's suggestion! We have provided additional details on blinding as follows:

The mechanical allodynia and CPP/CPA test were conducted in a double-blind manner. The experimenters were blinded to experimental groups (In part of **Constructs, Viral Packaging, and Stereotactic Injection**).
An evaluator blinded to the group assignments performed the assessment and analyzed the data (In part of **Data Analysis**).

7. Please include information on whether selection of mice for injury or virus was conducted in a randomized fashion. If so, what process was used for randomization.
Response: Thanks for the reviewer's suggestion! Experimental and control animals were randomized throughout the study. We have made supplementation in the **methods part** as follows:
We randomly tagged each mouse and then randomly assigned them to groups. Four or five mice were housed per cage.

8. Suggest that raw data be included in publication as a supplemental data set and/or on GitHub or OSF.
Response: Thanks for the reviewer's suggestion!
We will organize the raw data and upload it to the webpage as a corrected version.

9. For statistics – Please indicate whether statistical tests were pre-determined (registered) before the start of data collection or where determined a posteriori?
Response: Thanks for the reviewer's question!
Statistical tests were determined post-experiment.

Minor:

1. For the figures you show a brain slice to indicate viral injection and then projection. These are helpful. However, I would suggest you flip the left and right to match the direction left to right in the B part of the figure. In other word, if MD is on the left in B then it should be on the left in A (or vice versa). Either direction is fine but make them the same for ease of interpretation.

Response: Thank you for the reviewer's suggestion! We have adjusted the images of virus expression accordingly, ensuring consistency in the direction of virus expression as per your recommendation.

2. Intro lines 61-63 – The way FFI is described is a bit confusing as written and will likely confuse many readers. Please re-write and clarify. For example, the last part of the sentence refers to a "post synaptic neuron" but this could be either the inhibitory neuron OR another neuron in the circuit.

Response: Thank you for the reviewer's suggestion! We have revised the sentence as follows:

The feedforward inhibition (FFI) is one of the basic motifs of neuronal circuits, it includes an excitatory neuron, upon being activated, simultaneously sends signals to both a target neuron and an inhibitory interneuron, the inhibitory interneuron then suppresses the activity of the target neuron, modulating its response to the incoming excitatory signal [15].

3. Intro lines 65-72 – The organization of this paragraph is odd and doesn't flow well. Please restructure to carefully and intentionally layout the existing and unknown aspects of this circuitry.

Response: Thanks for the reviewer's suggestion! We have rephrased the paragraph as follows:

Thalamocortical projections from the MD excite the pyramidal neurons in layer III (L3) [15,17] and layer V (L5)[16,18] of the ACC, forming the FFI circuits. Studies have shown that prolonged nerve injury generates silent synapses in the MD-to-ACC projections [19]. Furthermore, the peripheral nerve damage weakened the MD inputs to the L5 cingulate neurons, contributing to pain aversion [16,18], indicating the involvement of MD-to-ACC projections in the development of pathological pain [20]. Neurons in L3 of the ACC are the primary recipients of the MD inputs [21–24] and construct the FFI microcircuits [25]. However, the alterations in the responses of L3 ACC neurons to MD inputs and their impact on FFI remain unknown.

4. Suggest removing "MP" as an acronym for membrane potentials. Please just write out.

Response: Thanks for the reviewer's suggestion! We replace "MP" with its full name membrane potential in the paper.

5. Line 199 – Please clarify whether photostimulation was of the MD or the terminals in the ACC.

Response: Thanks for the reviewer's suggestion! We re-wrote this sentence:

By photostimulating MD terminals into the ACC, we recorded the EPSC and IPSC with membrane potentials held at -70 mV and 0 mV, respectively.

6. Line 247-249 – Re-write – Sentence fragment

Response: Thanks for the reviewer's suggestion! We re-wrote this sentence as follows:

Similar to previous studies , we identified two predominant action potential firing patterns based on the presence or absence of afterdepolarization (ADP) in individual action potential: regular spiking (RS) neurons without ADP (Fig. 3A), and intermediate firing (IM) neurons characterized by evident ADP.

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

The authors have addressed all of my concerns.

Reviewer #2 (Remarks to the Author):

The authors have addressed almost all concerns and issues and the manuscript is substantially better.

There are two issues that need to be address:
1. I asked about statistical planning (a priori or a posteriori). The authors indicated in the rebuttal that stats were determined a posteriori. However, that is not written in the manuscript. It must be included in the stats section prior to publication.

2. The only other minor issue is with the raw data request. There appears to be raw data used in the figures which is appreciated but the rebuttal indicates that the placement of these data is not yet determined and the manuscript still says "All data reported in this paper will be shared by the lead contact upon request." In other words, it is unclear if the data will be included in the published version as they should be.

Reviewers' comments:

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There are two issues that need to be address:

1. I asked about statistical planning (a priori or a posteriori). The authors indicated in the rebuttal that stats were determined a posteriori. However, that is not written in the manuscript. It must be included in the stats section prior to publication.

Response: Thanks for the reviewer's suggestion! We added the statistical planning in the section of Statistics and Reproducibility：

Before starting experiments, based on our previous behavioral studies, we estimated the sample size by using G*Power software. Offline analysis of whole-cell patch-clamp data was performed using Clampfit 10. 6 and MiniAnalysis 6.03. GraphPad 8.0 was used to plot and fit the data. An evaluator blinded to the group assignments performed the assessment and analyzed the data. Statistical tests were determined post-experiment. Statistical comparisons were made using the unpaired two-sample *t*-test, One-way or two-way ANOVA (the Student-Newman-Keuls test was used for post hoc comparison). All data were presented as the mean ± standard error of the mean (SEM). In all cases, n.s, not significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

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Response: Thanks for the reviewer's suggestion! We mentioned that the raw data were uploaded as supplementary data in the section of Data availability：

Numerical source data for all figures are provided in Supplementary Data 1. Additional data supporting the findings of this study can be obtained from the lead corresponding author upon reasonable request.