

Contralateral Projection of Anterior Cingulate Cortex Contributes to Mirror-Image Pain

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Long-term limb nerve injury often leads to mirror-image pain (MIP), an abnormal pain sensation in the limb contralateral to the injury. Although it is clear that MIP is mediated in part by central nociception processing, the underlying mechanisms remain poorly understood. The anterior cingulate cortex (ACC) is a key brain region that receives relayed peripheral nociceptive information from the contralateral limb. In this study, we induced MIP in male mice, in which a unilateral chronic constrictive injury of the sciatic nerve (CCI) induced a decreased nociceptive threshold in both hind limbs and an increased number of c-Fos-expressing neurons in the ACC both contralateral and ipsilateral to the injured limb. Using viral-mediated projection mapping, we observed that a portion of ACC neurons formed monosynaptic connections with contralateral ACC neurons. Furthermore, the number of cross-callosal projection ACC neurons that exhibited c-Fos signal was increased in MIP-expressing mice, suggesting enhanced transmission between ACC neurons of the two hemispheres. Moreover, selective inhibition of the cross-callosal projection ACC neurons contralateral to the injured limb normalized the nociceptive sensation of the uninjured limb without affecting the increased nociceptive sensation of the injured limb in CCI mice. In contrast, inhibition of the non-cross-callosal projection ACC neurons contralateral to the injury normalized the nociceptive sensation of the injured limb without affecting the MIP exhibited in the uninjured limb. These results reveal a circuit mechanism, namely, the cross-callosal projection of ACC between two hemispheres, that contributes to MIP and possibly other forms of contralateral migration of pain sensation.

Key words: anterior cingulate cortex; chronic constrictive injury of the sciatic nerve; excitatory pyramidal neuron; mirror-image pain; projection

Significance Statement

Mirror-image pain (MIP) refers to the increased pain sensitivity of the contralateral body part in patients with chronic pain. This pathology requires central processing, yet the mechanisms are less known. Here, we demonstrate that the cross-callosal projection neurons in the anterior cingulate cortex (ACC) contralateral to the injury contribute to MIP exhibited in the uninjured limb, but do not affect nociceptive sensation of the injured limb. In contrast, the non-cross-callosal projection neurons in the ACC contralateral to the injury contribute to nociceptive sensation of the injured limb, but do not affect MIP exhibited in the uninjured limb. Our study depicts a novel cross-callosal projection of ACC that contributes to MIP, providing a central mechanism for MIP in chronic pain state.

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Introduction

Mirror-image pain (MIP) is a clinical phenomenon of the increased nociceptive sensibility that occurs in the intact body or limb contralateral to the injured or inflamed part (Watkins and Maier, 2002; Huang and Yu, 2010; Senba et al., 2012). It commonly occurs to patients with chronic pain, such as neuropathic pain (Konopka et al., 2012; Giglio and Gregg, 2018), complex regional pain syndrome (Huge et al., 2011), and headache (Mathew et al., 2008). Although appearing later (Arguis et al., 2008), MIP is a distressing clinical problem, which leads to more suffering and declines life quality for patients. However, the mechanisms through which MIP is generated are elusive.

Studies have detected MIP in animal models of inflammatory pain and neuropathic pain. Lidocaine intravenous injection obviously reduced ipsilateral allodynia but did little to MIP in chronic pain state (Sinnott et al., 1999), suggesting different mechanisms underlying MIP and the original pain. Accumulating evidence demonstrated multiple inflammatory factors contributing to MIP at peripheral and spinal levels, including glial activations and inflammatory secretions releases (Choi et al., 2015, 2018; Su et al., 2018; Yuan et al., 2020). In addition to these, MIP initiation and maintenance are possibly a result of mechanisms involved in central sensitization (Huang and Yu, 2010; Senba et al., 2012; Ishikawa et al., 2018; W. Wang et al., 2019), yet the neural contributors are less known. One hypothesis is that pain signals may be transferred to the other side in the brain (Milligan et al., 2003). Given that the nervous system is largely symmetrical and there are connections between the two sides of brain, especially cortical areas (Shepherd, 2013), it is not clear whether MIP results from the pain signals transmitting through these neural connections.

Bilateral pain behaviors induced by peripheral mononeuropathy were accompanied by bilateral blood flow changes in some cortex regions, including cingulate cortex (Paulson et al., 2000). Similarly, unilateral spared nerve injury led to bilateral neuronal hyperactivity in the anterior cingulate cortex (ACC), as directly seen via calcium imaging (Zhao et al., 2018). Of note, ACC is a well-established region that responds to physiological and pathologic pain signals (Zhuo, 2008; J. X. Yang et al., 2015; Kummer et al., 2020; Y. Q. Wang et al., 2021). Its rostral part is critical for pain-induced negative emotions, while the caudal part, for pain perception (Zhuo, 2008; Kummer et al., 2020). The ACC receives nociceptive signals from medial thalamus, primary somatosensory cortex (S1), and other supraspinal areas associated with pain regulation (J. W. Yang et al., 2006; Eto et al., 2011). Meanwhile, it sends projections to an array of supraspinal areas and spinal cord, as well as contralateral cortices (Bliss et al., 2016), thus serving as an important relay for pain signals transmitting. Specifically, the predominant excitatory pyramidal neurons dwelled in layer L2/3 and L5 show increased activities and hence may serve a pro-nociceptive role in chronic pain processing (Kummer et al., 2020). These findings suggested that the bilateral hyperactivity in ACC might be responsible for the MIP behaviors induced by chronic pain.

To test this, we used a chronic neuropathic pain model induced by unilateral chronic constrictive injury of the sciatic nerve (CCI) in male mice and studied the role of ACC in mediating MIP in chronic neuropathic pain state. We demonstrated that neuronal hyperactivity of the ACC both ipsilateral and contralateral to the CCI surgery contributed to MIP. In particular, the ACC neurons contralateral to the surgery mediated MIP through the population of cross-callosal projection neurons, and mediated the original pain produced by CCI through the population of non-cross-callosal projection neurons. Our findings depict a novel cross-callosal projection of ACC between two hemispheres that contributes to MIP, which may provide a better understanding for central processing of chronic pain.

Materials and Methods

Animals

Male C57BL/6J mice (7–8 weeks) were supplied by the Experimental Animal Center of Xuzhou Medical University, China. Mice (5 per cage at most) were housed in a vivarium (22°C–25°C) with free access to food and water under a 12 h light/dark cycle. Mice were randomly grouped

and subjected to experiments during the light cycle. Investigators were blinded to experimental conditions during testing. An online randomization tool (<https://www.random.org/lists/>) was used in mice grouping and data collection. Experiments were approved by the Animal Care and Use Committee of Xuzhou Medical University and performed in accordance with the *Guide for the Care and Use of Laboratory Animals* of National Institutes of Health.

CCI

To establish a neuropathic pain model, CCIs were conducted as we previously reported (Xia et al., 2020). Briefly, mice were anesthetized by 1% pentobarbital (40 mg/kg, i.p.). After fur removal and disinfection, the left sciatic nerve at the mid-thigh level was exposed by blunt dissection. Three nonabsorbable 4–0 silks were loosely tied around the sciatic nerve at ~1.0 mm intervals. Sham surgery without such constrictive injury was performed as controls. After suture, erythromycin ointment was applied locally. Finally, mice were placed in a cage with a heating pad underneath, and returned to their home cage when fully awake.

Adenovirus-associated virus (AAV) vectors

The viral vectors were purchased from Brain VTA or OBIO Technology: AAV-CaMKII-mCherry (2/9, 5.88E + 12 viral genome/ml [vg/ml]); AAV-CaMKII-hm4D(Gi)-mCherry (2/9, 6.72E + 12 vg/ml); AAV-CaMKII-cre (2/R, 6.24E + 12 vg/ml); AAV-Eflα-DIO-mCherry (2/9, 5.67E + 12 vg/ml); AAV-Eflα-DIO-hm4D(Gi)-mCherry (2/9, 2.63E + 12 vg/ml); AAV-CaMKII-taCasp3-TEVp (2/9, 5.46E + 12 vg/ml); AAV-flex-taCasp3-TEVp (2/9, 5.13E + 12 vg/ml); AAV-vGAT1-mCherry (2/9, 2.04E + 12 vg/ml); AAV-CaMKII-cre (2/9, 5.63E + 12 vg/ml); AAV-Eflα-DIO-TVA-EGFP (2/9, 5.03E + 12 vg/ml); AAV-Eflα-DIO-oRVG (2/9, 2.00E + 12 vg/ml); RV-EnvA-ΔG-DsRed (2.00E + 08 IFU/ml); AAV-Eflα-DIO-HK-EGFP (2/9, 3.62E + 12 vg/ml); herpes simplex virus (HSV)-ΔHK-tdTomato (1.50E + 09 PFU/ml); AAV-Eflα-DIO-EGFP (2/9, 6.86E + 12 vg/ml); AAV-CaMKII-cre (2/1, 1.13E + 13 vg/ml); and AAV-CaMKII-DO-hm4D (Gi)-mCherry (2/9, 2.23E + 12 vg/ml). For one experiment, the used virus vectors were of the same batch, titer, and volume.

Stereotaxic surgery and microinjection

Mice were anesthetized with 1% pentobarbital (40 mg/kg, i.p.), and fixed on a small-animal stereotaxic apparatus (RWD). After disinfection, the scalp skin was cut lengthwise to expose the cranium; 3% hydrogen peroxide was used to remove the periosteum, and the residual was washed off with normal saline. We mainly focused the posterior part of the dorsal ACC. For microinjection, AAV vectors of 200–300 nl volume were injected into the ACC (AP: 1.20 mm; ML: ±0.25 mm; DV: –1.70 mm) by a Hamilton syringe needle (33 gauge) at a rate of 0.1 μl/min, followed by a 10 min pause to minimize backflow. To prevent dryness, a small amount of normal saline was added to the operative field. Erythromycin ointment was applied locally to avoid infection. After the surgery, mice were cared in a recovery cage.

Behavioral tests

Paw withdraw threshold (PWT). To estimate 50% PWT, a simplified up-and-down method with von Frey filaments (North Coast) was used (Bonin et al., 2014). In brief, mice were placed in polyethylene cages separately on an elevated metallic wire mesh platform. Before testing, mice were allowed to acclimatize to the environment for 1–2 h (room temperature: 23 ± 2°C). Testing started with the midrange filament of 0.4 g strength. Subsequent filaments were decided according to the up-down method, and 5 consecutive touches were applied at 5 min intervals for rest. The filaments were pressed against the plantar surface and held for ~3 s. Positive responses were noted when mice withdrew their hind paw during this time. Finally, 50% PWTs were calculated through the formula described previously (Bonin et al., 2014).

Paw withdraw latency (PWL). PWLs were measured using the Hargreaves test (Hargreaves et al., 1988) with an IITC plantar analgesia meter (IITC Life Science). In a quiet environment (room temperature: 23 ± 2°C), mice were placed in polyethylene cages separately on a glass platform and allowed to accommodate to the apparatus for 1–2 h. A radiant heat source beneath the glass was used to stimulate the plantar

surface of the hind paw. Before testing, heat intensity was adjusted to produce a baseline of 10–15 s. A cutoff time was set at 20 s to prevent tissue damage. Flinching, flicking, and trembling were considered as positive responses. The measurements were in triplicate at 5 min intervals, and the mean was calculated as the PWL.

Tail flick test (TFT). Using the same apparatus for PWL measurements, the latencies until tail flicking in TFT were determined following a method modified from D'Amour and Smith (1941). The tail tip (1–2 cm at the end) of each mouse was subjected to a radiant heat. The heat intensity was adjusted to obtain a baseline of 3–5 s, and the cutoff time was set at 12 s. A positive response was noted if a tail flick was elicited. The measurements were repeated 3 times at intervals of 5 min, and the mean was noted finally.

Face mechanical threshold test (FMT). To determine the face mechanical thresholds, mice were placed in separate custom-made cages with the top and walls made of silver wire mesh on an elevated metallic wire mesh plate. Following the method described by Rodriguez et al. (2017), a series of graded von Frey filaments (North Coast) were applied to the skin surface of vibrissa pad. A positive response was noted when a withdrawal of head or paw was elicited. Tests were repeated for 3 times with at least 2 positive responses out of three trials representing a positive result. The mechanical thresholds were defined as the minimum force to produce positive responses.

Sucrose preference test (SPT). SPT was performed as we previously reported (Xia et al., 2020). Mice were singly housed with two identical leak-resistant bottles containing tap water for 3 d before testing. At testing day, mice first underwent water restriction for 8 h and then were given two identical leak-resistant bottles, one containing tap water and the other containing 2% sucrose solution, for 24 h. The two bottles of each mouse were exchanged every 12 h. Following the testing, the percentage of sucrose solution intake was calculated to reflect the sucrose preference.

Open field test (OFT). OFT was conducted with a white plastic open-field apparatus (40 cm × 40 cm). This field was artificially divided into a 20 cm × 20 cm center zone and a rest peripheral zone. After sterilization by 75% alcohol, mice were put into the center zone and allowed to travel freely within 10 min. The traveling trace, number of entries into the center zone, and time spent in the center zone for each mouse were recorded by the ANY-maze tracking system.

Elevated plus maze (EPM). An elevated apparatus with a digital camera above was used to perform EPM test. The maze, 70 cm above the floor, consists of two open arms (30 × 5 cm) and two closed arms (30 × 5 cm) with 15-cm-high opaque walls. When testing, mice were placed in the center area facing an open arm and allowed to travel freely for 5 min. The traveling trace, number of entries into the open arms, and time spent in the open arms for each mouse were recorded by the ANY-maze tracking system.

Tail suspension test (TST). Mice were suspended 50 cm above the floor with the tail tip adhered to a special apparatus for 6 min. The immobility time of each mouse during the last 4 min was noted.

Chemogenetic manipulations of ACC excitatory pyramidal neurons

To chemogenetically inhibit the unilateral ACC excitatory pyramidal neurons, 200 nl CaMKII-promoted AAV vector expressing hM4D(Gi)-mCherry was injected into the ACC. Four weeks following the virus injection, PWTs were conducted and PWLs were conducted at the next day. At each day, ~1 h after baseline detection, clozapine-N-oxide (CNO, HY-17366, MCE) was dissolved by normal saline and intraperitoneally injected at a dose of 1 mg/kg 20–30 min before a second round of testing. For long-term manipulations, the viral vector was injected likewise. Twenty-four days after the microinjection, mice were subjected to a 5 d CNO intraperitoneal administration at a dose of 1 mg/kg twice per day (8:00 A.M. and 8:00 P.M.). On the next day, behavioral tests began, and repeated every other day. For manipulating the cross-callosal projection neurons in the ACC contralateral to the CCI, the injections included 200 nl retrograde AAV-CaMKII-cre into the ACC ipsilateral to the CCI and 200 nl cre-dependent AAV vector carrying hM4D(Gi)-mCherry into the ACC contralateral to the CCI. To chemogenetically inhibit the non-cross-callosal projection neurons in the ACC contralateral to the

CCI, 200 nl retrograde AAV-CaMKII-cre was injected into the ACC ipsilateral to the CCI and 200 nl cre-independent AAV vector carrying hM4D(Gi)-mCherry into the ACC contralateral to the CCI. Other experimental procedures were the same as forementioned. After behavioral tests, mice were killed, and the expression of mCherry was verified. Behavioral results of the mice without proper ACC mCherry expression (e.g., off-target, poor expression) were excluded accordingly.

Genetic ablation of ACC excitatory pyramidal neurons

To genetically deplete the excitatory pyramidal neurons in the ACC contralateral to the CCI, 200 nl CaMKII-promoted AAV vector delivering caspase 3 was injected into the ACC contralateral to the CCI 21 d before the CCI surgery. To ablate the cross-callosal projection excitatory pyramidal neurons in the ACC contralateral to the CCI, the ACC ipsilateral to the CCI was injected with 200 nl retrograde CaMKII-promoted AAV vector, and the ACC contralateral to the CCI was injected with 200 nl cre-dependent AAV vector carrying caspase 3 21 d before the CCI surgery. PWTs and PWLs were determined at days 3, 7, 14, and 21 following the CCI surgery. Cell death was confirmed by the presence of autofluorescent cell bodies in the target area (H. Koga et al., 1988). In addition, neuronal nuclei (NeuN) staining was used to estimate cell loss (Velier et al., 1999).

Monosynaptic tracing

In order to trace the downstream cells receiving monosynaptic projections from the ACC contralateral to the CCI, 250 nl virus mix of AAV-CaMKII-cre and AAV-DIO-thymidine kinase (TK)-EGFP was injected into the ACC contralateral to the CCI; 21 d after that, 200 nl HSV- Δ TK-tTomato was injected into the same site. Because of the neurotoxicity, mice were closely observed and killed 3–5 d following the secondary injection. For mapping the monosynaptic inputs to the ACC ipsilateral to the CCI, 250 nl virus mix of AAV-CaMKII-cre, AAV-DIO-RG, and AAV-DIO-RV-EGFP was injected into the ACC ipsilateral to the CCI 21 d before the secondary injection of 200 nl RV-EnVA- Δ G-DsRed into the same site. Seven days later, mice were killed to obtain brain sections. For both cases, the individuals were eliminated when the two injections did not overlap or the starter cells (the initial HSV or rabies-infected cells) extended out the ACC area (Callaway and Luo, 2015). The obtained confocal images were manually mapped to corresponding coronal sections within a reference atlas (Franklin and Paxinos, 2008), and the brain regions were identified accordingly.

Labeling the GABA neurons in ACC

Because of the poor quality for immunofluorescent staining of GABA neurons in ACC, we labeled these neurons by injecting 300 nl AAV-vGAT1-mCherry into ACC bilaterally. Fourteen days following the injection, CCI surgery was conducted. Fourteen days after that, the ACC sections were prepared. The mCherry-positive cells in ACC were considered as GABA neurons.

Labeling the cross-callosal projection neurons in the ACC contralateral to the CCI

To label the cross-callosal projection neurons in the ACC contralateral to the CCI, we injected 200 nl retrograde AAV-CaMKII-cre into the ACC ipsilateral to the CCI and 200 nl cre-dependent AAV-DIO-mCherry into the ACC contralateral to the CCI. Fourteen days following the injection, sham or CCI surgery was conducted. Fourteen days after that, the ACC sections were prepared. The mCherry-positive cells in ACC were considered as the cross-callosal projection neurons in the ACC contralateral to the CCI.

Labeling the ACC neurons ipsilateral to the CCI that receive contralateral cross-callosal projection

To label the ACC neurons ipsilateral to the CCI that receive contralateral cross-callosal projection, 200 nl AAV2/1-CaMKII-cre was injected into the ACC contralateral to the CCI and 200 nl AAV-DIO-mCherry was injected into the ipsilateral side. Four weeks following the injection, the ACC sections were prepared. The mCherry-positive cells in the ACC ipsilateral to the CCI were considered as those receive cross-callosal projection from ACC neurons contralateral to the CCI.

Morphologic validation of the cre-off strategy

To label the cross-callosal projection and non-cross-callosal projection neurons in the ACC contralateral to the CCI in one individual, mice were injected with 200 nl retrograde AAV-CaMKII-cre into the ACC ipsilateral to the CCI and 300 nl virus mix of AAV-DIO-EGFP and AAV-DO-mCherry into the ACC contralateral to the CCI. Four weeks later, the ACC sections were prepared. The cre-off strategy would be considered successful if there was a negligible number of colabeled neurons in the ACC contralateral to the CCI.

Immunohistology and confocal imaging

With a deep anesthesia by 1% pentobarbital (40 mg/kg, i.p.), mice were subjected to the intracardial perfusion with 40 ml PBS, pH 7.4, and 20 ml 4% PFA. Subsequently, the brains were removed and postfixed in 4% PFA at 4°C for 6–8 h, followed by being kept in 30% sucrose solution for 48 h. Coronal brain sections (40 μ m thick) were prepared by a frozen section microtome (VT1000S, Leica Microsystems). Sections (from bregma 1.18 to 0.86) were reserved for counting at an interval of one section, and the rest of the sections were used for controls. Bregma 1.18 was used as a landmark since it is the rostral-most point at which the corpus callosum crosses the midline. Two to four sections were sampled from each mouse (the detailed *N* values can be found in the figure legends). The brain sections with fluorescent protein expression were directly coverslipped in mounting medium and visualized by a laser scanning confocal microscope (LSM 880, Carl Zeiss). For immunofluorescent staining, the sections were washed in PBS for 15 min, incubated with a quick antigen retrieval solution (P0090, Biyuntian) for 5 min, and subsequently blocked for 1 h with a PBS solution containing 1% BSA and 0.25% Triton X-100. According to the experimental needs, the sections were incubated with the primary antibodies overnight, including mouse anti-CaMKII α (1:500, MA1-048, Thermo Fisher Scientific), rabbit anti-c-Fos (1:500, 2250, Cell Signaling Technology), and mouse anti-NeuN (1:1000, MAB377, Millipore). After being washed in PBS for 15 min, the sections were tagged with secondary antibodies for 2 h, including anti-rabbit Alexa-594 (1:200, A21207, Thermo Fisher Scientific), anti-rabbit Alexa-488 (1:200, A-21206, Thermo Fisher Scientific), or anti-mouse Alexa-488 (1:200, A21202, Thermo Fisher Scientific). As controls, adjacent sections were incubated without primary antibody or without antibodies. Finally, the sections were mounted onto glass slides; 20 $\times$ and 40 $\times$ images were obtained using a confocal microscope (LSM 880, Carl Zeiss). To acquire the full area, image tiles were performed using Zeiss software. For fluorescent imaging, the same settings, including laser power, gain value, pinhole size, and line scans, were used to acquire a series of sections.

Cell counting

Sections (40 μ m, from bregma 1.18 to 0.86) were reserved for counting at an interval of one section. Two to four sections were sampled from each mouse (the detailed *N* values can be found in the figure legends). The dorsal area of ACC was delineated according to a reference atlas (defined as cingulate cortex, area 1) (Franklin and Paxinos, 2008). All of the labeled cells within this area were counted. Manual counting by Adobe Photoshop software was used to count the c-Fos-positive cells, NeuN-positive cells, and double-labeled cells, during which the investigator was blinded to the experimental conditions. The labeled neurons were identified independently for each fluorescent channel, among which those with colocalization of two kinds of fluorescence were considered as double-labeled neurons.

Data analysis and statistics

All experiments were replicated in 4–18 mice, with the data randomly collected and processed. We did not predetermine sample sizes by statistical methods, but our sample sizes were similar to an array of previous publications with similar experimental designs (Rodriguez et al., 2017; Zhang et al., 2017; Meda et al., 2019; Pan et al., 2019; An et al., 2020; Luan et al., 2020). Data were analyzed offline, and experimenters were not blinded to experimental group during the analyses.

A total of 391 mice were used in current study, among which 43 mice were excluded from data collection and analysis because of the

following reasons: (1) 32 mice were excluded because of off-target or poor expression for virus vectors; and (2) 11 mice were excluded because of health problems after surgeries (e.g., body weight drops >20% in a day) and experimental failures. No data points were excluded after data acquisition was accomplished.

Statistical analyses were conducted with GraphPad Prism 7.0 software (GraphPad Software). All data were analyzed with two-tailed tests and expressed as mean $\pm$ SEM. Two-way ANOVA with repeated measures followed by Bonferroni post-tests was used to compare the differences of PWTs and PWLs with multiple time points. *t* tests were used in comparing the differences between two groups, and Welch's correction was used when the variance was not equal. Detailed descriptions can be found in the figure legends. Statistical significance was defined as $p < 0.05$.

Results

MIP induced by chronic neuropathic pain

Using a unilateral CCI-induced neuropathic pain model in male mice (Xia et al., 2020), we evaluated the pain behaviors of hind paws bilaterally at multiple time points following the sham or CCI surgery (Fig. 1*A,B*). Compared with sham mice, CCI mice showed the long-lasting decreased PWTs and PWLs in the injured hind paw (Fig. 1*C*). Consistent with previous literature (Cao et al., 2014; Choi et al., 2018), PWTs and PWLs in the uninjured hind paw of CCI mice began to decline 7 d after the CCI surgery (Fig. 1*D*), indicating an occurrence of MIP in the uninjured limb of CCI mice. In addition, the onset of MIP in the uninjured limb was later than the onset of the hypersensitivity observed in the injured limb of CCI mice (Fig. 1*C,D*). Since this MIP phenomenon tended to be stable 14 d after the CCI surgery, we chose this time point in the following experiments.

Is MIP an outcome of the reduced general pain thresholds? To answer this question, we conducted two additional behaviors, TFT and FMT test 14 d after the sham or CCI surgery, to assess general pain thresholds (Fig. 1*A*). The following results showed that sham and CCI mice did not differ in behaviors in the TFT or FMT test (Fig. 1*E,F*), which do not support an altered general pain response in CCI mice. Chronic pain often causes affective disorders, such as anxiety and depression (McWilliams et al., 2004; Lerman et al., 2015). In order to explore whether MIP is associated with the CCI-induced negative emotions, SPT, OFT, EPM, and TST were conducted from day 14 after the sham or CCI surgery (Fig. 1*A*). The CCI mice showed similar behaviors in these tests to the sham mice (Fig. 1*G–N*), suggesting that the CCI mice did not exhibit anxiety-like or depressive behaviors at this stage of chronic neuropathic pain.

Bilateral c-Fos expression in ACC after CCI

To study the potential role of ACC in mediating MIP, we first detected ACC neuronal activities through c-Fos staining. ACC sections were prepared from sham or CCI mice 14 d following the surgery. The following staining results showed that the number of the c-Fos-positive cells in ACC was bilaterally increased in CCI mice compared with their sham counterparts (Fig. 2*A,B*). This result suggests that CCI induces hyperactivity bilaterally in the ACC, which is in line with previous findings (Paulson et al., 2000; Zhao et al., 2018). To further identify the cell type of c-Fos-positive neurons in CCI mice, we performed immunofluorescent staining in ACC sections from CCI mice. Interestingly, >90% c-Fos-positive cells in both sides of ACC were colabeled by CaMKII α (Fig. 2*C,D*), suggesting that these activated cells were mainly excitatory pyramidal neurons (X. Wang et al., 2013; Kang et al., 2015). There was no difference between the two sides in

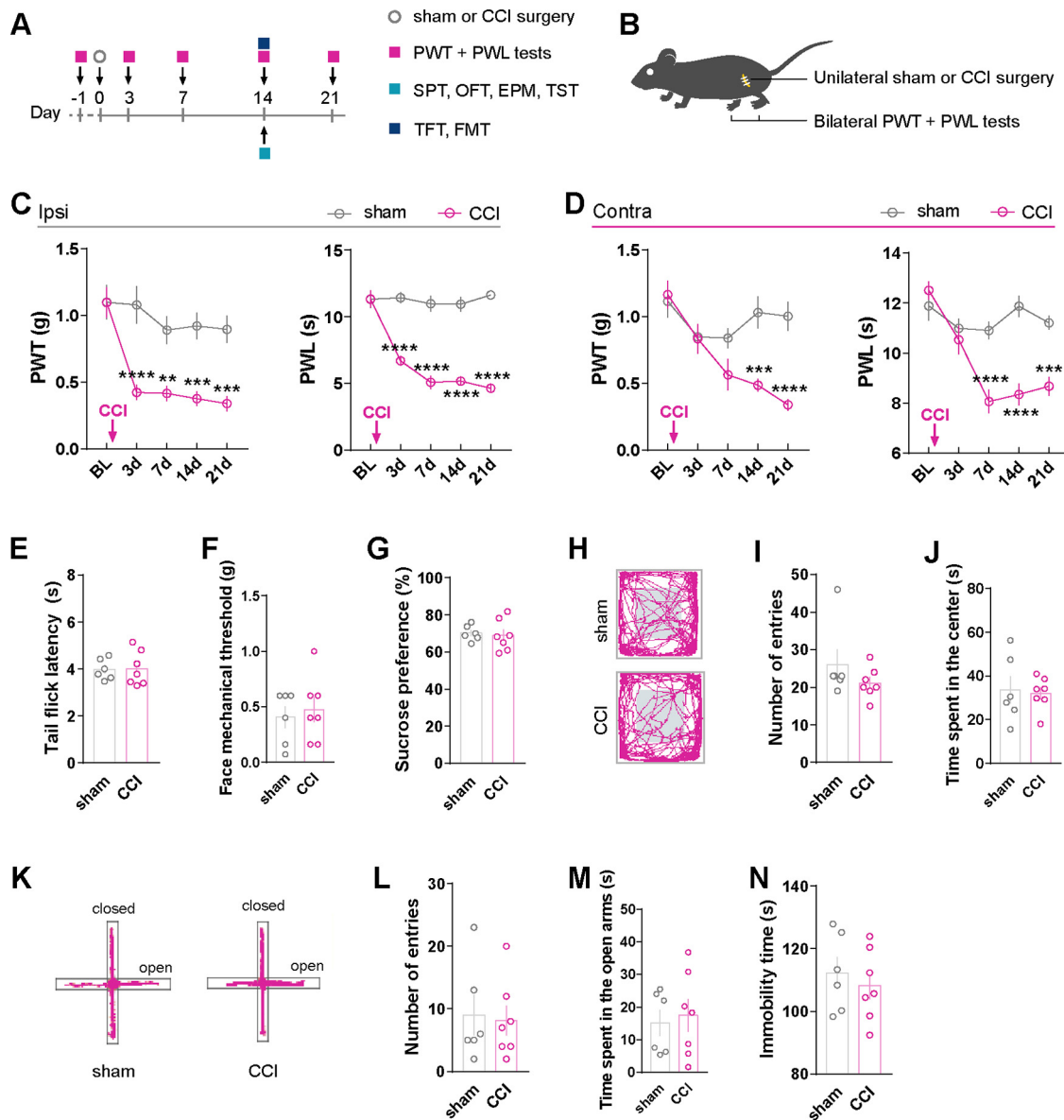


Figure 1. CCI-induced chronic neuropathic pain produces MIP in the contralateral paw. **A**, Experimental timeline. PWTs and PWLs of the two hind paws in mice were assessed at day 1 before, and at days 3, 7, 14, and 21 after the sham or CCI surgery. TFT and FMT tests, and SPT, OFT, EPM, and TST were conducted in different batches of mice from day 14 after the CCI surgery. **B**, Schematic for the unilateral sham or CCI surgery and bilateral pain threshold measurements. **C**, Statistics showing that, compared with the sham mice, CCI mice exhibited decreased PWTs and PWLs in the injured paw at days 3, 7, 14, and 21 following the CCI surgery. $n_{\text{sham}} = 14$; $n_{\text{CCI}} = 18$. PWT: group, $F_{(1,30)} = 113.2$, $p < 0.0001$; sham versus CCI, 3 d $p < 0.0001$, 7 d $p = 0.0017$, 14 d $p = 0.0002$, 21 d $p = 0.0002$. PWL: group, $F_{(1,30)} = 263.1$, $p < 0.0001$; 3 d $p < 0.0001$, 7 d $p < 0.0001$, 14 d $p < 0.0001$, 21 d $p < 0.0001$. **D**, Statistics showing that, compared with sham mice, CCI mice showed decreased PWTs at days 14 and 21, and decreased PWLs at days 7, 14, and 21 in the contralateral paw, suggesting an occurrence of MIP. $n_{\text{sham}} = 14$; $n_{\text{CCI}} = 18$. PWT: group, $F_{(1,30)} = 14.46$, $p = 0.0007$; sham versus CCI, 14 d $p = 0.0006$, 21 d $p < 0.0001$. PWL: group, $F_{(1,30)} = 36.87$, $p < 0.0001$; sham versus CCI, 7 d $p < 0.0001$, 14 d $p < 0.0001$, 21 d $p = 0.0004$. **E**, **F**, Summary data showing that sham and CCI mice did not differ in (**E**) tail flick latency or (**F**) FMT. TFT: mean $\pm$ SD, sham, 3.982 ± 0.4416 ; CCI, 4.011 ± 0.7325 ; $t_{(11)} = 0.08524$, $p = 0.9336$. FMT: sham, 0.4050 ± 0.2393 ; CCI, 0.4743 ± 0.2934 ; $t_{(11)} = 0.461$, $p = 0.6538$. **G**, Statistics showing that, in SPT, the percent of sucrose preference did not differ between sham (70.14 ± 4.162) and CCI mice (68.95 ± 3.867). $t_{(11)} = 0.3163$, $p = 0.7577$. **H**, Typical traveling trace of sham and CCI mice in open field. **I**, **J**, Summary data showing that, compared with sham mice, CCI mice exhibited similar levels of (**I**) number of entries into the center and (**J**) time spent in the center zone in the OFT. OFT: time spent in the center, sham, 33.67 ± 15.22 ; CCI, 32.07 ± 7.523 ; $t_{(11)} = 0.2463$, $p = 0.8100$; number of entries, sham, 26 ± 9.92 ; CCI, 21.14 ± 4.1 ; $t_{(11)} = 1.189$, $p = 0.2594$. **K**, Typical traveling traces of sham and CCI mice in EPM test. **L**, **M**, Statistics showing that sham and CCI mice exhibited similar levels of (**L**) number of entries into the open arm and (**M**) time spent in the open arm in EPM test. EPM: entries into the open arms: sham, 9 ± 7.772 ; CCI, 8.143 ± 6.176 ; $t_{(11)} = 0.2218$, $p = 0.8286$; time spent in the open arms, sham, 15.12 ± 9.636 ; CCI, 17.44 ± 13.08 ; $t_{(11)} = 0.3591$, $p = 0.7263$. **N**, Summary results showing that the immobility time in TST did not differ between sham (112.2 ± 12.38) and CCI mice (108.2 ± 11.37). $t_{(11)} = 0.6028$, $p = 0.5588$. $^{**}p < 0.01$. $^{***}p < 0.001$. $^{****}p < 0.0001$. Error bars indicate SEM. Data analyzed by (**C**, **D**) two-way repeated-measures ANOVA with Bonferroni post-tests, or (**E**–**G**, **I**, **J**, **L**–**N**) unpaired t test. Ipsi, ipsilateral; contra, contralateral; BL, baseline.

the ratio of c-Fos-positive cells that were also labeled for CaMKII α (Fig. 2C,D). Apart from excitatory pyramidal neurons, ACC possesses a large number of GABA inhibitory neurons, which have also been implicated in pain regulation (Kang et al., 2015; Juarez-Salinas et al., 2019). Here, we injected an AAV

vector, AAV-vGAT1-mCherry, into the ACC to label GABA neurons. We found that few c-Fos-positive cells were labeled for GABA and that there was no significant difference between the two sides in the proportion of c-Fos-labeled cells that were also labeled for GABA (Fig. 2C,E).

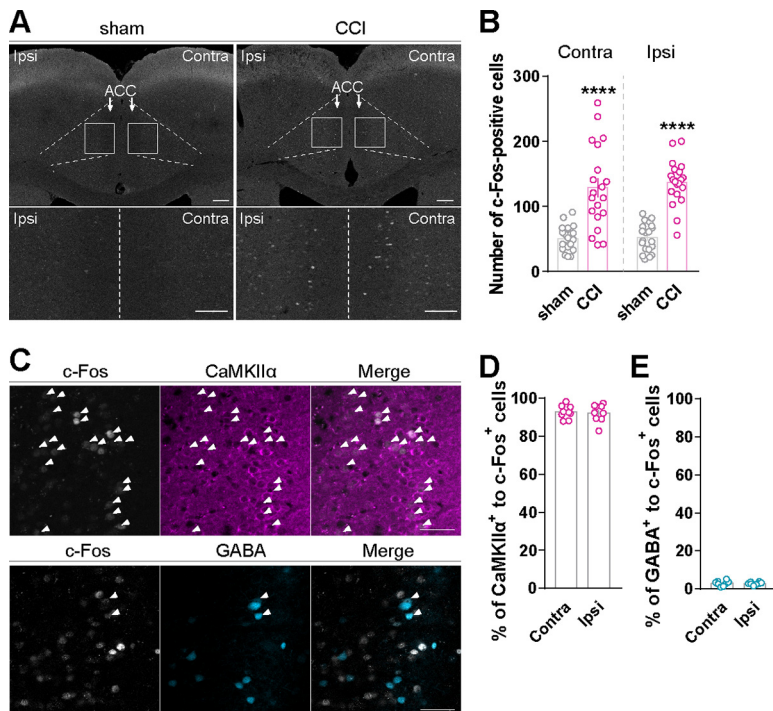


Figure 2. c-Fos signals are increased bilaterally in ACC on chronic neuropathic pain induced by CCI. **A**, Representative confocal images of c-Fos staining in the two sides of ACC from sham and 14 d CCI mice. Scale bars: top column, 200 μ m; bottom column, 100 μ m. **B**, Quantitative summary of c-Fos-positive neurons in two sides of ACC, indicating that both sides of the ACC are hyperactivated on CCI-induced chronic neuropathic pain. $n = 20$ from 6 mice/group. Contra: sham, 50.65 ± 19.18 ; CCI, 128.9 ± 63.7 ; Ipsi: sham, 52.5 ± 21.84 ; CCI, 136.6 ± 34.29 . Group, $F_{(1,76)} = 96.67$, $p < 0.0001$, two-way ANOVA; sham versus CCI, Contra $p < 0.0001$, Ipsi $p < 0.0001$; Bonferroni post-tests. **C**, Representative confocal images for cell type identification of c-Fos-positive neurons in ACC from 14 d CCI mice. White arrows indicate colabeled neurons. Scale bar, 50 μ m. **D**, Quantitative results showing that the majority of the c-Fos-positive neurons in the two sides of ACC from CCI mice were colabeled by CaMKII. $n = 9$ from 4 mice/group. Sham, 92.7 ± 3.428 ; CCI, 92.26 ± 4.658 . $t_{(8)} = 0.1973$, $p = 0.8485$; paired t test. **E**, Quantitative results showing that few c-Fos-positive neurons were labeled by GABA. GABA neurons were labeled by the local injection of AAV-vGAT1-mCherry. $n = 8$ from 4 mice/group. Sham, 2.912 ± 1.331 ; CCI, 2.783 ± 0.7378 . $t_{(7)} = 0.3075$, $p = 0.7674$; paired t test. **** $p < 0.0001$. Error bars indicate SEM. Ipsi, ipsilateral; contra, contralateral.

Roles of the two sides of ACC in regulating MIP

To explore whether neuronal hyperactivity of ACC is involved in CCI-induced MIP, we decreased the activities of the ACC excitatory pyramidal neurons by chemogenetics and assessed the pain thresholds in mice. Specifically, AAV-CaMKII-hM4D(Gi)-mCherry (an AAV vector that expresses mCherry and hM4D (Gi) behind a CaMKII promoter) or AAV-CaMKII-mCherry (as control) was injected into the ACC contralateral to the CCI 14 d before the sham or CCI surgery (Fig. 3*A,B*). PWTs and PWLs were determined at days 14 and 15, respectively, after the sham or CCI surgery (Fig. 3*A*). At least 1 h following the first determination of PWTs or PWLs, mice were subjected to an intraperitoneal injection of CNO (1 mg/kg). Twenty to 30 min after that, PWTs or PWLs were examined again. After CNO injection, the CCI mice injected with AAV-CaMKII-hM4D(Gi)-mCherry exhibited the increased PWTs and PWLs in the injured hind paw (Fig. 3*C,D*), and unchanged PWTs and PWLs in the uninjured hind paw (Fig. 3*E,F*), suggesting that inhibition of the ACC excitatory pyramidal neurons contralateral to the CCI relieved the nociceptive sensation in the injured limb but might not exert effects on CCI-induced MIP in the uninjured limb. Similarly, we chemogenetically inhibited the ACC excitatory pyramidal neurons ipsilateral to the CCI and studied the pain behaviors (Fig. 3*G,H*). In contrast with the previous findings, this CNO treatment did not change the PWTs or PWLs in the injured hind paw (Fig. 3*I,J*) but increased the pain thresholds in

the uninjured hind paw in CCI mice injected with AAV-CaMKII-hM4D(Gi)-mCherry (Fig. 3*K,L*). This result suggests that inhibition of the ACC excitatory pyramidal neurons ipsilateral to the CCI can relieve MIP in the uninjured limb without changing the pain thresholds in the injured limb in CCI mice.

In addition, we ablated the excitatory pyramidal neurons contralateral to the CCI by injecting AAV-CaMKII-caspase 3 into the ACC contralateral to the CCI 21 d before the sham or CCI surgery (Fig. 4*A,B*). The caspase 3 expression will initiate the apoptosis procedure in cells (C. F. Yang et al., 2013). We confirmed cell death by the presence of autofluorescent cell bodies and by the loss of NeuN-stained neurons (H. Koga et al., 1988; Velier et al., 1999) (Fig. 4*B*). PWTs and PWLs were determined at days 3, 7, 14, and 21 following the sham or CCI surgery (Fig. 4*A*). In the injured hind paw of CCI mice injected with AAV-CaMKII-caspase 3, the PWTs and PWLs were increased at days 14 and 21 after the CCI surgery (Fig. 4*C*). To our surprise, the pain thresholds in the uninjured hind paw of the CCI mice injected with AAV-CaMKII-caspase 3 did not decline as those of the CCI control mice did after the CCI surgery (Fig. 4*D*). These findings suggest that, like those ipsilateral to the CCI, the ACC neurons contralateral to the CCI may also contribute to MIP induced by CCI. This seems to conflict with our previous findings that chemogenetic inhibition of the ACC neurons contralateral to the CCI did not affect MIP; we next performed another experiment to solve this puzzle.

We injected AAV-CaMKII-hM4D(Gi)-mCherry into the ACC contralateral to the CCI, and 14 d later, performed sham or CCI surgery. Ten days after the sham or CCI surgery, CNO at a 1 mg/kg dose was intraperitoneally injected twice per day for 5 d (Fig. 4*E,F*). Thus, excitatory pyramidal neurons in the ACC contralateral to the CCI were repeatedly inhibited. After accomplishment of the CNO injection, PWTs and PWLs were measured at days 1, 3, and 5 (Fig. 4*E*). The behavioral results showed that, in CCI mice injected with AAV-CaMKII-hM4D(Gi)-mCherry, the PWTs and PWLs in both hind paws were increased and this increase disappeared at day 5 (Fig. 4*G,H*). These results demonstrate that long-term inhibition of the ACC neurons contralateral to the CCI is required to relieve MIP in the uninjured hind paw of CCI mice, reflecting a slow effect of the ACC neurons contralateral to the CCI on regulating MIP. Together with our previous results, these findings demonstrate that, apart from contributing to the nociceptive sensation in the injured limb, the hyperactivity of ACC excitatory pyramidal neurons contralateral to the CCI also contributes to CCI-induced MIP in the uninjured limb.

Direct cross-callosal projection from the ACC contralateral to the CCI

In order to decipher how ACC excitatory pyramidal neurons contralateral to the CCI transmit pain signals to the other side,

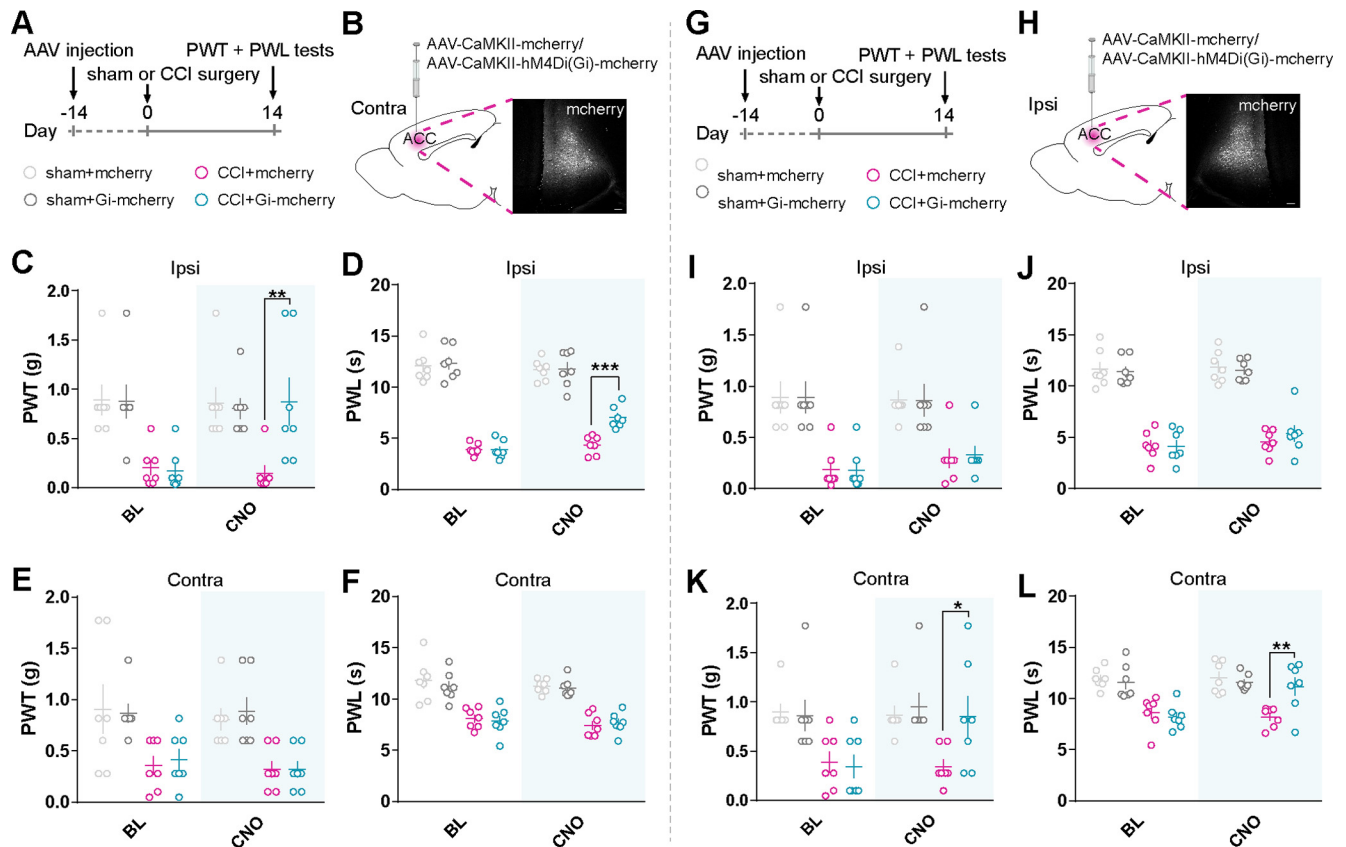


Figure 3. Chemogenetic inhibition of the two unilateral ACC exerts different effects on the original pain and MIP induced by CCI. **A, G**, Experimental timeline and figure legends for behavioral summary. Mice were injected with AAV vectors into the ACC contralateral or ipsilateral to the CCI at day 14 before the CCI surgery. PWTs and PWLS were assessed in 2 d from day 14 after the CCI surgery. **B, H**, Schematic of virus injection and typical confocal image for mCherry expression in the ACC contralateral or ipsilateral to the CCI. Scale bar, 100 μ m. **C, D**, Statistics demonstrating that chemogenetic inhibition of the ACC excitatory pyramidal neurons contralateral to the CCI relieved (**C**) mechanical allodynia and (**D**) thermal hyperalgesia in the ipsilateral paw induced by CCI. PWT: group, $F_{(3,24)} = 11.61$, $p < 0.0001$; CCI+mCherry versus CCI+Gi-mCherry, CNO $p = 0.0047$. PWL: group, $F_{(3,24)} = 110$, $p < 0.0001$; CCI+mCherry versus CCI+Gi-mCherry, CNO $p = 0.0009$. **E, F**, Statistics showing that chemogenetic inhibition of the ACC excitatory pyramidal neurons contralateral to the CCI did not change the (**E**) PWTs and (**F**) PWLS in the contralateral paw of CCI mice. PWT: group, $F_{(3,24)} = 10.92$, $p = 0.0001$; CCI+mCherry versus CCI+Gi-mCherry, CNO $p > 0.9999$. PWL: group, $F_{(3,24)} = 33.96$, $p < 0.0001$; CCI+mCherry versus CCI+Gi-mCherry, CNO $p > 0.9999$. **I, J**, Summary showing that chemogenetic inhibition of the ACC excitatory pyramidal neurons ipsilateral to the CCI did not change the (**I**) PWTs and (**J**) PWLS in the ipsilateral paw of CCI mice. PWT: group, $F_{(3,24)} = 25.67$, $p < 0.0001$; CCI+mCherry versus CCI+Gi-mCherry, CNO $p > 0.9999$. PWL: group, $F_{(3,24)} = 69.76$, $p < 0.0001$; CCI+mCherry versus CCI+Gi-mCherry, CNO $p > 0.9999$. **K, L**, Summary showing that chemogenetic inhibition of the ACC excitatory pyramidal neurons ipsilateral to the CCI relieved MIP in the contralateral paw induced by CCI. PWT: group, $F_{(3,24)} = 7.472$, $p = 0.0011$; CCI+mCherry versus CCI+Gi-mCherry, CNO $p = 0.0486$. PWL: group, $F_{(3,24)} = 17.72$, $p < 0.0001$; CCI+mCherry versus CCI+Gi-mCherry, CNO $p = 0.0024$. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. **** $p < 0.0001$. **C-F, I-L**, Data analyzed by two-way repeated-measures ANOVA with Bonferroni post-tests. Error bars indicate SEM. Ipsi, ipsilateral; contra, contralateral; BL, baseline.

we mapped the output pathways of these neurons by a modified HSV-mediated anterograde monosynaptic tracing strategy (Lo and Anderson, 2011). In brief, the mix of AAV-CaMKII-cre and AAV-DIO-EGFP-TK was injected into the ACC contralateral to the CCI to express TK and EGFP in the excitatory pyramidal neurons. Twenty-one days later, HSV- Δ TK-tdTomato was injected into the same site of the ACC (Fig. 5A). Thus, the starter cells (the initial HSV-infected cells) in the ACC contralateral to the CCI were colabeled by EGFP and tdTomato (Fig. 5B), and the output neurons were labeled by a single tdTomato. Interestingly, we found that many neurons in the ACC ipsilateral to the CCI were labeled by tdTomato (Fig. 5B). Furthermore, the CaMKII α -positive population constituted 87.9% of these tdTomato-positive neurons (Fig. 5E,F). These findings suggest that there is a direct cross-callosal projection from the ACC contralateral to the CCI. In addition, tdTomato-positive neurons were also found in the lateral occipital cortex, PFC, thalamic areas, and other regions (Fig. 5C,D). It should be noted that, except for the bilaterally projecting

neurons in the lateral occipital cortex, these tdTomato-positive neurons lie in the same side of the starter cells.

Given that HSV has high toxicity (Lo and Anderson, 2011; Wojaczynski et al., 2015), we performed an additional anterograde tracing experiment to confirm the cross-callosal projection from the ACC contralateral to the CCI (Fig. 5G). To do this, we injected AAV 2/1-CaMKII-cre into the ACC contralateral to the CCI and AAV-DIO-mCherry into the ACC ipsilateral to the CCI. Thus, the ACC neurons ipsilateral to the CCI that receive contralateral cross-callosal projection would be labeled by mCherry. The following results showed that there was a portion of mCherry-positive neurons in the ACC ipsilateral to the CCI (Fig. 5H). Immunofluorescent staining results further showed that the majority of the labeled neurons coexpressed CaMKII α (Fig. 5H,I).

To further verify this ACC-to-ACC projection, we used a modified rabies virus (RV)-mediated retrograde monosynaptic tracing strategy (Wickersham et al., 2007a,b). To do this, we injected the mix of AAV-CaMKII-cre, AAV-DIO-TVA-EGFP, and AAV-DIO-RG into the ACC ipsilateral to the CCI, and 21 d

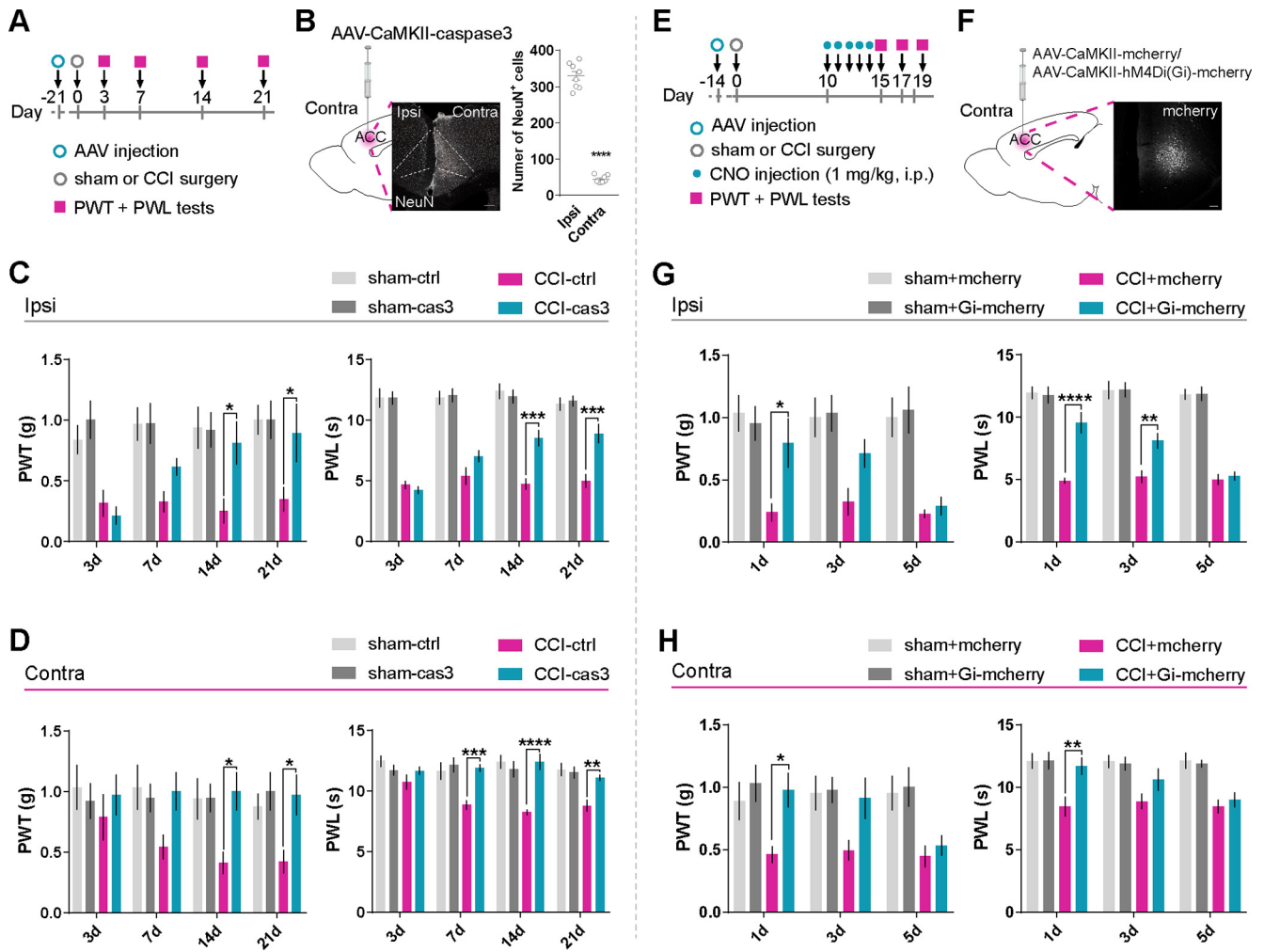


Figure 4. Genetic ablation or long-term inhibition of the excitatory pyramidal neurons in the ACC contralateral to the CCI relieves MIP induced by CCI. **A**, Timeline for the microinjections, CCI surgery, and PWT and PWL tests. **B**, Schematic of the virus injection for genetic ablation of the excitatory pyramidal neurons in the ACC contralateral to the CCI and typical confocal image indicating the efficacy of caspase 3. Scale bar, 200 μ m. Summary showing that, compared with that in the ACC ipsilateral to the CCI, the number of NeuN-labeled cells was significantly decreased in the ACC contralateral to the CCI. $n = 8$ from 4 mice. Ipsi, 329.9 ± 34.98 ; Contra, 45.13 ± 9.553 . $t_{(7)} = 24.67$, $p < 0.0001$. **C**, Summary results showing that, compared with their control counterparts, the CCI mice with caspase 3 expression exhibited increased PWTs and PWLs at days 14 and 21 following the CCI surgery. PWT: group, $F_{(3,23)} = 18.21$, $p < 0.0001$; CCI-ctrl versus CCI-cas3, 14 d $p = 0.0247$, 21 d $p = 0.0309$. PWL: group, $F_{(3,23)} = 321.3$, $p < 0.0001$; CCI-ctrl versus CCI-cas3, 14 d $p = 0.0001$, 21 d $p < 0.0001$. **D**, Summary showing that depleting the ACC excitatory pyramidal neurons contralateral to the CCI increased the PWTs at days 14 and 21, and the PWLs at days 7, 14, and 21 following the CCI surgery. PWT: group, $F_{(3,23)} = 5.111$, $p = 0.0074$; CCI-ctrl versus CCI-cas3, 14 d $p = 0.0235$, 21 d $p = 0.0398$. PWL: group, $F_{(3,23)} = 25.33$, $p < 0.0001$; CCI-ctrl versus CCI-cas3, 7 d $p = 0.0002$, 14 d $p < 0.0001$, 21 d $p = 0.0064$. **E**, Timeline for the behavioral tests in mice with repeated CNO administrations. Five day CNO injections (1 mg/kg, i.p., twice per day, 8:00 A.M. and 8:00 P.M.) began at day 10 following the CCI surgery. PWTs and PWLs were evaluated at days 1, 3, and 5 after the CNO injection. **F**, Schematic of the virus injection and typical confocal image showing the mCherry expression in the ACC contralateral to the CCI. Scale bar, 100 μ m. **G**, Summary showing that repeated inhibition of the excitatory pyramidal neurons in the ACC contralateral to the CCI increased the PWTs and PWLs in the ipsilateral paw in CCI mice. Ipsilateral PWT: group, $F_{(3,25)} = 11.79$, $p < 0.0001$; CCI+mCherry versus CCI+Gi-mCherry, 1 d $p = 0.0261$. Ipsilateral PWL: group, $F_{(3,25)} = 108.6$, $p < 0.0001$; CCI+mCherry versus CCI+Gi-mCherry, 1 d $p < 0.0001$, 3 d $p = 0.0025$. **H**, Summary showing that the repeated inhibition relieved MIP in the contralateral paw in CCI mice. Contralateral PWT: group, $F_{(3,25)} = 6.458$, $p = 0.0022$; CCI+mCherry versus CCI+Gi-mCherry, 1 d $p = 0.0257$. Contralateral PWL: group, $F_{(3,25)} = 15.19$, $p < 0.0001$; CCI+mCherry versus CCI+Gi-mCherry, 1 d $p = 0.0033$. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. **** $p < 0.0001$. Data analyzed by **(B)** paired t test and **(C,D,G,H)** two-way repeated-measures ANOVA with Bonferroni post-tests. Error bars indicate SEM. Ipsi, Ipsilateral; contra, contralateral; BL, baseline; cas3, caspase 3.

later, injected RV-EnVA- Δ G-DsRed into the same site (Fig. 5J). Accordingly, we labeled the starter cells (the initial RV-infected cells) with EGFP and DsRed (Fig. 5K) and the input cells with a single DsRed. DsRed-positive cells were found in the ACC contralateral to the CCI (Fig. 5K), providing more evidence supporting a direct ACC-to-ACC projection. Specifically, 95.1% of the DsRed-labeled neurons in the ACC contralateral to the CCI were colabeled by CaMKII α (Fig. 5N,O). Moreover, DsRed-positive cells were also found in the PFC, thalamus, S1, and other regions at the same side of the starter cells (Fig. 5L,M).

c-Fos expression in the cross-callosal projection neurons in the ACC contralateral to the surgery after CCI

We next asked whether the cross-callosal projection was involved in mediating CCI-induced MIP. To answer this question, we first explored the activities of these cross-callosal projection neurons in the ACC contralateral to the CCI in CCI mice. The cross-callosal projection neurons in the ACC contralateral to the CCI were labeled by mCherry through the injections of retrograde AAV-CaMKII-cre into the ACC ipsilateral to the CCI and AAV-DIO-mCherry into the ACC contralateral to the CCI (Fig. 6A).

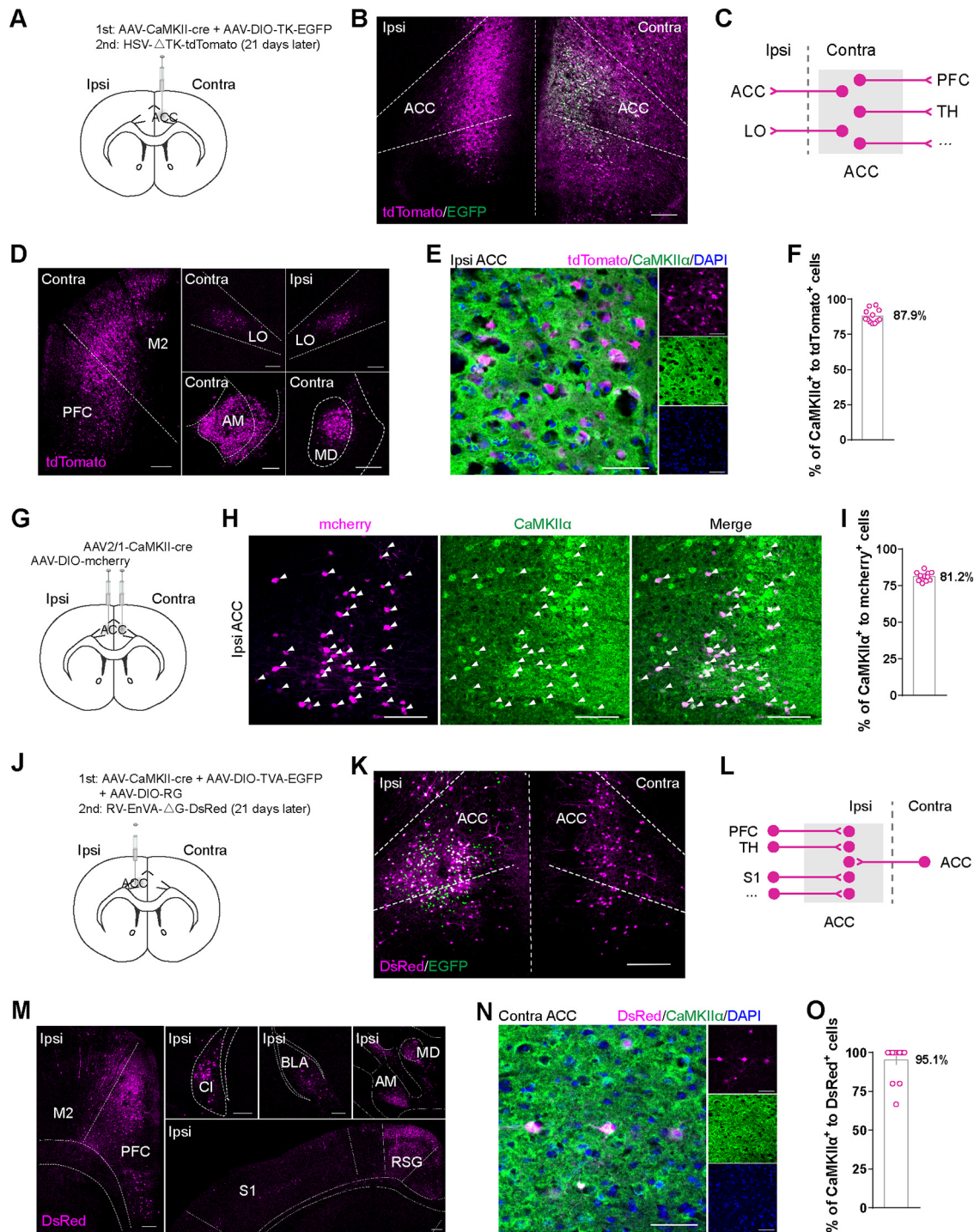


Figure 5. ACC contralateral to the CCI sends direct projection to the ACC ipsilateral to the CCI. **A**, Schematic of virus injections into the ACC contralateral to the CCI. The virus injections consisted of two stages: (1) mix of AAV-CaMKII-cre and AAV-DIO-TK-EGFP; and (2) 21 d later, HSV- Δ TK-tdTomato. **B**, Representative confocal images showing the tdTomato expression in the two sides of ACC and EGFP expression in the ACC contralateral to the CCI. The neurons labeled by both tdTomato and EGFP in the ACC contralateral to the CCI were defined as starter cells. Scale bar, 200 μ m. **C**, Schematic of the main pain-associated brain regions that receive monosynaptic inputs from the starter cells. **D**, Example confocal images of the tdTomato-positive neurons from the PFC, secondary motor cortex (M2), lateral occipital cortex (LO), anteromedial thalamus (AM), and mediodorsal thalamus (MD). Scale bar, 200 μ m. **E**, Example confocal images of the CaMKII α -stained section of the ACC ipsilateral to the CCI. Scale bar, 50 μ m. **F**, Quantified summary of the percent of CaMKII α -positive neurons to tdTomato-positive cells in the ACC ipsilateral to the CCI: 87.9 ± 4.71 ; $n = 12$ from 4 mice. **G**, Schematic of virus injections for labeling the ACC neurons ipsilateral to the CCI that receive contralateral inputs. **H**, Example images showing the mCherry-labeled ACC sections stained with CaMKII α . White arrows indicate colabeled neurons. Scale bar, 100 μ m. **I**, Summary showing the percent of CaMKII α -positive neurons to mCherry-positive cells: 81.2 ± 2.96 ; $n = 12$ from 4 mice. **J**, Schematic of virus injections into the ACC ipsilateral to the CCI. The virus injections consisted of two stages: (1) mix of AAV-CaMKII-cre, AAV-DIO-TVA-EGFP, and AAV-DIO-RG; and (2) 21 d later, RV-EnVA- Δ G-DsRed. **K**, Example confocal images showing the tdTomato expression in the two sides of ACC and EGFP expression in the ACC ipsilateral to the CCI. The neurons labeled by both DsRed and EGFP in the ACC ipsilateral to the CCI were defined as starter cells. Scale bar, 200 μ m. **L**, Schematic of the main pain-associated brain regions that send monosynaptic projections onto the starter cells. **M**, Representative confocal images of the DsRed-positive neurons from the M2, PFC, claustrum (CI), BLA, AM, MD, S1, and retrosplenial granular cortex (RSG). Scale bar, 200 μ m. **N**, Example confocal images of the CaMKII α -stained section of ACC contralateral to the CCI. Scale bar, 50 μ m. **O**, Quantified summary of the percent of CaMKII α -positive neurons to DsRed-positive cells in the ACC contralateral to the CCI: 95.1 ± 10.5 ; $n = 15$ from 5 mice. Error bars indicate SEM. Ipsi, ipsilateral; contra, contralateral.

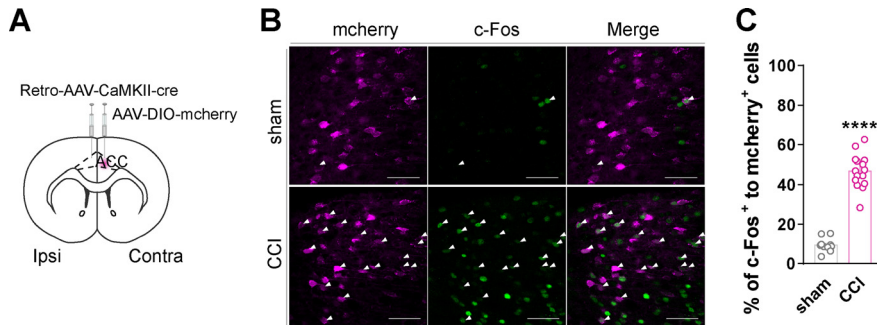


Figure 6. c-Fos signals of the cross-callosal projection excitatory pyramidal neurons in the ACC contralateral to the CCI are increased on chronic neuropathic pain induced by CCI. **A**, Schematic of virus injections for labeling the cross-callosal projection excitatory pyramidal neurons in the ACC contralateral to the CCI. **B**, Example confocal images showing the ACC sections stained with c-Fos from sham and 14 d CCI mice. White arrows indicate colabeled neurons. Scale bar, 50 μ m. **C**, Statistics showing that the percent of c-Fos-positive neurons to mCherry-labeled population were higher in 14 d CCI mice than that in sham mice: sham, 9.455 ± 3.514 , $n = 10$ from 4 mice; CCI, 46.69 ± 8.616 , $n = 15$ from 5 mice ($t_{(19,93)} = 14.97$, $p < 0.0001$, unpaired t test with Welch correction). **** $p < 0.0001$. Error bars indicate SEM. Ipsi, Ipsilateral; contra, contralateral.

Fourteen days after the injections, sham or CCI surgery was conducted. Fourteen days after that, mice were killed to obtain ACC sections. Subsequently, the ACC sections were stained with c-Fos. Compared with that in sham mice, the ratio of c-Fos-positive cells to total mCherry-positive neurons in the ACC contralateral to the CCI was higher (Fig. 6B,C), suggesting that the cross-callosal projection neurons in the ACC contralateral to the CCI represent the increased activities in CCI mice. These findings led us to hypothesize that the cross-callosal projection neurons in the ACC contralateral to the CCI might contribute to MIP in the uninjured limb of CCI mice.

Roles of the cross-callosal projection neurons in the ACC contralateral to the CCI

To determine whether the increased activities of the cross-callosal projection neurons in the ACC contralateral to the CCI are contributable to MIP in CCI mice, we chemogenetically decreased the activities of these neurons. To do this, we injected retrograde AAV-CaMKII-cre into the ACC ipsilateral to the CCI and cre-dependent AAV-DIO-hM4D(Gi)-mCherry or AAV-CaMKII-mCherry (as control) into the ACC contralateral to the CCI 14 d before the sham or CCI surgery (Fig. 7A,B). PWTs and PWLs, respectively, were assessed at days 14 and 15 after the CCI surgery (Fig. 7A). After a single CNO injection (1 mg/kg, i.p.), the CCI mice with Gi-mCherry expression exhibited unchanged PWTs and PWLs in the bilateral hind paws compared with the CCI mice with mCherry expression (Fig. 7C–F).

Next, we repeatedly inhibited the cross-callosal projection neurons in the ACC contralateral to the CCI. The AAV vectors, same to those in Figure 7B, were injected 14 d before the sham or CCI surgery (Fig. 8A–C). CNO was administered (5 d, twice per day, 1 mg/kg, i.p.) from day 10 after the surgery (Fig. 8A). After this CNO treatment, PWTs and PWLs were assessed at days 1, 3, and 5. The CCI mice with Gi-mCherry expression showed the unchanged PWTs and PWLs in the injured hind paw (Fig. 8D), and increased PWTs and PWLs in the uninjured hind paw compared with the CCI mice with

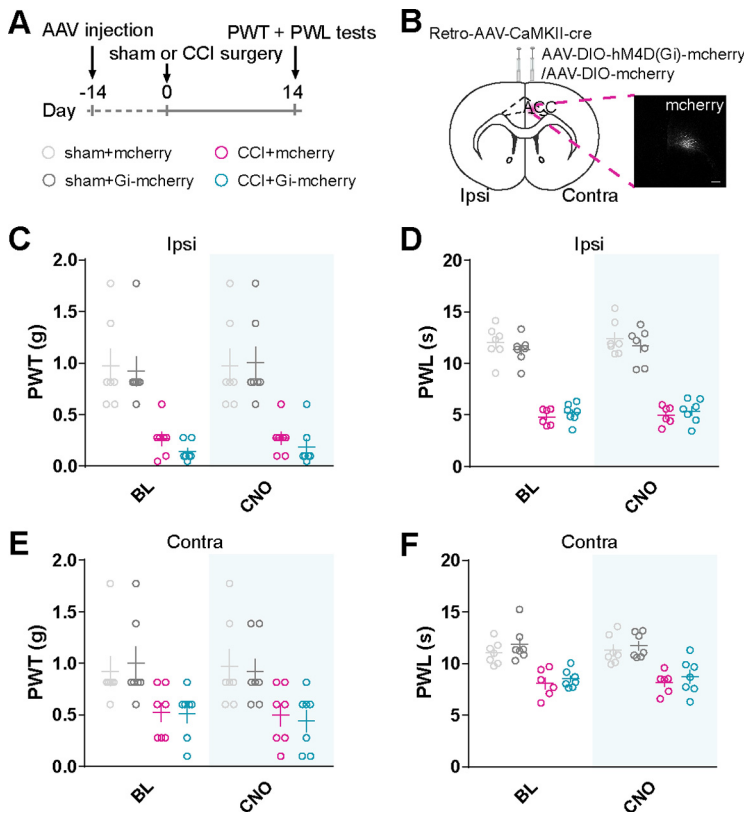


Figure 7. Chemogenetic inhibition of the cross-callosal projection excitatory pyramidal neurons in the ACC contralateral to the CCI with a single CNO injection does not affect nociceptive sensation of CCI mice. **A**, Timeline of microinjections, CCI surgery, and behavioral tests, and figure legends for behavioral summary. PWTs and PWLs, respectively, were assessed in 2 d from day 14 after the CCI surgery. **B**, Schematic of virus injections to express Gi-mCherry or mCherry (as control) in the cross-callosal projection excitatory pyramidal neurons in the ACC contralateral to the CCI, and typical image showing the mCherry expression in the ACC contralateral to the CCI. Scale bar, 100 μ m. **C–F**, Behavioral summary showing that inhibition of these projecting neurons by a single CNO injection (1 mg/kg, i.p.) did not change the pain thresholds of the two sides of paws in CCI mice. Ipsilateral PWT: group, $F_{(3,24)} = 18.67$, $p < 0.0001$; CCI+mCherry versus CCI+Gi-mCherry, CNO $p = 0.9611$. Ipsilateral PWL: group, $F_{(3,23)} = 117.8$, $p < 0.0001$; CCI+mCherry versus CCI+Gi-mCherry, CNO $p = 0.9510$. Contralateral PWT: group, $F_{(3,24)} = 9.347$, $p = 0.0003$; CCI+mCherry versus CCI+Gi-mCherry, CNO $p = 0.9894$. Contralateral PWL: group, $F_{(3,23)} = 18.58$, $p < 0.0001$; CCI+mCherry versus CCI+Gi-mCherry, CNO $p = 0.8634$. **C–F**, Data analyzed by two-way repeated-measures ANOVA with Bonferroni post-tests. Error bars indicate SEM. Ipsi, Ipsilateral; contra, contralateral; BL, baseline.

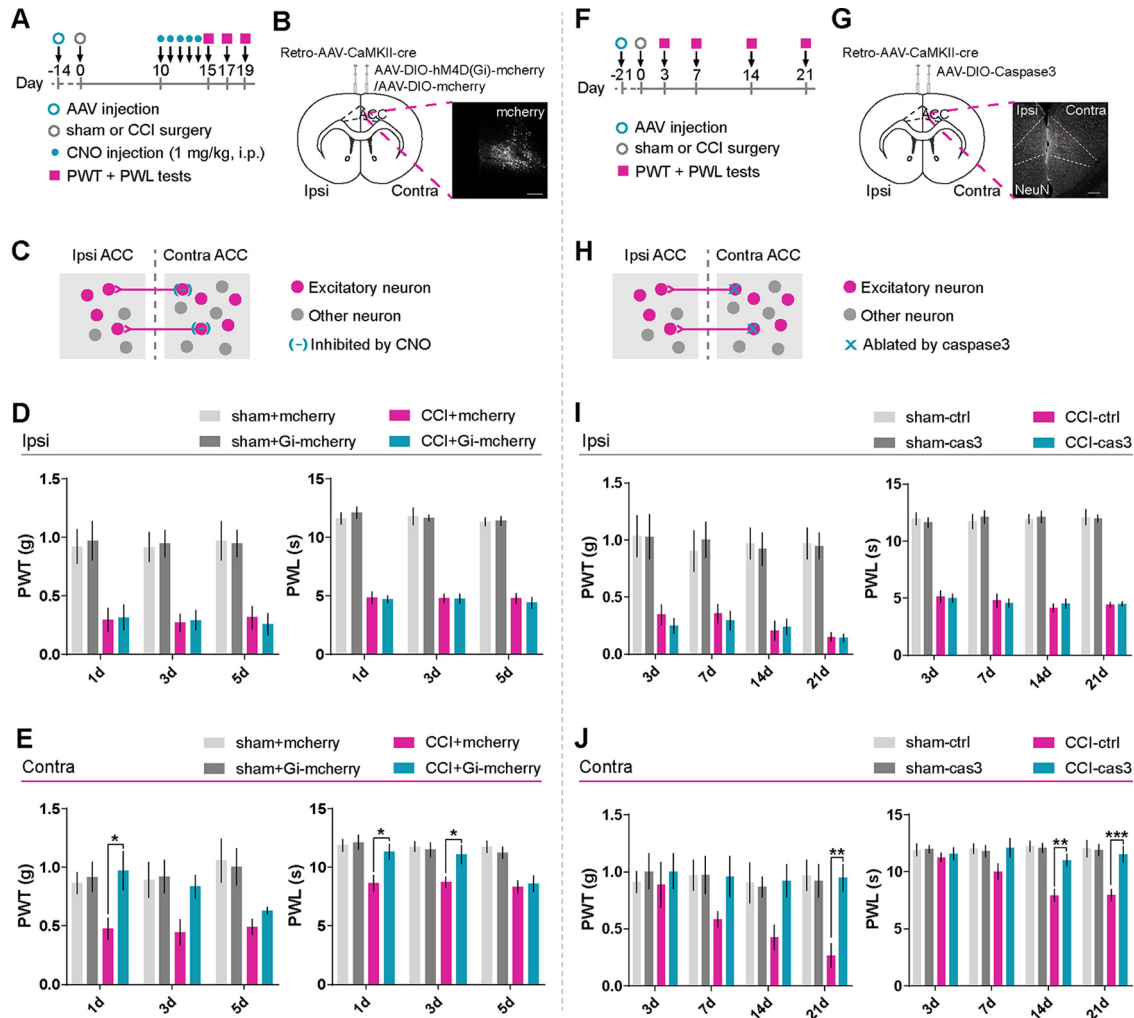


Figure 8. Long-term inhibition or genetic ablation of the cross-callosal projection excitatory pyramidal neurons in the ACC contralateral to the CCI relieves MIP without changing the original pain in CCI mice. **A**, Timeline of microinjections, CCI surgery, CNO injection, and behavioral tests. CNO was given twice every day for 5 d (1 mg/kg, i.p.). **B**, Schematic of the virus injection and typical confocal image showing the mCherry expression in putative cross-callosal projection excitatory pyramidal neurons in the ACC contralateral to the CCI. Scale bar, 100 μ m. **C**, Schematic of the inhibited neurons in the ACC contralateral to the CCI. **D**, Summary showing that the repeated inhibition had no effect on PWTs and PWLs in the ipsilateral paw in CCI mice. Ipsilateral PWT: group, $F_{(3,23)} = 11.79$, $p < 0.0001$; ipsilateral PWL: group, $F_{(3,23)} = 190.9$, $p < 0.0001$. **E**, Summary showing that the repeated inhibition of these pyramidal neurons relieved MIP in the contralateral paw in CCI mice. Contralateral PWT: group, $F_{(3,23)} = 4.722$, $p = 0.0012$; CCI+mCherry versus CCI+Gi-mCherry, 1 d $p = 0.0458$. Contralateral PWL: group, $F_{(3,23)} = 18.25$, $p < 0.0001$; CCI+mCherry versus CCI+Gi-mCherry, 1 d $p = 0.0162$, 3 d $p = 0.0452$. **F**, Timeline for the microinjections, CCI surgery, and PWT and PWL tests. **G**, Schematic of the virus injection for genetic ablation of the cross-callosal projection excitatory pyramidal neurons in the ACC contralateral to the CCI, and example confocal image indicating the efficacy of caspase 3. Scale bar, 200 μ m. **H**, Schematic of the ablated neurons in the ACC contralateral to the CCI. **I**, Summary results showing that this genetic ablation had no effect on pain thresholds in the ipsilateral paw of CCI. PWT: group, $F_{(3,22)} = 55.46$, $p < 0.0001$. PWL: group, $F_{(3,22)} = 195.8$, $p < 0.0001$. **J**, Summary showing that depleting the cross-callosal projection excitatory pyramidal neurons in the ACC contralateral to the CCI increased the PWTs at day 21, and the PWLs at days 14 and 21 following the CCI surgery. PWT: group, $F_{(3,22)} = 13.1$, $p < 0.0001$; CCI-ctrl versus CCI-cas3, 21 d $p = 0.0063$. PWL: group, $F_{(3,22)} = 14.85$, $p < 0.0001$; CCI-ctrl versus CCI-cas3, 14 d $p = 0.0013$, 21 d $p = 0.0002$. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. **D**, **E**, **I**, **J**, Data analyzed by two-way repeated-measures ANOVA with Bonferroni post-tests. Error bars indicate SEM. Ipsi, ipsilateral; contra, contralateral; BL, baseline; cas3, caspase 3.

mCherry expression (Fig. 8E). These results suggest that, like chemogenetic inhibition of the ACC contralateral to the CCI (Fig. 4E–H), a long-term inhibition was required for the cross-callosal projection neurons in the ACC contralateral to the CCI to produce antinociception effects in the uninjured limb of CCI mice.

We reasoned that similar MIP-relieving effects might be acquired by genetic removal of the cross-callosal projection neurons in the ACC contralateral to the CCI. To test this, retrograde AAV-CaMKII-cre was injected into the ACC ipsilateral to the CCI and cre-dependent AAV-DIO-caspase 3 was injected into the ACC contralateral to the CCI 21 d before the sham or CCI surgery (Fig. 8F–H). Pain thresholds were detected at days 3, 7, 14, and 21 after the surgery

(Fig. 8F). Compared with the CCI control mice, the CCI mice with caspase 3 expression showed the unchanged PWTs and PWLs in the injured hind paw (Fig. 8I), and increased PWTs at day 21 and PWLs at days 14 and 21 in the uninjured hind paw (Fig. 8J). These results suggest that genetic depletion of the cross-callosal projection neurons in the ACC contralateral to the CCI had no effects on the increased nociceptive sensation of the injured limb but could abolish the establishment of nociceptive sensation in the uninjured limb of CCI mice. Together, these findings demonstrate that the cross-callosal projection neurons in the ACC contralateral to the CCI contribute to CCI-induced MIP in the uninjured limb without changing the nociceptive sensation in the injured limb.

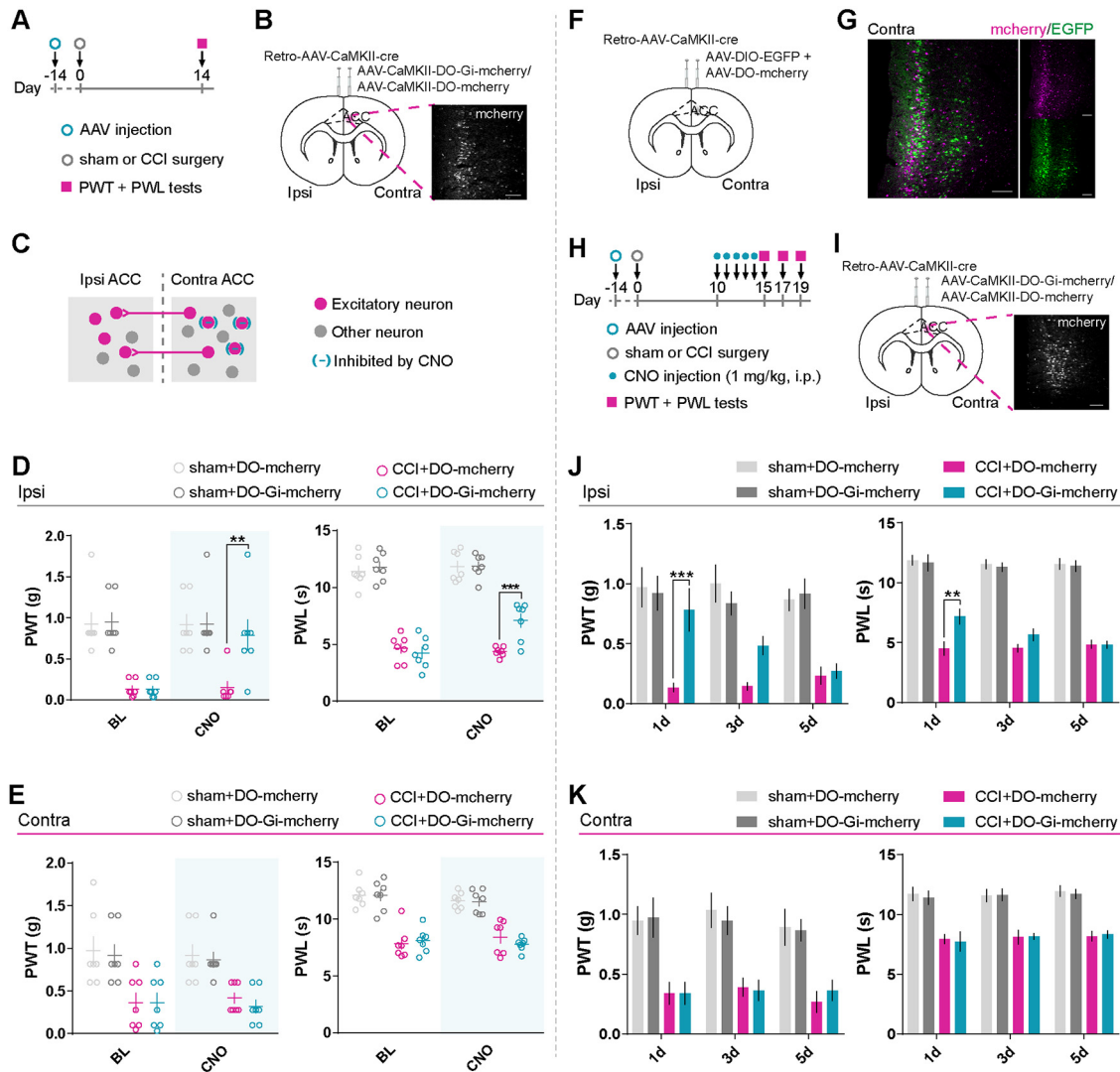


Figure 9. Chemogenetic inhibition of the non-cross-callosal projection excitatory pyramidal neurons in the ACC contralateral to the CCI reduces nociception in the ipsilateral paw but has no effect on contralateral MIP in CCI mice. **A**, Timeline of microinjections, CCI surgery, and behavioral tests. PWTs and PWLs were assessed in 2 d from day 14 after the CCI surgery. **B**, Schematic of virus injections and typical confocal image showing the mCherry expression. Scale bar, 100 μ m. **C**, Schematic of the inhibited non-cross-callosal projection excitatory pyramidal neurons in the ACC contralateral to the CCI. **D**, Statistics showing that chemogenetic inhibition by the CNO injection (1 mg/kg, i.p.) reduced the pain thresholds in the ipsilateral paw of CCI mice. PWT: group, $F_{(3,24)} = 22$, $p < 0.0001$; CCI+DO-mCherry versus CCI+DO-Gi-mCherry, CNO $p = 0.0026$. PWL: group, $F_{(3,24)} = 112$, $p < 0.0001$; CCI+DO-mCherry versus CCI+DO-Gi-mCherry, CNO $p = 0.0008$. **E**, Summary results suggesting that MIP was not affected by inhibiting the non-cross-callosal projection excitatory pyramidal neurons in the ACC contralateral to the CCI. PWT: group, $F_{(3,24)} = 10.62$, $p = 0.0001$; CCI+DO-mCherry versus CCI+DO-Gi-mCherry, CNO $p = 0.9328$. PWL: group, $F_{(3,24)} = 36.49$, $p < 0.0001$; CCI+DO-mCherry versus CCI+DO-Gi-mCherry, CNO $p = 0.6897$. **F**, Schematic of virus injections for labeling the cross-callosal projection neurons with EGFP and non-cross-callosal projection neurons with mCherry in the ACC contralateral to the CCI. **G**, Typical confocal image showing the mCherry and EGFP expression in the ACC contralateral to the CCI. Scale bar, 100 μ m. **H**, Timeline of microinjections, CCI surgery, CNO injections, and behavioral tests. CNO was injected twice per day for 5 d (1 mg/kg, i.p.). **I**, Schematic of virus injections and typical confocal image showing the mCherry expression. Scale bar, 100 μ m. **J**, Summary showing that the repeated inhibition increased PWTs and PWLs in the ipsilateral paw in CCI mice at day 1. Ipsilateral PWT: group, $F_{(3,24)} = 25.47$, $p < 0.0001$; CCI+DO-mCherry versus CCI+DO-Gi-mCherry, 1 d $p = 0.0008$. Ipsilateral PWL: group, $F_{(3,24)} = 130$, $p < 0.0001$; CCI+DO-mCherry versus CCI+DO-Gi-mCherry, 1 d $p = 0.0011$. **K**, Summary showing that the repeated inhibition had no effect on MIP in the contralateral paw in CCI mice. Contralateral PWT: group, $F_{(3,24)} = 13.66$, $p < 0.0001$. Contralateral PWL: group, $F_{(3,24)} = 28.51$, $p < 0.0001$. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. **D, E, J, K**, Data analyzed by two-way repeated-measures ANOVA with Bonferroni post-tests. Error bars indicate SEM. Ipsi, ipsilateral; contra, contralateral; BL, baseline; cas3, caspase 3.

Roles of the non-cross-callosal projection neurons in the ACC contralateral to the CCI

So far, our findings suggest that the cross-callosal projection neurons in the ACC contralateral to the CCI are involved in regulation of MIP in CCI-induced chronic neuropathic pain state. We next asked whether the non-cross-callosal projection neurons in the ACC contralateral to the CCI were involved in this process as well. To manipulate this population, we used a “cre-off” system where the elements tagged by “DO” (a stop sequence) cannot express in the presence of cre recombinase (Saunders et al., 2012).

Based on this system, we injected retrograde AAV-CaMKII-cre into the ACC ipsilateral to the CCI, and AAV-CaMKII-DO-hM4D(Gi)-mCherry or AAV-CaMKII-DO-mCherry (as control) into the ACC contralateral to the CCI 14 d before the sham or CCI surgery (Fig. 9A–C). These viral expressions allowed the CNO injection to trigger inhibition of the non-cross-callosal projection neurons in the ACC contralateral to the CCI. PWTs and PWLs, respectively, were determined at days 14 and 15 following the sham or CCI surgery. After a single CNO administration (1 mg/kg, i.p.), the CCI mice with Gi-mCherry expression

showed the increased PWTs and PWLs in the injured hind paw (Fig. 9D), and unchanged PWTs and PWLs in the uninjured hind paw compared with CCI mice with mCherry expression (Fig. 9E).

Furthermore, by labeling the cross-callosal projection neurons with EGFP and non-cross-callosal projection neurons with mCherry in the ACC contralateral to the CCI, we observed separated distributions of these two populations in one individual (Fig. 9F,G), providing morphologic validations for the used cre-off strategy.

We next studied the effect of repeated inhibition of the non-cross-callosal projection neurons in the ACC contralateral to the CCI on pain behaviors. AAV vectors, same as those in Figure 9B, were injected 14 d before the sham or CCI surgery (Fig. 9H,I). CNO was repeatedly injected (1 mg/kg, 5 d, twice per day, i.p.) from day 10 after the surgery. PWTs and PWLs were determined at days 1, 3, and 5 following the injection (Fig. 9H). Compared with the CCI mice with mCherry expression, the CCI mice with Gi-mCherry expression exhibited the increased PWTs and PWLs in the injured hind paw at day 1 (Fig. 9J), and unchanged PWTs and PWLs in the uninjured hind paw (Fig. 9K). These data suggest that the non-cross-callosal projection neurons in the ACC contralateral to the CCI mediate the nociceptive sensation in the injured limb but are not involved in mediating CCI-induced MIP in the uninjured limb. Together, these findings represent a dissociation scenario where two populations of neurons in the ACC contralateral to the CCI underpin CCI-induced two distinct pain components: the original pain in the injured limb and the MIP in the uninjured limb (Fig. 10).

Discussion

MIP has been reported in many clinical cases, mostly under neuropathic pain backgrounds (Huge et al., 2011; Konopka et al., 2012; Giglio and Gregg, 2018; Maatman et al., 2019; Masgoret et al., 2020). MIP is sporadic in human but common in animal models of neuropathic pain (Arguis et al., 2008; Cao et al., 2014; C. L. Wang et al., 2017), and inflammatory pain (Choi et al., 2015, 2017, 2018). This discrepancy may possibly result from the ignorance of MIP in clinic (Huang and Yu, 2010). Despite this, a previous study showed that all animals with unilateral CCI-induced neuropathic pain exhibited bilateral hyperalgesia and allodynia (Paulson et al., 2000). On this basis, we chose the CCI model in the present study. Our current results demonstrate a monosynaptic connection between the two sides of ACC engaging in mediating MIP induced by CCI. Combined with the finding that the ACC excitatory pyramidal neurons ipsilateral to the CCI mediate MIP as well (Fig. 3G–L), we suggest that the ACC excitatory pyramidal neurons contralateral to the CCI may act their roles through facilitating the ACC neurons ipsilateral to the CCI. Nonetheless, we still do not know exactly whether the cross-callosal projection of ACC enrolls the ACC excitatory pyramidal neurons ipsilateral to the CCI and how this projection remodels these neurons during this process. Thus, future studies

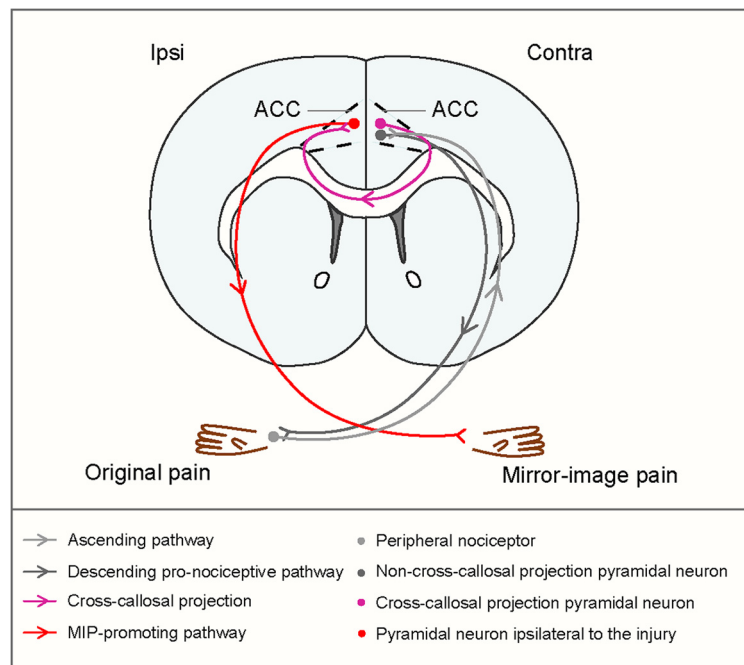


Figure 10. Schematic of the theoretical pathways through which the ACC contralateral to the CCI mediates the original pain and MIP induced by CCI. The ACC excitatory pyramidal neurons contralateral to the CCI receive nociceptive information via the ascending pathway, and exert pro-nociceptive effect on the original pain processing through the non-cross-callosal projection excitatory pyramidal neurons via the descending pro-nociceptive pathway. After receiving the nociceptive information, the ACC contralateral to the CCI conveys this information to the other side via the cross-callosal projection, and the ACC ipsilateral to the CCI sequentially transmits the pain signals, thus producing MIP at the contralateral side via the descending MIP-promoting pathway. Ipsi, ipsilateral; contra, contralateral.

are warranted, and synaptic plasticity can be considered as a research direction (Jaggi and Singh, 2011; Bliss et al., 2016).

One of the intriguing features of MIP is that it appears later than the primary pain syndrome (Cao et al., 2014; Choi et al., 2018). Such delay phenomenon is also reported in our current study, as demonstrated by the evidence that MIP appeared ~7 d following the establishment of the unilateral CCI surgery (Fig. 1D). Inhibition of the ACC excitatory pyramidal neurons ipsilateral to the CCI triggers a reverse of MIP immediately (Fig. 3G–L), whereas a long-term manipulation of the cross-callosal projection neurons in the ACC contralateral to the CCI is required to reverse MIP (Figs. 7 and 8). The effect of the cross-callosal projection neurons in the ACC contralateral to the CCI on MIP seems to be a slow process. We propose that this slow effect of the cross-callosal projection neurons in the ACC contralateral to the CCI may account for the later occurrence of MIP.

Several lines of studies have been dedicated to unraveling the mystery of how pain spreads to the contralateral intact territory. Milligan et al. (2003) showed that both primary allodynia and mirror-image allodynia were created by glial and proinflammatory cytokine actions in the spinal cord. They also proposed that these factors contributed to the mirror-image effects because glia might communicate with distant glia via gap junction, calcium waves, and their releases (Milligan et al., 2003). This proposal was supported by later research (Cheng et al., 2014; Choi et al., 2015, 2018; Su et al., 2018). However, it is not likely that the MIP is entirely ascribed to inflammatory processes in the spinal cord. After all, without fine control, these actions may possibly cause pain responses in uncertain regions rather than the precise mirror-image territory (Huang and Yu, 2010). Therefore, neural controls may serve a more important role in the MIP process. The ACC receives nociceptive information from the medial

thalamus, which carries the pain signals from the spinal cord (J. W. Yang et al., 2006; Shyu and Vogt, 2009). It also receives nociceptive inputs from the S1, a well-known pain-related area (Singh et al., 2020). Pain signals from the ACC can be conveyed to the spinal cord through the descending pathways (Zhuo, 2017). Thus, ACC serves as an important relay node for pain signals transmitting. Our current findings suggest that the direct cross-callosal projection of ACC is activated and may build a bridge for the pain signals transmitting (Figs. 6–8), which promotes the contralateral effects. These findings provide evidence supporting the neural theory that central sensitization in the brain can lead to the spread of pain to the contralateral part. In addition, we propose a loop for MIP processing, the injured side, to the ACC contralateral to the injury, to the ACC ipsilateral to the injury, to the uninjured side. Apart from the ACC, the S1 possesses similar cross-callosal connection, which has been suggested to activate astrocyte and inhibitory neurons and prime MIP after peripheral nerve injury (Ishikawa et al., 2018). In addition to these, symmetrical nerve connections have also been observed in the spinal cord (Koltzenburg et al., 1999), yet it remains unclear whether the establishment and development of MIP involve this connection.

Beyond local factors, the spinal plasticity can be shaped by the descending pathways arising from some supraspinal regions, including the ACC, which in turn influences the pain responses (Millan, 2002; Sandkuhler, 2007; Zhuo, 2017). Noticeably, the ACC can also directly facilitate spinal excitatory transmission through a top-down descending pathway independent of the rostral ventromedial medulla (RVM) and promotes pain responses consequently (Chen et al., 2018). Furthermore, spinal LTP is linked to the MIP behaviors (Xu et al., 2020). As described in our current study, two distinct populations of neurons in the ACC contralateral to the CCI, cross-callosal projection and non-cross-callosal projection neurons, respectively, contribute to the original pain sensation in the injured limb and the contralateral MIP in the uninjured limb in chronic neuropathic pain state (Figs. 8 and 9). According to the potential actions of ACC on spinal plasticity, we raise a possibility that, after receiving information from the cross-callosal projection neurons in the ACC contralateral to the CCI, the activated neurons in the ACC ipsilateral to the CCI may cause an increase of excitatory synaptic transmission in the spinal cord and hence relay the pain signals to the mirror-image region. In addition, the non-cross-callosal projection ACC neurons contralateral to the CCI may serve as a nociceptive amplifier for the primary pain through the direct (RVM-independent) and indirect (RVM-dependent) descending pathways (Zhuo, 2017).

One interesting finding in the present study is that pain thresholds of the injured paw were barely affected at the early stage of CCI-induced neuropathic pain when we ablated the ACC excitatory pyramidal neurons contralateral to the CCI before the CCI surgery (Fig. 4A–D). Such result indicates that the ACC may play a limited role in maintaining nociceptive sensation at the initial stage of pain processing. In line with this, although these neurons respond to acute pain (Hutchison et al., 1999; K. Koga et al., 2010), optical inhibition of the ACC CaMKII-positive excitatory pyramidal neurons is not sufficient to relieve pain behaviors in mice with acute inflammatory pain (precisely 1 h following an intraplantar injection of complete Freund's adjuvant) (Smith et al., 2021). In contrast, similar optical inhibition reversed the increase in mechanical sensitivity on chronic inflammatory pain (3 d after complete Freund's adjuvant treatment) (Kang et al., 2015). In concert with this finding, we

demonstrated that both genetic depletion of the ACC excitatory pyramidal neurons contralateral to the CCI and chemogenetic inhibition of these neurons significantly increased pain thresholds in the injured paw of CCI mice at a stage where neuropathic pain persisted (Fig. 4). Together, we suggest that the ACC plays a more crucial role in chronic pain state and its adaptations, in particular, hyperactivity, may contribute to pain chronification.

It is important to point out that, apart from promoting nociception sensation, hyperactivity of the ACC is heavily implicated in driving negative emotions associated with chronic pain (Price, 2000; Shackman et al., 2011). Although we did not detect obvious changes in affective behaviors in present study, we cannot ensure that negative emotions will not show up as pain is prolonged. Thus, the possibility exists that MIP appears ahead of pain-associated affective disorders in chronic pain processing and MIP may provide predicting value for the following comorbid affective disorders, which may extend the clinical significance of the MIP behaviors in patients with chronic pain.

A limitation of our study is that we did not use stereologically unbiased cell counting methods. The counting results may be affected by sampling biases, errors in estimating the numbers of neurons, and potential experimental procedures altering the sizes of the area we counted (e.g., differences in the size of the injection site). In addition, errors may be caused by lipofuscin-induced autofluorescence. Nevertheless, combined with specific neural manipulations, immunofluorescent staining, and behavioral tests, our current data can support the notion that the cross-callosal projection neurons in ACC contralateral to the CCI contribute to the MIP induced by CCI.

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