nature portfolio

Peer Review File

Berberine alleviates chronic pain-induced anxiety-like behaviors by inhibiting the activation of VLT-projecting cACC (Cg2) neurons

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This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Communications Biology.

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Thank you for precise revision you have done. Now the paper is ready for publication.
Good luck

REVIEWER COMMENTS
Reviewer #1 (Comments to the Author):

This study found that neurons in cingulate area 2 (Cg2) of the cACC, but not in cingulate area 1 of the cACC, projected to the VLT. Next, authors induced chronic inflammatory pain by plantar injection of complete Freund's adjuvant (CFA) and observed anxiety-like behaviors until two weeks postinjection and found that berberine alleviates comorbid anxiety symptoms in chronic pain by inhibiting the activation of VLT-projecting cACC (Cg2) neurons. However, the following shortcomings would dampen my enthusiasm for the manuscript.

1.In VLT-projecting cACC (Cg2) neurons, which type of neuron mediates anxiety-like behaviors and anxiolytic effects of berberine in mice treated with CFA?
Re: We thank you for your suggestion. We believe that such neurons may be glutamatergic neurons. In general, the cortex is dominated by glutamatergic neurons, which account for about 80% of the total, with about 20% of other GABAergic neurons. Further, the remote projection neurons in the cortex are generally dominated by glutamatergic neurons, and it is the GABAergic neurons in the subcortical brain regions that are capable of remote projection. Our team has previously observed the projection of GABAergic neurons in the anterior cingulate cortex and found that only some of them projected to the dorsal side of the caudate putamen (striatum) and did not continue to project downstream (no projection fibers were observed in the VLT). Taken together, we believe that cACC (Cg2) glutamatergic neurons may mediate the anxiety-like behaviors and anxiolytic effects of berberine in mice treated with CFA.
Thank you again for your suggestion, we have added the above relevant content to the Discussion section of the revised manuscript (see lines 379-391 for details).

2.Authors found that one week after CFA injection, anxiety-like behaviors could be evoked by the activation of VLT-projecting cACC (Cg2) neurons. Authors also found that activation of VLT-projecting cACC (Cg2) neurons induced anxiety-like behaviors in normal mice. These two results seem contradictory. The author should explain it clearly.
Re: Thank you for your suggestion. We have focused on two main time points in our observation of the CFA-induced chronic pain model. One was one week after CFA injection, and at this time point, although the mice showed a significant decrease in pain threshold, they did not show anxiety-like behaviors (Figure 2F, H). It was not until two weeks after CFA injection that we found anxiety-like behaviors in the CFA mice (Figure 2D, F). The above results suggest that CFA-induced chronic pain needs to last for a certain period of time (two weeks) to induce anxiety-like behaviors.
Based on the above characteristics of the CFA chronic pain model, we performed the activation of VLT-projecting cACC (Cg2) neurons one week after CFA injection to observe whether anxiety-like behaviors could be induced earlier. Moreover, we also observed whether the activation of the VLT-projecting cACC (Cg2) neurons could directly induce anxiety-like behaviors under normal conditions.
Thank you again for your suggestion, we have added the above relevant content to the Discussion section of the revised manuscript (see lines 352-364 for details).

3.The abstract is not concise enough and should be rewritten.

Re: Thanks to your suggestion, we have simplified and rewritten the abstract. Please see the Abstract section of the revised manuscript for more details (highlighted in red).

4.The discussion section should be further enriched. e.g why berberine acts on VLT-projecting cACC rather that other regional neurons, and etc.

Re: Thank you for reviewing our manuscript and for your valuable comments. Your question about whether berberine has an effect on neurons in other brain regions is a good one that relates to the depth and breadth of our study.

In the present study, we focused on exploring the effect of berberine on VLT-projecting cACC (Cg2) neurons, which is based on the foundation of our previous studies and the relevant neural circuitry observed in pathological states. Our experiments were designed to verify whether berberine could intervene in this specific neural circuit, and our results show that berberine does have an effect on VLT-projecting cACC (Cg2) neurons.

Regarding whether berberine is involved or not in the regulation of pain associated anxiety by other brain regions, our current study did not address this and therefore cannot provide direct evidence. However, we believe this is an important question that deserves to be further explored in future studies.

In response to your concerns, we have added the following to the Discussion section (see lines 438-446 for details):

“Although our findings suggest that berberine can significantly affect VLT-projecting cACC neurons, we recognize that it is unclear whether berberine has similar effects on neurons in other brain regions. This is an important question that may affect the clinical use of berberine. We suggest that future studies could explore the effects of berberine on the whole brain, such as brain regions, nerual circuits, as well as the molecular mechanisms and functional consequences of these potential effects. Such studies will contribute to a comprehensive understanding of berberine's mechanism of action and provide additional information for clinical treatment.”

Thank you again for your valuable time and professional advice, which provides very important guidance for our team's subsequent research.

5.There are some spelling errors in manuscript. e.g. DREADD hM3D, Cre-independent, and etc.

Re: First of all, I would like to express my sincere admiration and gratitude for your rigorous and meticulous attitude. In the newly revised manuscript, we have changed “hM3D” to “hM3Dq” and “Cre-independent” to “Cre-dependent”. We have also changed “hM4D” to “hM4Di”. All relevant descriptions, both in the main text section and in the figure notes section (which are labeled in red). Corresponding descriptions in the figures have also been revised (including the text image and supplementary figures).

Reviewer #2 (Comments to the Author):

The review of the manuscript entitled: “Berberine alleviates comorbid anxiety symptoms in chronic pain by inhibiting the activation of VLT-projecting cACC (Cg2) neurons in male mice"

The study aimed to investigate the effect of Berberine in alleviating comorbid anxiety symptoms (CAS) with chronic pain. For this purpose, authors first clarified that those neurons of the caudal ACC (Cg2), but not the (Cg1), project to the ventral lateral thalamus (VLT). Then, a persistent pain model established by unilateral plantar injection of complete Freund’s adjuvant (CFA) and stable anxiety-like behaviors investigated until pain persisted for two weeks. Authors then activated VLT-projecting (Cg2) neurons in CFA-treated mice without anxiety-like behaviors and in normal mice to induce anxiety-like behaviors. They also inhibited the activation of VLT-projecting (Cg2) neurons in CFA-treated mice with anxiety-like behaviors and observed that these behaviors were alleviated. Finally, they screened the effective dose of BBR for anxiolysis in CFA-treated mice with anxiety-like behaviors and they reached this conclusion that BBR alleviates CAS in chronic pain by inhibiting the activation of VLT-projecting cACC (Cg2) neurons. The topic is interesting and the study is well designed and nicely written.

Comments for Authors:
Thank you for the valuable research you have done. The topic is interesting and novel in the field. The paper is nicely written and it is overall understandable. However, there is one comment to improve your work:
1) Unlike in abstract, the conclusions section is missed at the end of the paper. Please include conclusions after discussion.
Re: Thank you very much for your advice. We have added a conclusion section at the end of the discussion and highlighted it in red. Please see lines 448-451 for more details.