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

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Chronic pain is often accompanied by anxiety, and gradually increasing anxiety makes the pain itself more protracted. Berberine has been found to be able to cross the blood–brain barrier to treat psychiatric disorders, but its neurocirculatory mechanisms remain unclear. Here, we found that neurons in cingulate area 2 (Cg2) of the caudal anterior cingulate cortex (cACC), but not in Cg1 of the cACC, projected to the ventral lateral thalamus (VLT). Next, we induced chronic inflammatory pain by plantar injection of complete Freund's adjuvant (CFA) and observed stable anxiety-like behaviors until two weeks postinjection. We specifically activated VLT-projecting cACC (Cg2) neurons in one-week-old CFA-induced mice without anxiety-like behaviors and in normal control mice to induce anxiety-like behaviors. We inhibited the activation of VLT-projecting cACC (Cg2) neurons in two-week-old CFA-treated mice with anxiety-like behaviors and observed that their anxiety-like behaviors were alleviated. On this basis, we further screened the effective dose of berberine for anxiolysis in two-week-old CFA-treated mice. We observed that the effective dose of berberine obtained above decreased the activity of VLT-projecting cACC (Cg2) neurons. The activation of VLT-projecting cACC (Cg2) neurons abrogated the anxiolytic effect of berberine in two-week-old CFA-treated mice.

Comorbid anxiety symptoms (CAS) associated with chronic pain frequently occur<sup>1</sup>. Increasing evidence shows that CAS could be one of the important reasons for the chronification of pain<sup>2,3</sup>. CAS often appears first, and persistent anxiety further causes a series of more serious diseases, such as depression, sleep disturbance, and cognitive impairment<sup>4–6</sup>. To prevent CAS and chronic pain from interacting with each other and further resulting in more severe symptoms, greater clinical burden, and greater treatment difficulty, in-depth research on the pathological mechanisms of CAS is urgently needed.

The anterior cingulate cortex (ACC) is a key cortical area closely associated with the CAS in chronic pain<sup>7,8</sup>. Multiple afferent projections of the ACC have previously been shown to regulate CAS in chronic pain<sup>9,10</sup>. However, whether the efferent projections of the ACC are associated with CAS in chronic pain has rarely been reported.

The ACC is generally divided into rostral and caudal parts. In our previous studies, whole-brain efferent connections of the rostral ACC (rACC) revealed that the ventral lateral thalamus (VLT) is one of the main descending projections of the rACC<sup>11</sup>. Magnetic resonance studies of the human brain have shown a strong correlation between anxiety symptoms caused by painful experiences and VLT<sup>12</sup>. Deep brain stimulation via the VLT can alleviate anxiety and improve patient quality of life<sup>13</sup>. Our previous study also revealed that VLT-projecting rACC neurons are involved in the regulation of CAS in chronic pain. However, it is unclear whether the caudal ACC (cACC) projects to the VLT or whether VLT-projecting cACC neurons are involved in CAS in chronic pain.

Berberine (BBR) is a compound isolated from the Chinese medicinal plant *Coptis chinensis*<sup>14</sup>. BBR has received increasing attention because of its ability to cross the blood–brain barrier easily and its therapeutic effects on

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psychiatric disorders<sup>15</sup>. Previous studies on the anxiolytic properties of BBR have focused predominantly on its molecular mechanism<sup>16</sup>. However, it is unclear whether BBR has a therapeutic effect on CAS in chronic pain and whether BBR acts by interfering with related neural circuits.

In this study, we first microinjected anterograde and retrograde tracer adeno-associated virus (AAV) and clarified the VLT-projecting cACC subregion (cingulate area 1 (Cg1) and/or cingulate area 2 (Cg2)). Next, we imitated a persistent pain model by unilateral plantar injection of complete Freund's adjuvant (CFA) and investigated stable anxiety-like behaviors until the pain persisted for two weeks. We then specifically activated VLT-projecting cACC neurons in mice one week after CFA injection (without anxiety-like behaviors) and normal control mice to observe whether anxiety-like behaviors appeared and inhibited the activation of this circuit in mice with anxiety-like behaviors two weeks after CFA injection to determine whether anxiety-like behaviors could be alleviated. Finally, we investigated the effect of the intervention and effective dose of BBR on CAS in chronic pain and this circuit.

## Results

### cACC (Cg2) neurons project to the VLT

To determine whether cACC neurons project to the VLT, we first delivered an anterograde tracer AAV (AAV2/9-hSyn-EGFP) into the cACC (Cg1) or cACC (Cg2) (Supplementary Fig. 1a, b, d, e). We found that neurons in the cACC (Cg2), but not in the cACC (Cg1), projected to the VLT (Supplementary Fig. 1c, f). To further confirm these results, we delivered a retrograde tracer, AAV (AAV2/Retro-hSyn-tdTomato), into the VLT (Supplementary Fig. 1g, h). The somata in the cACC (Cg2), but not in the cACC (Cg1), were infected via retrograde tracing from the VLT (Supplementary Fig. 1i). These results suggest that cACC (Cg2) neurons project to the VLT.

### CFA injection evoked anxiety-like behaviors two weeks after injection

To explore the role of VLT-projecting cACC (Cg2) neurons in CAS associated with chronic pain, we established a persistent pain model via unilateral plantar injection of CFA (Fig. 1a, b). The results of the mechanical pain tests revealed that the mechanical hyperalgesia induced by CFA was maintained until 16 days after CFA injection (Fig. 1c). The results of the

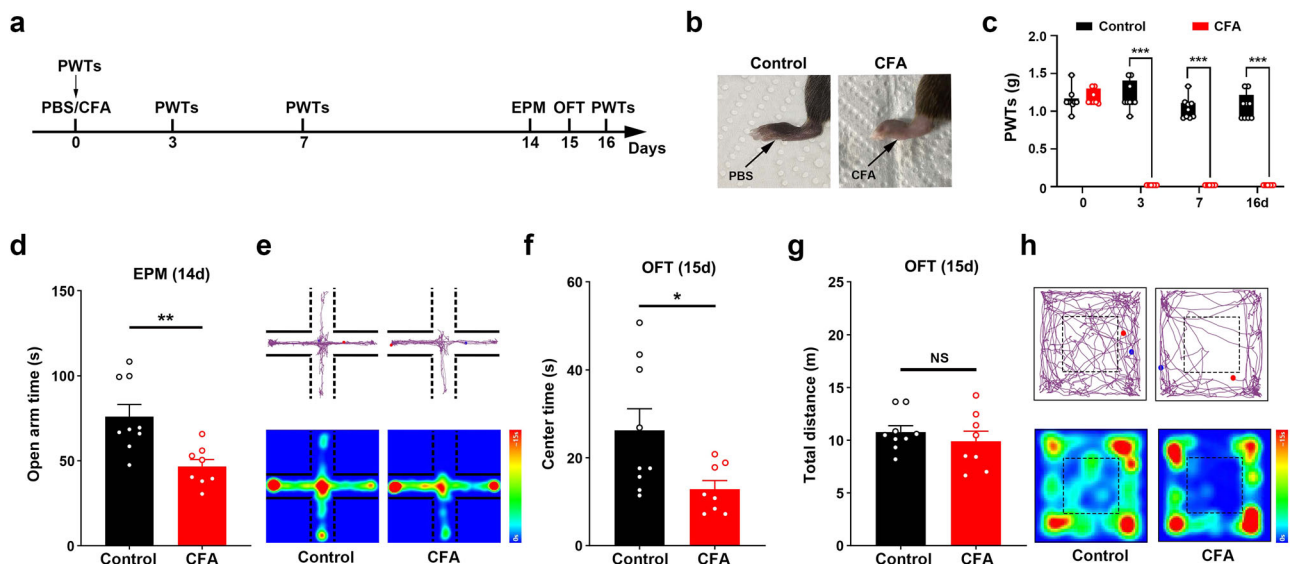
elevated plus maze (EPM) test revealed that the mice spent significantly less time moving in the open arms on Day 14 after the injection of CFA (Fig. 1d). The open field test (OFT) results revealed that the mice spent significantly less time moving in the center area on Day 15 after the injection of CFA (Fig. 1e). These results suggest that CFA caused significant anxiety-like behaviors two weeks after injection.

### One week after CFA injection, anxiety-like behaviors could be evoked by the activation of VLT-projecting cACC (Cg2) neurons

To observe the effects of VLT-projecting cACC (Cg2) neurons on chronic pain without CAS, we first examined anxiety-like behavioral performance in mice one week after CFA injection. The results of both the EPM test and OFT revealed that CFA did not induce significant anxiety-like behaviors one week after injection (Fig. 2f–j). To specifically activate VLT-projecting cACC (Cg2) neurons one week after CFA injection, we delivered the retrograde transport virus AAV2/Retro-hSyn-Cre into the VLT, and a Cre-dependent virus encoding the neuronal activator DREADD hM3Dq was injected into the cACC (Cg2) (Fig. 2a–c). An examination of the coexistence of c-Fos with AAVs (mCherry-labeled), revealed a significant increase in neuronal activity in VLT-projecting cACC (Cg2) neurons labeled with hM3Dq (Fig. 2d, e). The results of the EPM test revealed that mice, with specifically activated VLT-projecting cACC (Cg2) neurons, spent significantly less time moving in the open arms on Day 7 after injection of CFA (Fig. 2f, g). The results of the OFT revealed that mice, with specifically activated VLT-projecting cACC (Cg2) neurons, spent significantly less time moving in the center area on Day 8 after injection of CFA (Fig. 2h–j). These results suggest that the activation of VLT-projecting cACC (Cg2) neurons induces anxiety-like behaviors in chronic pain model mice without CAS.

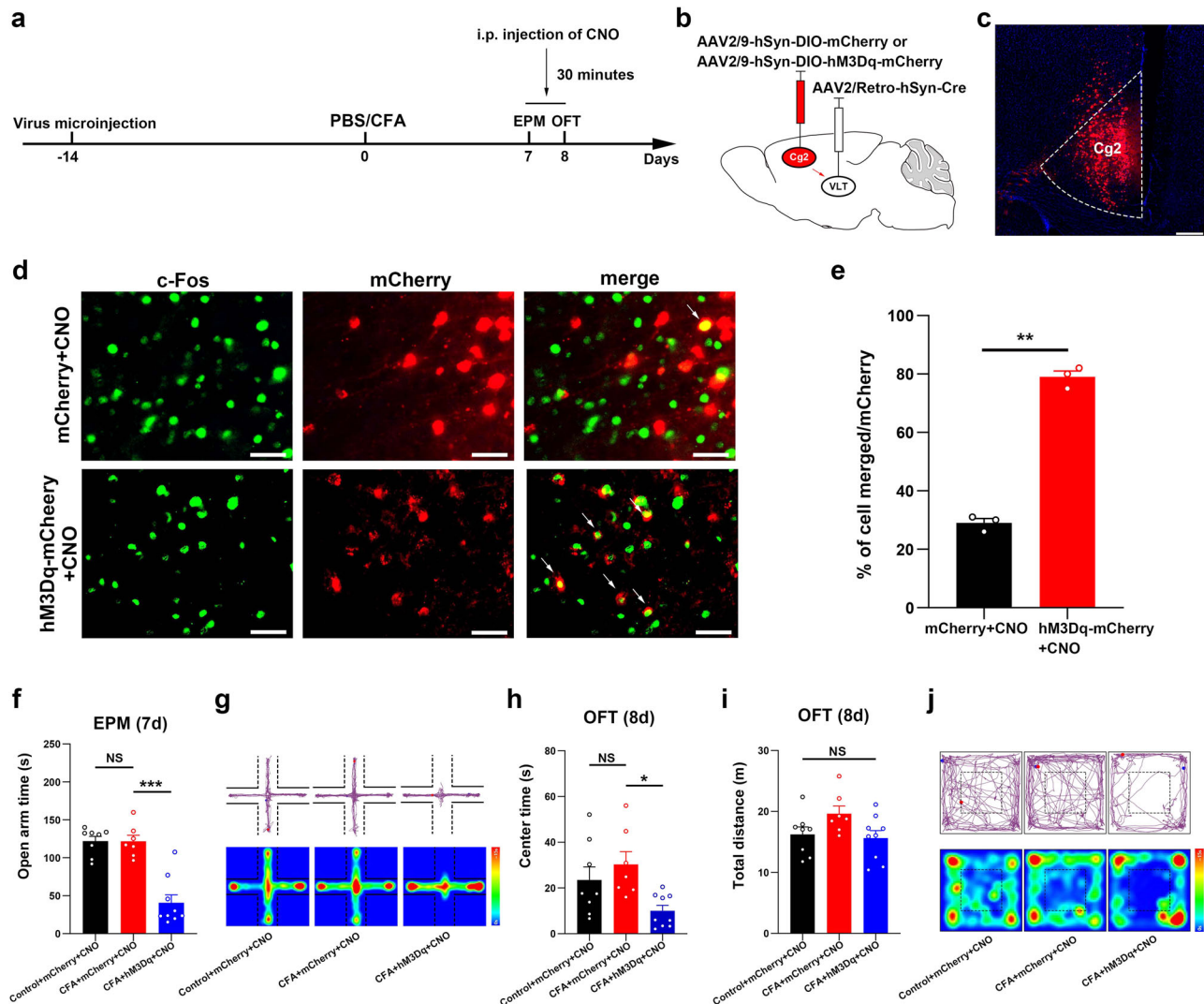
### Activation of VLT-projecting cACC (Cg2) neurons induced anxiety-like behaviors in normal mice

To observe whether the activation of VLT-projecting cACC (Cg2) neurons induces anxiety-like behaviors in normal mice, we delivered the retrograde transport virus AAV2/Retro-hSyn-Cre into the VLT, and a Cre-dependent virus encoding the neuronal activator DREADD hM3Dq was injected into the cACC (Cg2) (Supplementary Fig. 2a–c). The results of the EPM and OFT revealed that mice, with specifically activated VLT-projecting cACC (Cg2) neurons, spent significantly less time in the open arms and center area



**Fig. 1 | Anxiety-like behaviors were evoked two weeks after CFA injection.** **a** Schematic of the experimental design. **b** Gross macroscopic view of left hindpaw after CFA or PBS injection into the left hindpaw of mice. **c** Statistical results of 50% paw withdrawal thresholds (PWTs). **d** Statistical results of time spent in the open arms in the EPM test. **e** Representative images of movement trajectory (top) and

activity time (bottom) in the EPM test. **f, g** Statistical results of time spent in the central area and total distance traveled in the OFT. **h** Representative images of movement trajectory (top) and activity time (bottom) in the OFT. Data are the mean  $\pm$  SEM; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.01$ . CFA complete Freund's adjuvant, EPM elevated plus maze, OFT open field test.



**Fig. 2 | After one week of CFA injection, anxiety-like behaviors could be evoked by activation of VLT-projecting cACC (Cg2) neurons.** **a** Schematic of the experimental design. **b, c** Schematic for specific infection of VLT-projecting cACC (Cg2) neurons with mCherry or hM3Dq-mCherry. Scale bar, 200  $\mu$ m. **d** Representative images showed that intraperitoneal injection of CNO induced c-Fos expression in VLT-projecting cACC (Cg2) neurons with mCherry or hM3Dq-mCherry. Scale bar, 20  $\mu$ m. **e** Percentage of labeled mCherry<sup>+</sup> neurons expressing c-Fos in mCherry+CNO group and hM3Dq-mCherry+CNO group. **f** Statistical

results of time spent in the open arms in the EPM test. **g** Representative images of movement trajectory (top) and activity time (bottom) in the EPM test. **h, i** Statistical results of time spent in the central area and total distance traveled in the OFT. **j** Representative images of movement trajectory (top) and activity time (bottom) in the OFT. Data are the mean  $\pm$  SEM; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , NS not significant. CFA complete Freund's adjuvant, VLT ventral lateral thalamus, cACC caudal anterior cingulate cortex, Cg2 cingulate area 2, CNO Clozapine N-oxide, EPM elevated plus maze, OFT open field test.

(Supplementary Fig. 2d–h). These results suggest that the activation of VLT-projecting cACC (Cg2) neurons induces anxiety-like behaviors in normal mice.

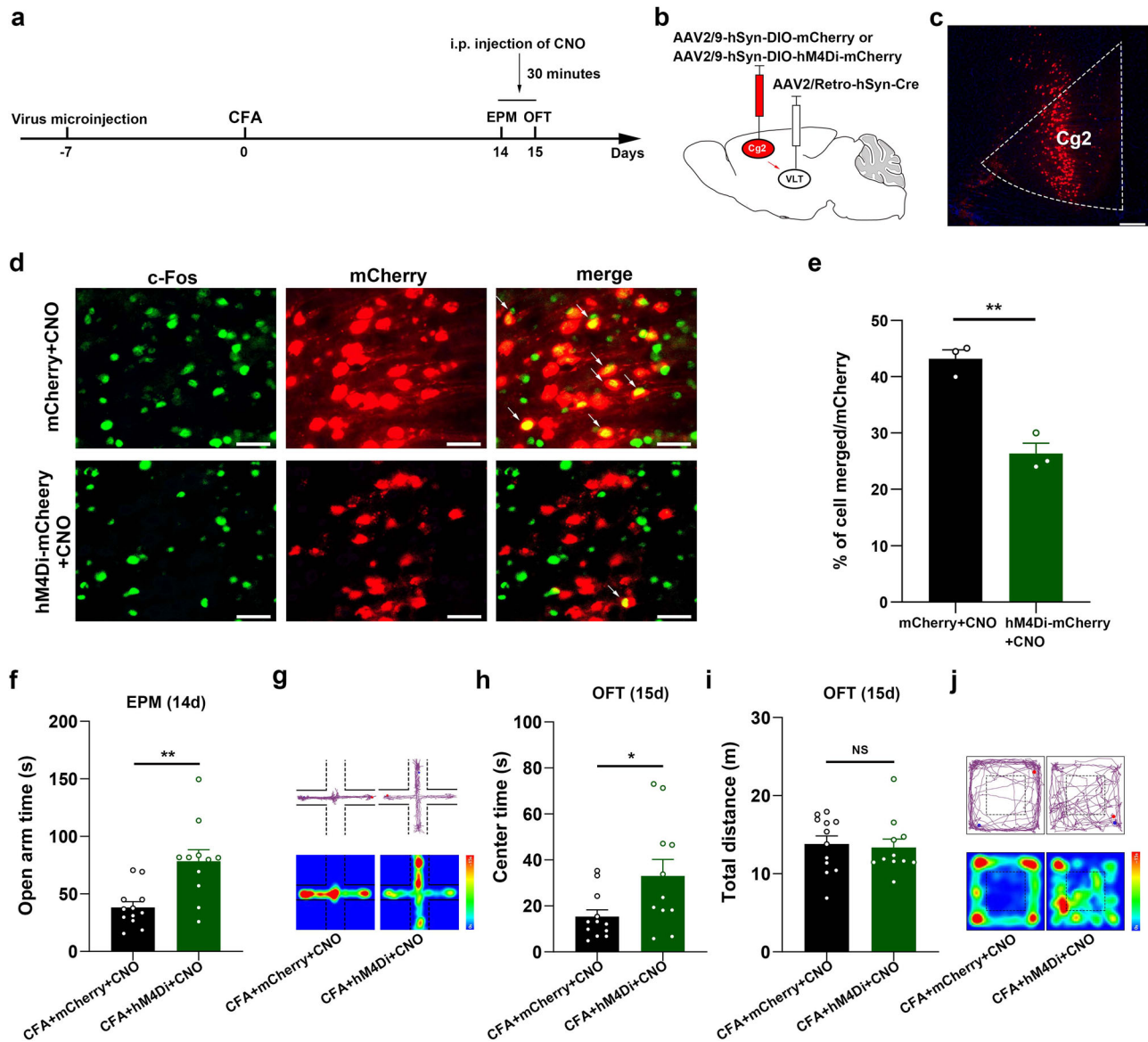
### Inhibition of VLT-projecting cACC (Cg2) neurons alleviated anxiety-like behaviors induced by CFA

To observe the effects of VLT-projecting cACC (Cg2) neuron activation on CAS in chronic pain, we delivered the retrograde transport virus AAV2/Retro-hSyn-Cre into the VLT, and a Cre-dependent virus encoding the neuronal inhibitor DREADD hM4Di was injected into the cACC(Cg2) (Fig. 3a–c). An examination of the coexistence of c-Fos with the AAVs (mCherry-labeled) revealed a significant decrease in neuronal activity in VLT-projecting cACC(Cg2) neurons labeled with hM4Di (Fig. 3d, e). The results of the EPM test revealed that mice in which VLT-projecting cACC (Cg2) neuron activation was specifically inhibited spent significantly more time moving in the open arms on Day 14 after injection of CFA (Fig. 3f, g). The OFT results revealed that mice in which VLT-projecting cACC (Cg2) neuron activation was specifically inhibited spent significantly more time

moving in the center area on Day 15 after injection of CFA (Fig. 3h–j). These results suggest that the inhibition of VLT-projecting cACC (Cg2) neuron activation alleviates anxiety-like behaviors in chronic pain model mice.

### BBR alleviated anxiety-like behaviors induced by CFA

To observe the effect of BBR on CAS in chronic pain, we selected different concentrations of BBR (2.5, 5 and 10 mg/kg/day) for effective dose screening (Fig. 4a). We first observed the effect of BBR on the mechanical pain threshold and found that different doses of BBR (2.5, 5 and 10 mg/kg/day) did not alleviate mechanical hyperalgesia (Fig. 4b). The results of the EPM test revealed a significant increase in the activity time in the open arms of the mice after intraperitoneal (i.p.) injection of 10 mg/kg BBR, but no significant changes were observed for either 2.5 mg/kg or 5 mg/kg BBR (Fig. 4c). The OFT results also revealed a significant increase in the activity time in the center area in mice after i.p. injection of 10 mg/kg BBR, but no significant changes were observed for either 2.5 mg/kg or 5 mg/kg BBR (Fig. 4d, e). These results suggest that 10 mg/kg BBR alleviated anxiety-like behaviors in mice after two weeks of CFA injection.



**Fig. 3 | Inhibition of VLT-projecting cACC (Cg2) neuron activation alleviated anxiety-like behaviors induced by CFA.** **a** Schematic of the experimental design. **b, c** Schematic for specific infection of VLT-projecting cACC (Cg2) neurons with mCherry or hM4Di-mCherry. Scale bar, 200  $\mu$ m. **d** Representative images showed that intraperitoneal injection of CNO induced c-Fos expression in VLT-projecting cACC (Cg2) neurons with mCherry or hM4Di-mCherry. Scale bar, 20  $\mu$ m. **e** Percentage of labeled mCherry<sup>+</sup> neurons expressing c-Fos in mCherry+CNO group and hM4Di-mCherry+CNO group. **f** Statistical results of time spent in the

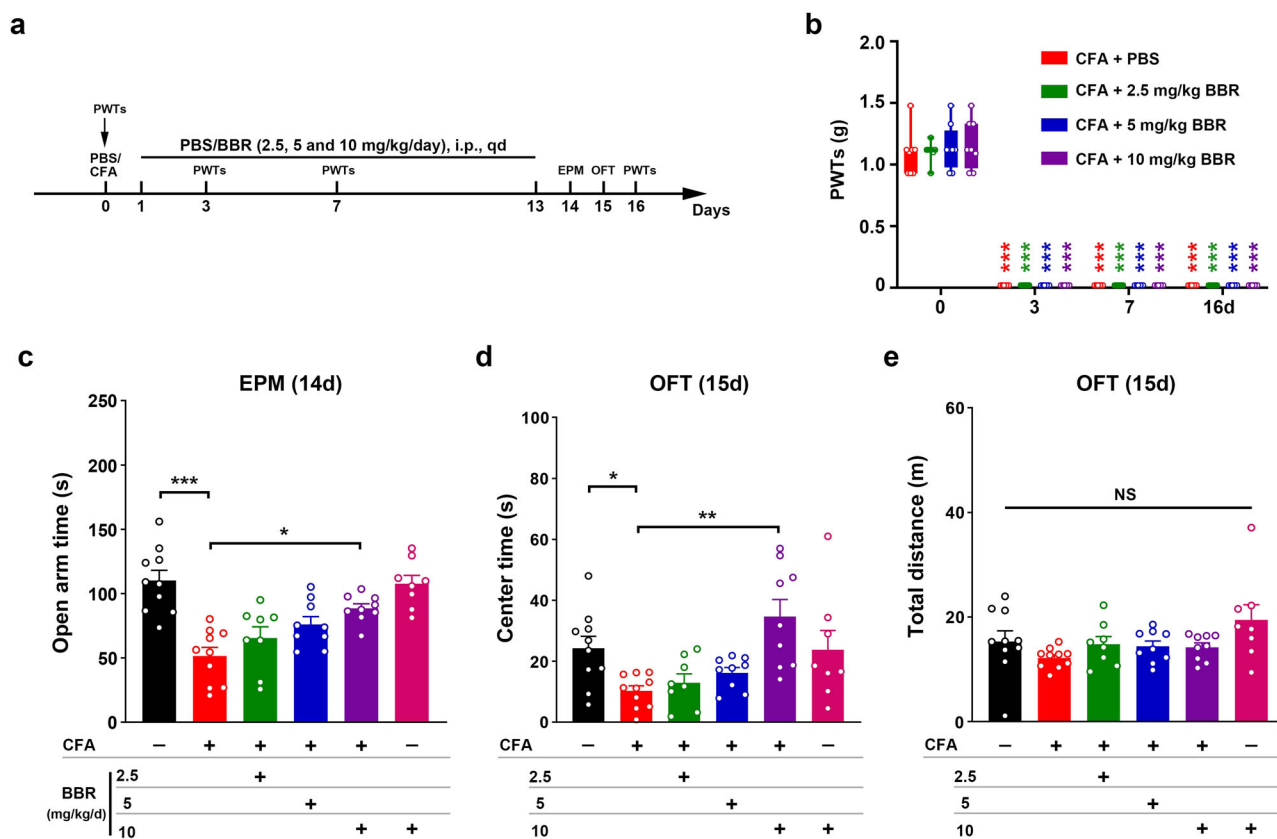
open arms in the EPM test. **g** Representative images of movement trajectory (top) and activity time (bottom) in the EPM test. **h, i** Statistical results of time spent in the central area and total distance traveled in the OFT. **j** Representative images of movement trajectory (top) and activity time (bottom) in the OFT. Data are the mean  $\pm$  SEM; \* $P < 0.05$ , \*\* $P < 0.01$ , NS not significant. CFA complete Freund's adjuvant, VLT ventral lateral thalamus, cACC caudal anterior cingulate cortex, Cg2 cingulate area 2, CNO Clozapine N-oxide, EPM elevated plus maze, OFT open field test.

**Inhibition of VLT-projecting cACC (Cg2) neurons is required for the ability of BBR to relieve anxiety-like behaviors induced by CFA**

To further demonstrate whether 10 mg/kg BBR exerts anxiolytic effects through VLT-projecting cACC (Cg2) neurons, we first observed changes in the activity of VLT-projecting cACC (Cg2) neurons after 10 mg/kg BBR intervention (Fig. 5a). We labeled VLT-projecting cACC (Cg2) neurons by injecting the retrograde transport virus AAV2/Retro-hSyn-Cre into the VLT and a Cre-dependent virus (mCherry-labeled) into the cACC (Cg2) (Fig. 5b, c). On the basis of the results of the EPM and OF tests, which demonstrated that BBR indeed alleviated anxiety-like behaviors (Fig. 5d–h), we examined the effect of BBR on the activity of VLT-projecting cACC (Cg2) neurons. When c-Fos was coexpressed with the AAVs (mCherry-labeled), a significant decrease in neuronal activity was observed in VLT-projecting cACC (Cg2) neurons after 10 mg/kg BBR intervention (Fig. 5i, j).

These results suggest that the activity of VLT-projecting cACC (Cg2) neurons decreases, when BBR alleviates anxiety-like behaviors in CFA-treated mice.

To confirm whether 10 mg/kg BBR exerts an anxiolytic effect by inhibiting VLT-projecting cACC (Cg2) neurons, we specifically activated VLT-projecting cACC (Cg2) neurons at the same time as BBR intervention to observe whether the anxiolytic effect of BBR was reversed (Fig. 5k–m). The results of the EPM test revealed that BBR increased the activity of CFA-treated mice in the open arms, but the activity of CFA-treated mice in the open arms was reduced again after VLT-projecting cACC (Cg2) neurons were activated (Fig. 5n, o). In the OFT, BBR increased the activity time of CFA-treated mice in the center area of the open field, but after VLT-projecting cACC(Cg2) neurons were activated, the activity time of CFA mice in the center area of the open field was significantly reduced (Fig. 5p–r).



**Fig. 4 | BBR alleviated anxiety-like behaviors induced by CFA. a** Schematic of the experimental design. **b** Statistical results of 50% paw withdrawal thresholds (PWTs). \*\*\**P* < 0.001 vs. baseline in the same group. **c–e** Statistical results of time spent in the open arms in the EPM test and the central area in the OFT after different gradient

concentrations of BBR (2.5, 5 and 10 mg/kg/day). Data are the mean ± SEM; \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, NS not significant. BBR Berberine, CFA complete Freund’s adjuvant, EPM elevated plus maze, OFT open field test.

The above results suggest that the anxiolytic effect of BBR in CFA-treated mice is eliminated by the activation of VLT-projecting cACC (Cg2) neurons. Therefore, BBR may exert its anxiolytic effect in CFA-treated mice by inhibiting VLT-projecting cACC (Cg2) neurons.

### Discussion

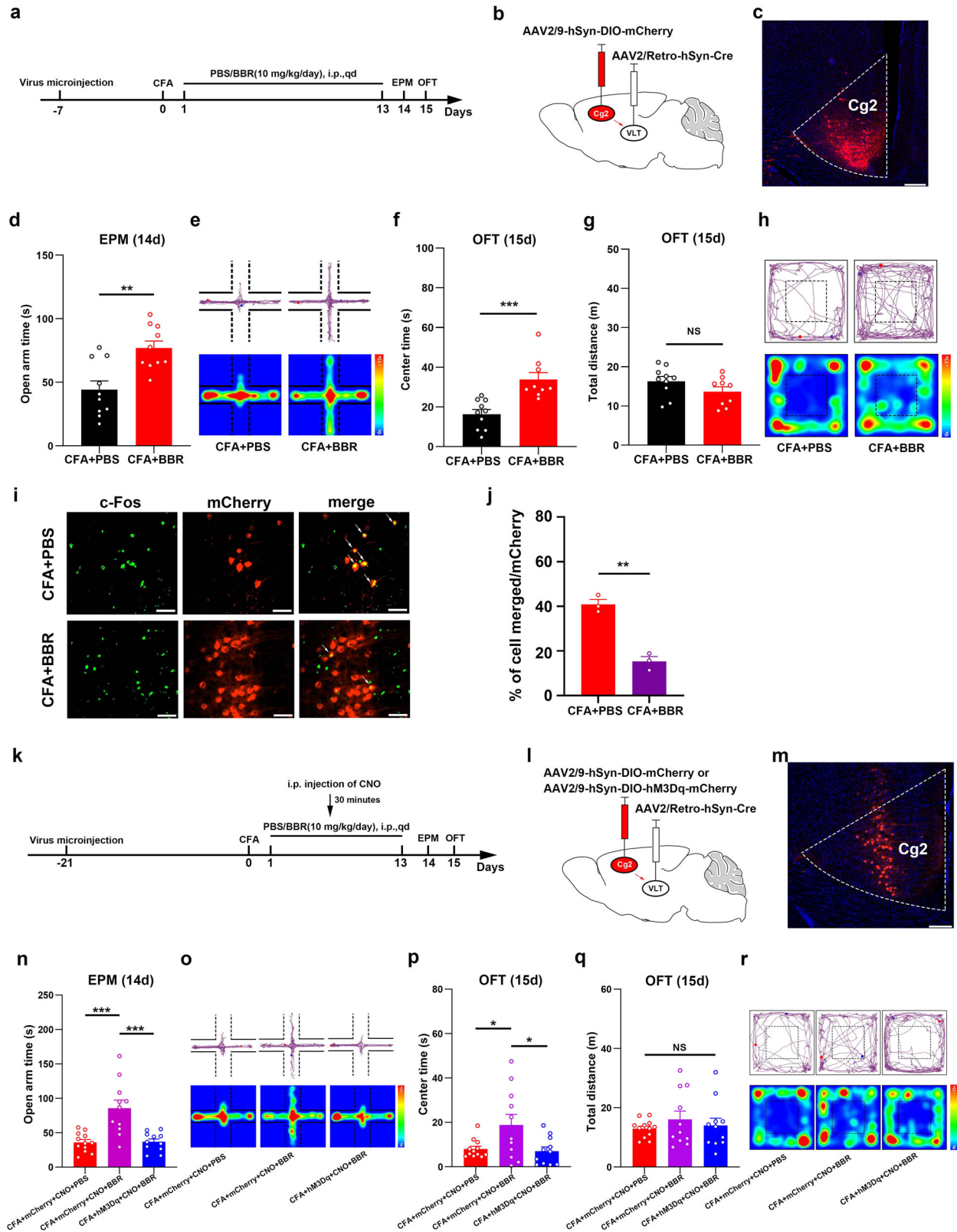
In this study, we first microinjected anterograde and retrograde tracer AAVs and clarified that neurons in the cACC (Cg2), but not in the rACC (Cg1), project to the VLT. Next, we established a persistent pain model by unilateral plantar injection of CFA and investigated stable anxiety-like behaviors until pain persisted for two weeks. We then specifically activated VLT-projecting cACC (Cg2) neurons in CFA-treated mice without anxiety-like behaviors and in normal mice to induce anxiety-like behaviors. We inhibited the activation of VLT-projecting cACC (Cg2) neurons in CFA-treated mice with anxiety-like behaviors and observed that these behaviors were alleviated. On this basis, we further screened the effective dose of BBR for anxiolysis in CFA-treated mice with anxiety-like behaviors. We observed that the effective dose of BBR obtained above decreased the activity of VLT-projecting cACC (Cg2) neurons. The activation of VLT-projecting cACC (Cg2) neurons reversed the anxiolytic effect of BBR in CFA-treated mice. Together, the data of this study suggest that BBR alleviates CAS in chronic pain by inhibiting the activation of VLT-projecting cACC (Cg2) neurons.

The ACC is a central hub for the development of comorbid mental disorders associated with chronic pain<sup>17</sup>. The ACC is divided into the rACC and cACC. The rACC is dominated by the Cg1 region. The cACC consists of two regions, Cg1 and Cg2, together. Our preliminary studies and others have shown that the rACC (Cg1) is strongly associated with pain-induced negative emotions, independent of pain itself<sup>18–20</sup>. Other studies have shown that in addition to the rACC (Cg1), the cACC (Cg1) is also involved in modulating pain affect<sup>21</sup>. Although the literature suggests that Cg2 is the

subregion in the ACC with the strongest connections to other brain regions and is recommended as a potential target for treating neuropsychiatric disorders<sup>22</sup>, relatively few in-depth studies on the involvement of the cACC (Cg2) in the development of CAS exist.

The ACC internal signaling and descending projections are the key parts of top-down processing<sup>23</sup>. Understanding these internal processing dynamics can help us better understand the expression processing of inner emotions. The VLT used to be considered an internal thalamic region that encodes motor information<sup>24</sup>. In addition to cerebellar and basal ganglia afferents (related to motor function), the VLT receives pronounced input from frontal areas and the prefrontal cortex<sup>25</sup>. The VLT could be a key hub that integrates multiple types of information to make motor decisions or choices<sup>26</sup>. Recent studies have shown that the VLT is necessary for preparing and planning movements<sup>27,28</sup>. CAS tends to cause avoidance behaviors in rodents, which could be caused by negative motor planning (anticipatory) induced by internal negative emotion<sup>29–31</sup>. Therefore, we hypothesize that there could be a corticothalamic connection between the cACC (Cg2) and VLT that manipulates the explicit expression of inner emotions.

BBR is a promising agent for anxiety symptoms in various neuropsychiatric diseases<sup>32,33</sup>. Xinlu Li et al.<sup>34</sup> indicated that BBR mitigated the degree of anxiety and anxiety-like behaviors in transgenic mice with Alzheimer’s disease. Yuan Fang et al.<sup>35</sup> observed that ovariectomy significantly aggravated anxiety-like behaviors and that BBR ameliorated ovariectomy-induced anxiety-like behaviors. Additionally, BBR could be used as a potential agent for the treatment of methamphetamine addiction-induced anxiety symptoms<sup>36</sup> and anxiety-like behaviors caused by post-traumatic stress disorder<sup>37</sup>. In the present study, we focused on the interventional effects of BBR on chronic pain-induced anxiety-like behaviors and reported that BBR significantly alleviated chronic inflammatory pain-induced



anxiety-like behaviors. This study expands the spectrum of diseases that can be treated with BBR.

Since the discovery of the ability of BBR to penetrate the blood-brain barrier, BBR has been increasingly researched for the treatment of disorders of brain function. Zongshi Qin et al.<sup>16</sup> reported that the anxiolytic and antidepressant effects of BBR were accompanied by the inhibition of the

NLRP3 inflammasome in the prefrontal cortex and hippocampus. Bombi Lee et al.<sup>38</sup> indicated that BBR attenuated anxiety-like behaviors by blocking the increase in hypothalamic corticotrophin-releasing factor expression and tyrosine hydroxylase expression in the locus coeruleus. Pingyuan Ge et al.<sup>39</sup> reported a therapeutic role of BBR in anxiety and depression through the downregulation of glutamate levels in the cortex and striatum. Currently,

**Fig. 5 | Inhibition of VLT-projecting cACC (Cg2) neuron activation was required for the effect of BBR on relieving anxiety-like behaviors induced by CFA.**

**a** Schematic of the experimental design. **b, c** Schematic for specific infection of VLT-projecting cACC (Cg2) neurons with mCherry. Scale bar, 200  $\mu$ m. **d–h** Statistical results of time spent in the open arms in the EPM test and the central area in the OFT after intraperitoneal injection of BBR (10 mg/kg/day). **i** Representative images showed that intraperitoneal injection of PBS or BBR induced c-Fos expression in VLT-projecting cACC (Cg2) neurons with mCherry. Scale bar, 20  $\mu$ m. **j** Percentage of labeled mCherry<sup>+</sup> neurons expressing c-Fos in CFA + PBS group and CFA + BBR group. **k** Schematic of the experimental design. **l, m** Schematic for specific

infection of VLT-projecting cACC (Cg2) neurons with mCherry or hM3Dq-mCherry. **n** Statistical results of time spent in the open arms in the EPM test. **o** Representative images of movement trajectory (top) and activity time (bottom) in the EPM test. **p, q** Statistical results of time spent in the central area and total distance traveled in the OFT. **r** Representative images of movement trajectory (top) and activity time (bottom) in the OFT. Data are the mean  $\pm$  SEM; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , NS not significant. VLT ventral lateral thalamus, cACC caudal anterior cingulate cortex, Cg2 cingulate area 2, BBR Berberine, CFA complete Freund's adjuvant, EPM elevated plus maze, OFT open field test, CNO Clozapine N-oxide.

studies on neurocircuitry related to the anxiolytic effects of BBR are lacking. The present study expands our understanding of the conventional anxiolytic targets of BBR. The Findings of this work prompted us to further explore the antipsychotic effects of BBR from the perspective of brain neural connections in the future.

## Methods

### Experimental animals

In the present study, we used male C57BL/6J mice (aged 8–10 weeks) to carry out the experiments. All the mice were purchased from Vital River Laboratories (Jiaxing, China). The mice were housed under standard environmental conditions at 23–25 °C, 40–60% humidity and a 12-h light–dark cycle (lights on at 8:00 am) and were given free access to food and water. All animal care and experimental studies followed the Guide for the Care and Use of Laboratory Animals. All animal experiments were approved by the Laboratory Animal Management and Welfare Ethical Review Committee of Zhejiang Chinese Medical University. We have complied with all relevant ethical regulations for animal use.

### Animal models of CAS induced by chronic inflammatory pain

CFA was used to establish a chronic inflammatory pain model and induce anxiety-like behaviors<sup>40,41</sup>. CFA (4 mg/mL heat-killed *Mycobacterium tuberculosis* H37 RA, Chondrex, Catalog #7001, USA) and phosphate-buffered saline (PBS, Solarbio, Catalog #P1020, China) were mixed at a ratio of 1:1.5 to form water-in-oil emulsions of immunogens. Under brief anesthesia with isoflurane, a 1 mL BD insulin syringe was used to inject the immunogens (20  $\mu$ L) into the plantar surface of the left hind paw of each mouse to establish a chronic inflammatory pain model and induce anxiety-like behaviors at two weeks after CFA injection (Figs. 1d–h and 2f–j). The control mice were injected in the same manner but with only 20  $\mu$ L of PBS.

### Anxiety-like behavior tests

Anxiety-like behaviors in the mice were evaluated by the EPM test and the OFT. The EPM test, which was performed 35 cm above the floor, consisted of two open arms (30  $\times$  6 cm), two opposing closed arms (30  $\times$  6  $\times$  15 cm) and a central platform (6  $\times$  6 cm). The open and closed arms were placed vertically in a cross. An ANY-maze video tracking system (ANY-maze, Stoelting, USA) was used to monitor and record the behaviors of the mice in the EPM. The activity of the mice in the EPM test was recorded for 5 min. To prevent olfactory cue bias, we used 75% alcohol to wash the apparatus after each test. Each behavioral test (including the EPM test, OFT, and mechanical pain test) was performed by a dedicated researcher.

The OFT was performed in a square acrylic box (40  $\times$  40  $\times$  40 cm). We divided the bottom evenly into 16 squares of equal area by the ANY-maze video tracking system. The outer 12 squares were defined as the peripheral region and the middle 4 squares were defined as the central region. The activity of the animals in the OFT were monitored and recorded in real time via an ANY-maze video tracking system. The activity of the mice was recorded for 5 min in the OFT. To prevent olfactory cue bias, we used 75% alcohol to wash the apparatus after each test.

### Mechanical pain tests

We used von Frey filaments (0.02–1.4 g, Touch-Test Sensory Evaluator, North Coast Medical, Inc., Gilroy, CA, USA) to test the 50% paw withdrawal thresholds (PWTs) in the left hind paws of the mice via the up-down method. For adaptation, the mice were individually placed in a transparent box on a raised wire mesh screen for 20 min. Filaments were applied to the central plantar surface of the hindpaw for 3 sec until buckling occurred. Positive responses were defined as paw withdrawal, flinching, and licking.

### AAV microinjection

The mice were fixed on a stereotaxic apparatus (RWD Life Science, 68513, China) under anesthesia with 0.3% pentobarbital sodium. AAVs were injected at a rate of 40 nL/min via a borosilicate glass capillary (Sutter Instrument, B100-58-10, USA) connected to a 10  $\mu$ L syringe (World Precision Instruments, USA) controlled by a motorized microinjector (KD Scientific, Legato 130, USA).

To determine the VLT-projecting cACC subregion (Cg1 and/or Cg2), we infused an anterograde tracer AAV (AAV2/9-hSyn-EGFP,  $5.04 \times 10^{12}$  vg/mL, 60 nL/injection, BrainVTA, China) into Cg1 (AP, +1.00 mm; ML,  $\pm 0.31$  mm; DV,  $-0.85$  mm) or Cg2 (AP, +1.00 mm; ML,  $\pm 0.31$  mm; DV,  $-1.40$  mm) and a nontranssynaptic retrograde AAV (AAV2/Retro-hSyn-tdTomato,  $1.31 \times 10^{13}$  vg/mL, 60 nL/injection, Shanghai Taitool, China) into the VLT (AP,  $-1.06$  mm; ML,  $\pm 1.13$  mm; DV,  $-3.56$  mm).

To specifically infect VLT-projecting cACC neurons with mCherry, hM3Dq-mCherry or hM4Di-mCherry, we delivered the retrograde transport virus AAV2/Retro-hSyn-Cre ( $5.40 \times 10^{12}$  vg/mL, 80 nL/injection, BrainVTA, China) into the VLT, and a Cre-dependent virus encoding the neuronal activator/inhibitor DREADD hM3Dq/hM4Di was injected into the cACC subregion (AAV2/9-hSyn-DIO-mCherry,  $5.40 \times 10^{12}$  vg/mL; AAV2/9-hSyn-DIO-hM3Dq-mCherry,  $5.27 \times 10^{12}$  vg/mL; AAV2/9-hSyn-DIO-hM4Di-mCherry,  $5.45 \times 10^{12}$  vg/mL; 60 nL/injection, BrainVTA, China).

To label VLT-projecting cACC neurons (colabeled with c-Fos to determine the effect of BBR on VLT-projecting cACC neurons), we delivered AAV2/Retro-hSyn-Cre into the VLT and AAV2/9-hSyn-DIO-mCherry into the cACC subregion.

### Chemogenetic manipulations of mouse behaviors

The chemogenetic manipulations were performed by i.p. injection of clozapine N-oxide (CNO, 2 mg/kg; BrainVTA, China) 30 min prior to each behavior session. To allow enough time for virus infection and expression on target circuits, the chemogenetic manipulations were executed at least 3 weeks after virus injection.

### Verification of viral infection and virus labeling

All the mice were anesthetized and perfused with 0.9% saline followed by 4% (w/v) paraformaldehyde to acquire the brains. All the brains were postfixed with 4% paraformaldehyde overnight at 4 °C, and dehydrated in 15% and 30% sucrose until sectioning with a cryostat (CryoStar NX50, Thermo Fisher Scientific, USA). Coronal brain slices (30  $\mu$ m) were mounted with fluorescent medium containing DAPI (ab104139; Abcam, United States) and imaged via a virtual slide microscope (VS120-S6-W; Olympus, Japan). Since Cre-dependent viruses (i.e., AAV2/9-hSyn-DIO-mCherry) can only

express fluorescent proteins by binding to Cre (i.e., AAV2/Retro-hSyn-Cre), it is necessary to check for viral infection at the injection site first. Mice with no fluorescence or very low fluorescence protein expression in the target brain region were excluded from this study.

The validity of the virus was verified via an immunofluorescence method. The effectiveness of the viral labeling was determined by detecting the colabeling rate of the virus-expressed fluorescent protein and the c-Fos-labeled fluorescent protein. The brain slices were incubated with rabbit anti-c-fos antibody (1:500, ab190289, Abcam, USA) overnight at 4 °C and an Alexa Fluor 488-conjugated secondary antibody (1:800, ab15001, Abcam, USA) for 1 h at 37 °C.

### BBR administration

BBR was administered by i.p. injection to the mice. In accordance with the experimental requirements, the doses of berberine used were 2.5, 5 and 10 mg/kg/day. The mice in the control and CFA groups received PBS without BBR. The mice were observed for 13 days (Days 1 to 13 after CFA or PBS injection).

### Statistics and reproducibility

All the data are expressed as the means ± standard errors of the means (SEMs). PWTs data were statistically analyzed by two-way repeated-measures analysis of variance followed by the Tukey post hoc test. We used independent-sample t-tests to statistically analyze the data between the two groups. One-way analysis of variance followed by Tukey's post hoc test was used to compare 3 or more groups.  $P < 0.05$  was considered a statistically significant difference.

### Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

### Data availability

The datasets have been uploaded in accordance with the journal's requirements (see Supplementary Data 1 file). All other relevant raw data that support the findings of the current study are available from the corresponding author upon reasonable request.

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## Author contributions

Z.S. designed the experiments and wrote the manuscript. J.G.L., J.Q.F. and X.M.S. provided guidance on the feasibility of the experiments. M.H. and Y.X.C. performed the viral injections. P.P.F. and C.X. conducted the behavioral experiments. J.C. and S.T.Z. carried out the animal modeling and berberine administration. B.Y.L. and X.F.H. were responsible for histology and microscopy. Z.S. and J.G.L. analyzed the data.

## Competing interests

The authors declare no competing interests.

## Additional information

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