Research Paper

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Neural pathways in medial septal cholinergic modulation of chronic pain: distinct contribution of the anterior cingulate cortex and ventral hippocampus

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Abstract

One specific behavior can be synergistically modulated by different neural pathways. Medial septal (MS) cholinergic system innervates widespread cortical and subcortical regions and participates in pain modulation, but the underlying neural pathways are not fully understood. This study examined the contribution of MS cholinergic neurons and 2 neural pathways: MS–rostral anterior cingulate cortex (rACC) and MS–ventral hippocampal CA1 (vCA1), in modulating perceptual and affective pain behaviors in a mouse model of chronic inflammatory pain. We found that chronic pain activated MS cholinergic neurons and pyramidal neurons in the rACC, but suppressed pyramidal neuronal activities in the vCA1, all of which contributed to the maintenance of pathological pain. Chemogenetic inhibition of MS cholinergic neurons or the MS–rACC pathway inhibited rACC pyramidal neuronal activities and attenuated perceptual and affective dimensions of chronic pain. By contrast, chemogenetic activation of MS cholinergic neurons also produced analgesia, but by rescuing hypofunctional pyramidal neurons in vCA1. These results clearly demonstrate that the MS cholinergic system differentially modulates chronic inflammatory pain through MS–rACC or MS–vCA1 pathways. More significantly, our research provides evidence for a novel paradigm of neural circuit modulation: MS cholinergic inhibition and activation induce similar analgesia but through distinct neural pathways.

Keywords: Pain, Medial septum, Acetylcholine, Hippocampus, Anterior cingulate cortex

1. Introduction

One neural pathway may participate in various behaviors and vice versa, 1 specific behavior can be synergistically modulated by different neural pathways. Medial septum (MS) and diagonal band complex are part of the basal forebrain cholinergic system crucial for information processing and various behaviors including chronic pain.[6,40] The MS receives nociceptive information directly from the spinal dorsal horn,[4,52] and more than 50% of MS neurons are excited by noxious stimuli from widespread peripheral regions.[20] An intriguing finding from previous reports is the constant analgesic effect of MS cholinergic manipulation in chronic pain, regardless of cholinergic inhibition or activation. Loss of MS cholinergic neurons in aging and Alzheimer disease[2,59] or after selective ablation with 192 IgG-saporin (SAP)[71] is accompanied by decreased pain sensitivity. On the other hand, a variety of physiological processes, such as environmental enrichment, exercise, and cognitive activities, promote forebrain cholinergic transmission and relieve pain.[27,29,33] Direct intraventricular injection of cholinergic agonists also produces analgesia.[30]

One explanation for these confusing findings is that both chronic pain per se and pain-modulatory neural processes implicate the MS cholinergic system, with different MS pathways recruited on cholinergic inhibition and activation. But there is no direct evidence supporting this hypothesis. Medial septal cholinergic neurons target widespread cortical and subcortical pain-relevant areas including the rostral anterior cingulate cortex (rACC) and hippocampus.[25,62] In this study, we applied chemogenetic manipulation on MS cholinergic pathways in a mouse model of chronic inflammatory pain induced by intraplantar injection of complete Freund adjuvant (CFA), and identified 2 cholinergic pathways that differentially participated in pain modulation: the MS–rACC pathway maintained pathological pain, whereas the MS–ventral hippocampal CA1 (vCA1) pathway contributed to pain relief induced by MS cholinergic activation.

2. Materials and methods

2.1. Animals

Adult male ChAT (choline acetyltransferase)-cre transgenic mice (Jax Lab #006410, subsequently referred to as ChAT mice) and C57BL/6 mice were 8 to 10 weeks of age at the beginning of experiments and housed in 4 to 6 cohorts. Temperature (22∼23°C), humidity (40%-60%), and circadian cycle (12 hours of light/dark

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cycles, starting at 07:00) were maintained in custom-designed ICV cabinets. Food and water were available ad libitum. All animal experimental procedures followed the Guidelines of the Committee for Research and Ethical Issues of International Association for the Study of Pain,[73] and were approved by the Animal Care and Use Committee of Peking University Health Science Center. All behavioral testing was performed in a blind manner.

2.2. Stereotaxic cannula implantation and intracranial virus injection

For intracranial virus injection, mice were anesthetized with 1% sodium pentobarbitone and placed in a stereotaxic frame (RWD, Shenzhen, China). Mice were injected with an AAV vector expressing hM3Dq or hM4Di receptors in the cre-dependent configuration (AAV5-hSyn-DIO-hM3D(Gq)-mCherry, AAV5-hSyn-DIO-hM4D(Gi)-mCherry)[3] (1×10^{12} virus particles/mL, 0.5 μL, the University of North Carolina Vector Core Facilities) into the MS (−1.2 mm anterior–posterior [AP], 0.73 mm medial–lateral [ML], and −4.13 mm dorsal–ventral [DV] 10° angle towards the midline, from the bregma) at a rate of 0.1 μL/minute through a 0.5 μL Hamilton microsyringe. After injection, needles were left in place for an additional 5 minutes before it was slowly withdrawn to minimize spread of viral particles along the injection tract. For pathway experiments, metal cannula (RWD) was implanted bilaterally in the rACC (+1.6 mm AP, ±0.4 mm ML, and −0.7 mm DV) or vCA1 (−3.0 mm AP, ±3.2 mm ML, and −2.7 mm DV) after virus injection. Animals were given 3 weeks for recovery from surgery and virus expression. Injection sites were verified histologically after behavioral testing.

2.3. Chemogenetics

For chemogenetic manipulation of MS cholinergic neurons, mice were injected intraperitoneally (i.p.) (1.0 mg/kg dissolved in normal saline)[45] or intracranially through an inner cannula (3.0 μM in artificial cerebral spinal fluid [aCSF], 0.5 μL) with clozapine N-oxide (CNO; Tocris, Bristol, United Kingdom)[15] 30 minutes before behavioral assessment. Same amounts of saline or aCSF were used as controls, respectively. This dosage of CNO was selected on the basis of both published studies and our pilot experiments.[69]

For chemogenetic manipulation of rACC or vCA1 pyramidal neurons, rAAV5-CaMKIIα-hM3D(Gq)-mCherry, or rAAV5-CaMKIIα-hM4D(Gi)-mCherry was injected into the rACC or vCA1 in C57BL/6 mice, and CNO was i.p. injected as described above.

2.4. Complete Freund adjuvant–induced inflammatory pain and spared nerve injury–induced neuropathic pain

To induce inflammatory pain, mice received intraplantar injection of 40 μL CFA into their left hind paws. For control animals, an equal volume of saline was injected instead.[66,72]

For spared nerve injury (SNI), mice were anaesthetized with 1% sodium pentobarbitone. The skin overlying the left calf was incised and the subcutaneous muscles were separated by blunt dissection to reveal the 3 branches of the sciatic nerve. The tibial nerve and common peroneal nerve were ligated and cut, leaving the sural nerve intact. For the animals in the sham group, the sciatic nerve was only exposed but not ligated or cut.[12]

2.5. Thermal and mechanical pain measurement

Each animal was handled for 10 minutes and adapted in a Plexiglas cube for 30 minutes for 3 days before experiments. Thermal or mechanical pain threshold was measured while the animal stayed calm and awake. Paw withdrawal latencies (PWLs) to thermal stimuli were measured with a focused radiant heat (15 W of power) applied onto the central region of the hind paw (Hargreaves Method, IITC 390).[72] Paw withdrawal latencies were measured 3 times at minimal 5-minute intervals and averaged. To avoid tissue damage, a cutoff time of 20 seconds was imposed.

Mechanical pain threshold was assessed by von Frey filaments (Stoelting, Wood Dale, IL). Each test started from an initial filament (0.07 g). The filaments were inserted through the mesh floor of the cage and applied to the central (CFA model) or lateral (SNI model) plantar surface of the paw in an ascending order. Each filament was applied 5 times at an interval of about 5 minutes. The threshold was taken as the lowest force required for a withdrawal reflex of the paw to 3 of 5 repetitive stimuli, with the cutoff set at 2.0 g.[57]

2.6. Complete Freund adjuvant–induced conditioned place aversion

Mice were tested in a conditioning apparatus consisting 2 equal rectangular compartments ($20 \times 15 \times 15$ cm)[64] with distinct visual and tactile features. The apparatus was cleaned with 75% ethanol after each test.

During the preconditioning phase (day 1), animals were allowed to freely explore the whole box for 15 minutes. Time spent in either chamber was recorded. Animals were excluded if they spent more than 70% of the time (>720 seconds) in either compartment. With this standard, 30 of 154 mice were excluded in this study.

During the conditioning phase (days 2 and 3), animals were first randomly restricted in one of the chambers for 30 minutes. On the next day, CFA was subcutaneously injected into the plantar surface of the left hind paw 6 hours before the mouse was placed in the other compartment for 30 minutes. Complete Freund adjuvant–induced inflammation appeared within 2 hours after the injection and peaked at 6 to 24 hours.[19] The chambers were counterbalanced among the subjects.

The postconditioned phase (day 4) repeated the procedure of day 1, with CNO or saline injected 0.5 hours before animal entrance into the apparatus.

A conditioned place aversion (CPA) score was calculated with the amount of time spent in the conditioning compartment (ie, compartment paired with CFA) on the postconditioning day (ie, the last day) subtracted from the amount of time spent in the same compartment on the preconditioning day.[37]

2.7. Food-induced conditioned place preference

The procedure of food–conditioned place preference (CPP) was similar to CFA-CPA, with the only difference in that on day 3, the mouse did not receive CFA injection but explored the second compartment containing favored food (small chocolate pellets).

2.8. Open-field test

Spontaneous motor activity was assessed in an open field ($60 \times 60 \times 60$ cm) exposed to 60 lux illumination. The area was divided into 16 quadrants (4 central and 12 peripheral). Mice were individually placed into the center of the field and allowed to explore for 5 minutes. Total distance travelled during this period was recorded using a digital video camera and measured using SMART software (v2.5.21; Panlab, Harvard Apparatus, Holliston, MA. SMART Video-tracking, RRID: SCR_002852). The apparatus was cleaned using 75% ethanol after each exploration.[18,69,72]

2.9. Immunostaining

Animals were deeply anesthetized with 1% pentobarbital (100 mg/kg, i.p.) and transcardially perfused with normal saline followed by 4% paraformaldehyde in 0.01 M phosphate buffer (PB, pH 7.4). After removal from the skull, the brain was postfixed for 4 to 6 hours and dehydrated in graded sucrose solutions (20%-30%). After embedded in optimal cutting temperature compound, the brain was sectioned coronally into 50 μm slices on a freezing microtome (Model 1950; Leica Instrument Co, Ltd, Solms, Germany).

For ChAT or c-Fos 3,3′-diaminobenzidine (DAB) staining,[7,38] free-floating sections were washed in phosphate-buffered saline (PBS) and incubated in 3% H_2O_2 in methanol for 1 hour to quench endogenous peroxidase activity. After several rinses in PBS, sections were incubated in 10% normal sheep serum in PBS for 60 minutes, followed by incubation in primary antibodies (rabbit anti-ChAT, 1:200, Millipore AB143; rabbit anti-cFos, 1:300, Santa Cruz sc-253) in 0.5% PBST (0.5% TritonX-100 in 0.1M PBS) for 24 hours at 4°C. Sections were rinsed and incubated with biotin-conjugated anti-rabbit (ChAT or c-Fos) antibodies (Beijing Zhong Shan Jin Qiao, China; 1 hour at 24°C). Sections were then washed in PBS, followed by several washes in PBS without the detergent added before immunoreactive profiles were generated using a DAB kit for peroxidase. Sections were then mounted, dehydrated, and coverslipped. Negative controls were performed by omitting the primary antibody. We did not detect nonspecific labeling in negative controls.

For ChAT, EAAC1, PV, and c-Fos immunofluorescence, sections were rinsed with 0.3% Triton-X in 0.1 M PBS and blocked with 10% bovine serum albumin for 1 hour. Sections were then incubated with primary antibodies (sheep anti-ChAT, 1:200, Millipore AB143; sheep anti-EAAC1, 1:200, Millipore AB1520; mouse anti-PV, 1:200, Sigma P3088; rabbit anti-c-Fos 1:500, Santa Cruz Biotechnology, sc-253) in 0.3% PBST for 36 hours at 4°C. Sections were rinsed and incubated with Alexa Fluor 594-conjugated donkey anti-rabbit antibody, Alexa Fluor 488-conjugated donkey anti-sheep antibody, Alexa Fluor 488-conjugated sheep anti-mouse antibody, (1:500; Abcam, Cambridge, United Kingdom, 1.5 hours at 24°C), rinsed in PBS, mounted, and coverslipped. Images were taken by a laser scanning confocal microscope (model FV1000; Olympus Co, Ltd, Tokyo, Japan).

2.10. Statistical analysis

Data were expressed as mean ± SEM. All statistical analyses were performed using GraphPad Prism 5 software. Differences between 2 or multiple groups were calculated using the paired or unpaired Student *t* test, or analysis of variance (ANOVA) followed by Bonferroni post hoc analyses, respectively. *P* values lower than 0.05 were considered to represent significant differences.

3. Results

3.1. Complete Freund adjuvant–induced inflammatory pain activates medial septal cholinergic neurons

We first examined whether persistent pain activated MS cholinergic neurons. Mice with intraplantar CFA injection showed significant paw inflammation with thermal hyperalgesia and mechanical allodynia, which peaked 6 to 24 hours after injection.[72] We detected a 3-fold increase in the number of c-Fos-positive neurons, indicating neuronal excitation, in the MS 6 hours after CFA injection ($t = 5.1$, $P < 0.001$, the unpaired *t* test, **Figs. 1A–C**). The majority (69.9 ± 11.0%) of these c-Fos-positive neurons colabelled with ChAT (**Fig. 1D**), a marker of cholinergic neurons, whereas others with EAAC1 or PV (**Figs. 1E and F**), markers of glutamatergic and GABAergic neurons, respectively. These findings indicate strong activation of MS cholinergic neurons in persistent inflammatory pain.

3.2. Chemogenetic inhibition of medial septal cholinergic neurons attenuates perceptual and affective dimensions of inflammatory pain

To elucidate the contribution of MS cholinergic activation to pain, we expressed inhibitory Gi-coupled receptors in MS cholinergic neurons in ChAT-cre mice, and 87.8 ± 6.1% of ChAT-positive MS neurons were transfected by the virus (mCherry) (**Fig. 2A**). The Gi-coupled receptor was activated exclusively by CNO to inhibit local cholinergic neurons, as shown in our previous work.[69]

Medial septal cholinergic inhibition significantly attenuated CFA-induced thermal hyperalgesia (time effect: $F_{(6, 72)} = 16.20$, $P < 0.001$; group effect: $F_{(1, 72)} = 30.81$, $P < 0.001$; interaction: $F_{(6, 72)} = 3.39$, $P < 0.01$, **Fig. 2B**) and mechanical allodynia (time effect: $F_{(6, 60)} = 40.26$, $P < 0.001$; group effect: $F_{(1, 60)} = 17.8$, $P < 0.01$; interaction: $F_{(6, 60)} = 4.27$, $P < 0.01$, 2-way ANOVA with the Bonferroni test, **Fig. 2C**). Paw withdrawal latencies and paw withdrawal thresholds of the contralateral paw remained unaffected throughout these procedures (data not shown). It was worth noting that chemogenetic inhibition affected neither baseline pain thresholds, nor those 28 days after CFA injection when pathological hyperalgesia and allodynia had recovered. The ineffectiveness on day 28 was unlikely resultant from lost virus efficiency or loss of cholinergic neurons because the same manipulation produced significant analgesia on day 28 in SNI, a more persistent neuropathic pain model (time effect: $F_{(5, 50)} = 32.76$, $P < 0.001$; group effect: $F_{(1, 50)} = 42.98$, $P < 0.001$; interaction: $F_{(5, 50)} = 3.47$, $P < 0.01$, Supplementary Fig. 1B, available online at http://links.lww.com/PAIN/A568), and no significant changes in the number of MS cholinergic neurons were observed up to 28 days after CFA injection ($F_{(4, 25)} = 0.55$, $P > 0.05$, 1-way ANOVA with the Bonferroni test, Supplementary Figs. 2A and B, available online at http://links.lww.com/PAIN/A568). The pathological pain-specific effect of MS cholinergic inhibition was further confirmed by the fact that PWLs did not change with intraplantar saline injection (interaction: $F_{(4, 64)} = 1.01$, $P > 0.05$, time effect: $F_{(4, 64)} = 3.41$, $P < 0.05$; group effect: $F_{(1, 64)} = 2.08$, $P > 0.05$, 2-way ANOVA with the Bonferroni test, Supplementary Fig. 3B, available online at http://links.lww.com/PAIN/A568). Clozapine N-oxide administration in wild-type instead of ChAT-cre mice with chemogenetic virus expression also confirmed specificity of our chemogenetic manipulation (interaction: $F_{(6, 108)} = 0.49$, $P > 0.05$; time effect: $F_{(6, 108)} = 80.30$, $P < 0.001$; group effect: $F_{(1, 108)} = 0.15$, $P > 0.05$; 2-way ANOVA with the Bonferroni test, Supplementary Fig. 3C, available online at http://links.lww.com/PAIN/A568).

In addition to evoked nociceptive responses, we also examined spontaneous pain affect with CFA-induced CPA (CFA-CPA). When hind paw CFA injection was paired with 1 compartment in the place-conditioning apparatus, mice spent significantly less time in this compartment on the postconditioning test day ($t = 2.6$, $P < 0.05$, the paired *t* test, **Fig. 2D**, Supplementary Fig. 3A, available online at http://links.lww.com/PAIN/A568), indicating aversion to pain-associated context. Inhibiting MS cholinergic neurons during probing significantly attenuated this aversion ($t = 2.5$, $P < 0.05$, the unpaired *t* test, **Fig. 2E**). This effect was not caused by memory deficits because a control food-induced CPP (food-CPP) paradigm was not affected ($t = 1.0$, $P > 0.05$, the unpaired *t* test,

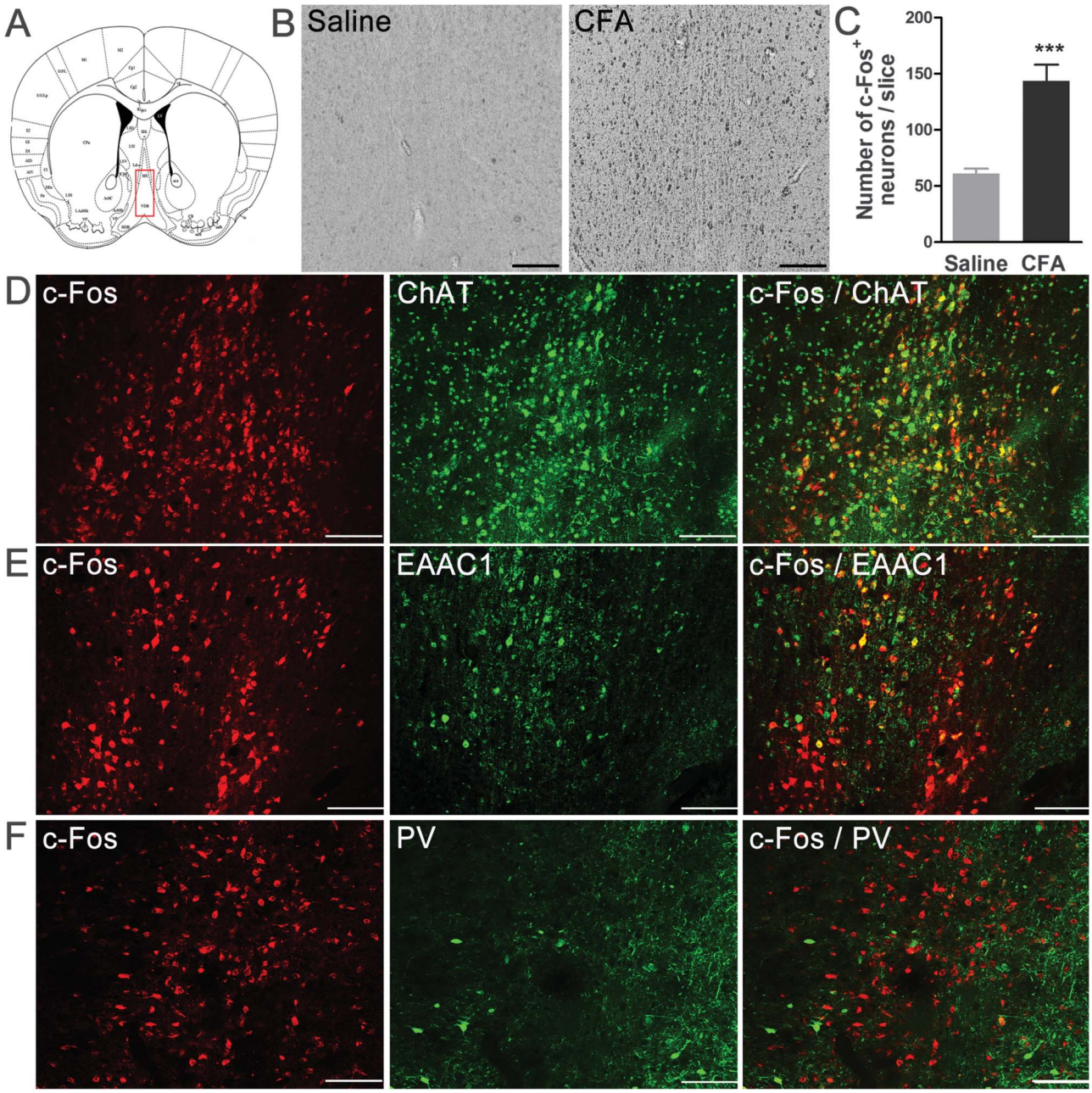

Figure 1. Complete Freund adjuvant–induced inflammatory pain activates MS cholinergic neurons in mice. (A) Illustration of the MS (red box). (B) Representative immunohistochemistry images showing enhanced c-Fos expression in the CFA (right) group (6 hours after CFA injection). Scale bar: 100 μm. (C) c-Fos increased significantly in CFA group compared with saline group ($t = 5.1$, $^{***}P < 0.001$, the unpaired t test). 69.9 ± 11.0% of c-Fos (red) in CFA group were colocalized with ChAT (green) (D), whereas the rest colabelled with EAAC1 (E) and PV (F). Four slices were taken from each mouse, 4∼5 mice in either group. Error bars indicated SEMs. Scale bar: 100 μm. CFA, complete Freund adjuvant; MS, medial septum.

Supplementary Figs. 3A and D, available online at http://links.lww.com/PAIN/A568).

Together, these findings indicate a crucial role of the MS cholinergic system in maintaining pathological pain.

3.3. Chemogenetic activation of medial septal cholinergic neurons attenuates perceptual and affective dimensions of inflammatory pain

A variety of physiological processes, including environmental enrichment, locomotion, and cognitive activities, promotes forebrain cholinergic transmission and modulates pain.[27,29,33,72] To directly explore the role of MS cholinergic activation in pain modulation, we chemogenetically activated MS cholinergic neurons with excitatory Gq expression. The excitatory effect was verified by a 4-fold increase of c-Fos immunofluorescence after CNO injection (63.1 ± 3.99 vs 14.0 ± 2.36, CNO group vs saline group, $t = 7.8$, $P < 0.001$, the unpaired t test), which colocalized with mCherry from the virus (**Figs. 3A–D**).

Cholinergic activation in the MS produced very similar effects to inhibition: both perceptual and affective dimensions of inflammatory pain were attenuated (thermal hyperalgesia: time effect: $F_{(6, 72)} = 29.94$, $P < 0.001$; group effect: $F_{(1, 72)} = 32.71$, $P < 0.001$; interaction: $F_{(6, 72)} = 3.10$, $P < 0.01$, **Fig. 4A**; mechanical allodynia: time effect: $F_{(6, 60)} = 29.22$,

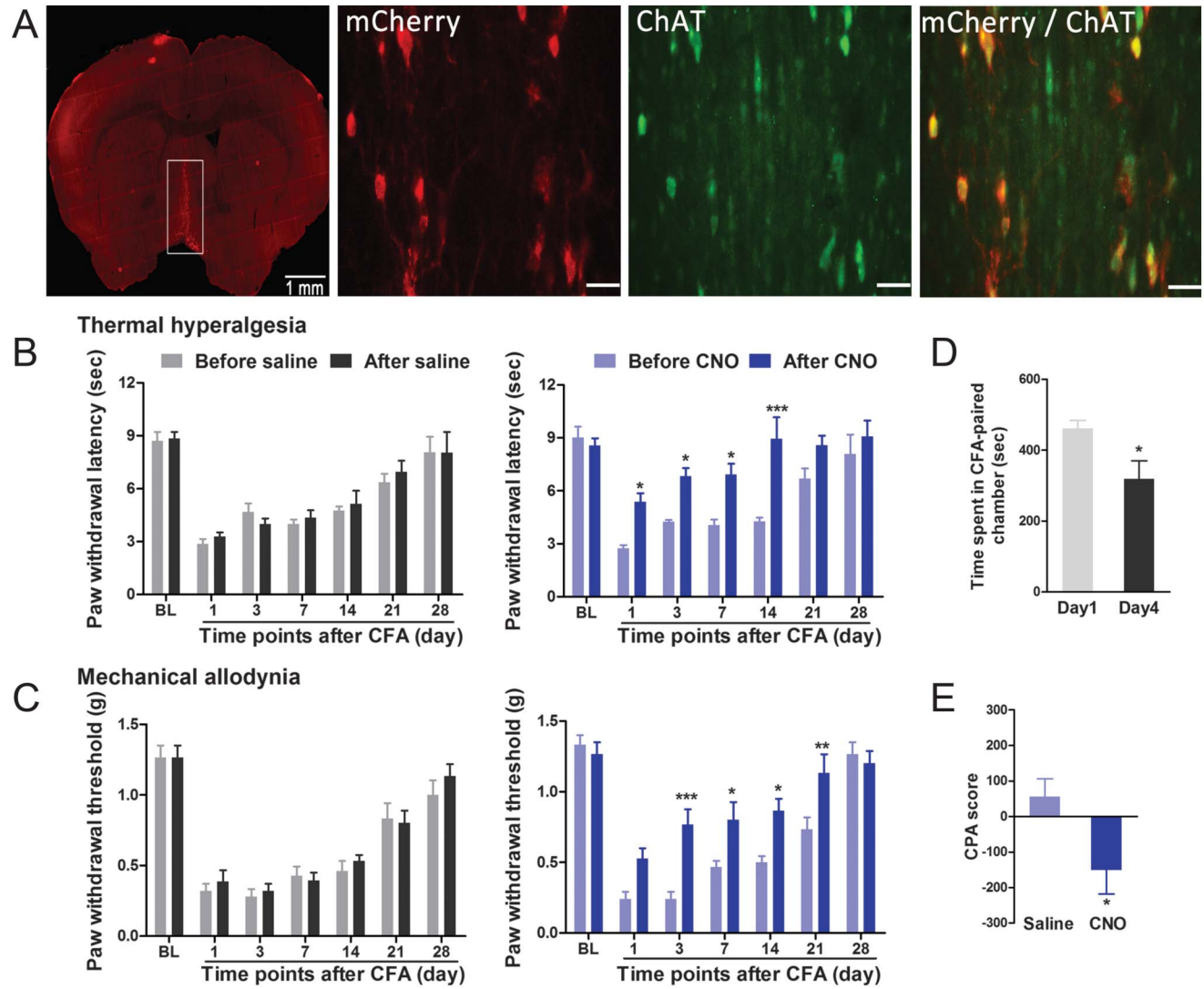

Figure 2. Chemogenetic inhibition of MS cholinergic neurons attenuates perceptual and affective dimensions of inflammatory pain in mice. (A) Colocalized expression of AAV5-hSyn-DIO-hM4D(Gi)-mCherry virus with ChAT (green) in the MS (white box) of ChAT-cre mice (87.8 ± 6.1%, 3 slices per mouse from 3 mice). Scale bar: 100 μm. Chemogenetic inhibition of MS cholinergic neurons significantly attenuated CFA-induced thermal hyperalgesia (B) and mechanical allodynia (C), without affecting physiological pain thresholds. n = 7∼8 in each group.*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, 2-way ANOVA with the Bonferroni test. BL: baseline. (D) Inflammatory mice spent significantly less time in CFA-paired compartment on the postconditioning test day (day 4), indicating successful establishment of CFA-CPA. n = 8 in each group. *$P < 0.05$, the paired *t* test. (E) Inhibiting MS cholinergic neurons during probing significantly attenuated CFA-CPA. n = 8 in each group. *$P < 0.05$, the unpaired *t* test. ANOVA, analysis of variance; CFA, complete Freund adjuvant; CNO, clozapine N-oxide; CPA, conditioned place aversion; MS, medial septum.

$P < 0.001$; group effect: $F_{(1, 60)} = 15.24$, $P < 0.01$; interaction: $F_{(6, 60)} = 2.32$, $P < 0.05$, 2-way ANOVA with the Bonferroni test, **Fig. 4B**; CPA model: $t = 3.9$, $P < 0.01$, the paired *t* test, **Fig. 4C**; CPA score: $t = 3.57$, $P < 0.01$, the unpaired *t* test, **Fig. 4D**). Locomotion was not affected by MS manipulations, indicated by normal performance in the open-field test (for the MS, $F_{(2, 36)} = 0.21$, $P > 0.05$, 1-way ANOVA, Supplementary Fig. 4A, available online at http://links.lww.com/PAIN/A568).

Thus, MS cholinergic activation also produces analgesic effects.

3.4. Inhibiting medial septal–rostral anterior cingulate cortex pathway relieves complete Freund adjuvant–induced inflammatory pain

Two major targets of the MS cholinergic system are the rACC and hippocampus, both involved in pain modulation.[11,67,72] We asked whether and how these MS neural pathways participate in the modulation of chronic pain. We first examined the MS–rACC pathway, by expressing AAV5-hSyn-DIO-hM4D(Gi)-mCherry or AAV5-hSyn-DIO-hM3D(Gq)-mCherry viruses in the MS of ChAT-cre mice and delivered CNO in the rACC through implanted cannulas.

Although baseline pain thresholds were not affected (**Figs. 5A and C**), cholinergic inhibition of the MS–rACC pathway alleviated thermal hyperalgesia in a similar manner to MS inhibition (time effect: $F_{(6, 96)} = 21.87$, $P < 0.001$; group effect: $F_{(1, 96)} = 50.31$, $P < 0.001$; interaction: $F_{(6, 96)} = 3.20$, $P < 0.01$, 2-way ANOVA with the Bonferroni test, **Fig. 5A**, right). No significant changes of PWLs were detected with aCSF injection (interaction: $F_{(6, 84)} = 0.24$, $P > 0.05$; time effect: $F_{(6, 84)} = 76.63$, $P < 0.001$; group effect: $F_{(1, 84)} = 0.62$, $P > 0.05$, 2-way ANOVA with the Bonferroni test, **Fig. 5A**, left). Complete Freund adjuvant–CPA (CFA-CPA) was also attenuated by inhibiting

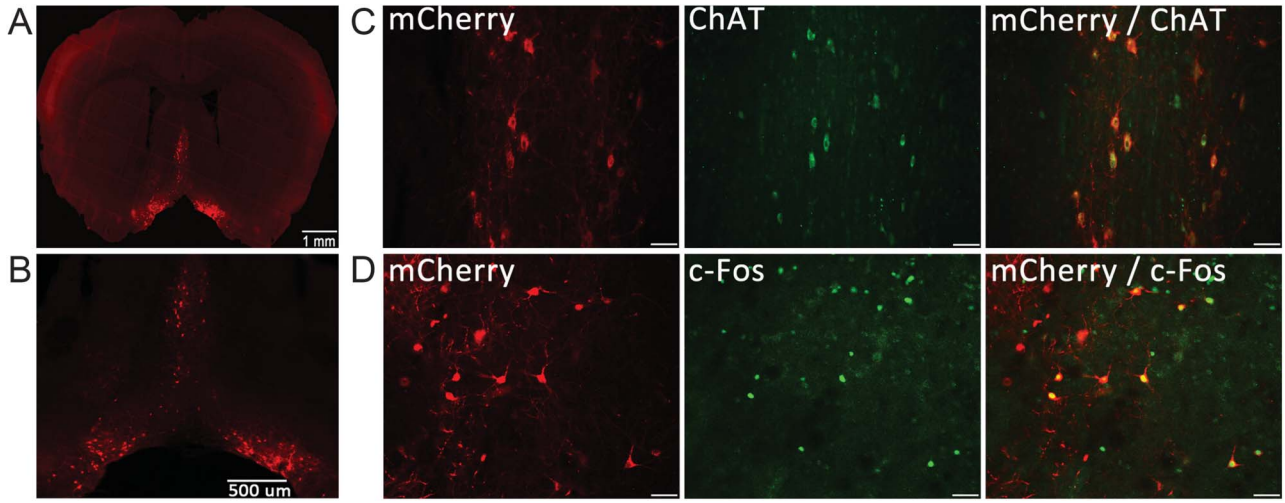

Figure 3. Validation of excitatory chemogenetic manipulation of MS cholinergic neurons in mice. (A) Expression of AAV5-hSyn-DIO-hM3D(Gq)-mCherry virus in the MS of ChAT-cre mice. (B) Higher magnification view of (A). (C) Gq virus colocalized with ChAT (green). (D) The excitatory effect was verified by increased c-Fos (green) immunofluorescence after CNO injection (number of c-Fos-positive neurons: 63.1 ± 3.99 vs 14.0 ± 2.36, CNO group vs saline group, $t = 7.8$, $^{***}P < 0.001$, the unpaired t test. Four slices were taken from each mouse, 4∼5 mice in each group), which colocalized with mCherry from the virus, scale bar: 100 μm. CNO, clozapine N-oxide; MS, medial septum.

MS–rACC cholinergic transmission (CPA score, $t = 2.2$, $P < 0.05$, the unpaired t test, **Fig. 5B**).

By contrast, activating the MS–rACC pathway affected neither pain perception (interaction: $F_{(6,\ 126)} = 0.95$, $P > 0.05$; time effect: $F_{(6,\ 126)} = 120.50$, $P < 0.001$; group effect: $F_{(1,\ 126)} = 0.01$, $P > 0.05$, 2-way ANOVA with the Bonferroni test, **Fig. 5C**), nor pain affect ($t = 0.5$, $P > 0.05$, **Fig. 5D**).

These results indicate that the MS–rACC cholinergic pathway maintains chronic pain.

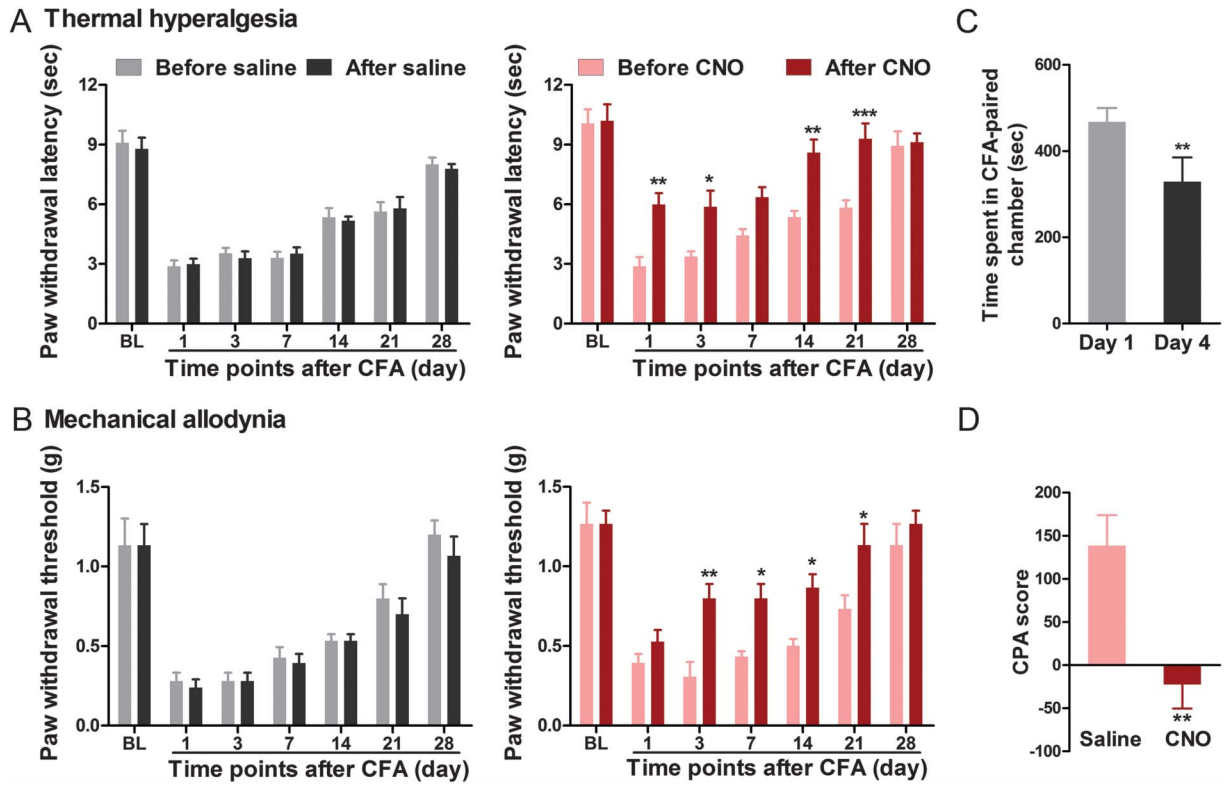

Figure 4. Chemogenetic activation of MS cholinergic neurons attenuates perceptual and affective dimensions of inflammatory pain in mice. Cholinergic activation of MS cholinergic neurons significantly attenuated CFA-induced thermal hyperalgesia (A) and mechanical allodynia (B), without affecting physiological pain thresholds. n = 7∼8 in each group.$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, 2-way ANOVA with the Bonferroni test. (C) Mice spent significantly less time in CFA-paired compartment on the postconditioning test day (day 4) showed that CFA-CPA model was successfully established. n = 7 in each group. $^{**}P < 0.01$, day 4 vs day 1, the paired t test. (D) Activating MS cholinergic neurons during probing significantly attenuated this aversion. n = 7 in each group. $^{**}P < 0.01$, CNO group vs saline group, the unpaired t test. ANOVA, analysis of variance; BL, baseline; CFA, complete Freund adjuvant; CNO, clozapine N-oxide; CPA, conditioned place aversion; MS, medial septum.

3.5. Medial septal cholinergic inputs maintain enhanced rostral anterior cingulate cortex pyramidal neuronal activities in inflammatory pain

We next explored the effects of MS cholinergic inputs on rACC neuronal activities. Consistent with previous reports of increased ACC neuronal activities in chronic pain,[32,55,68] we observed increased expression of c-Fos in the rACC 6 hours after CFA injection (71.0 ± 7.8 vs 24.9 ± 3.1 per slice, CFA group vs saline group, $t = 6.2$, $P < 0.001$, the unpaired t test). The majority (88.7 ± 4.9%) of these c-Fos-positive neurons colocalized with EAAC1 (**Figs. 6A and B**). These enhanced neuronal activities were completely reversed by chemogenetic inhibition of MS cholinergic neurons ($F_{(2, 15)} = 34.2$, $P < 0.001$, 1-way ANOVA, **Figs. 6A and D**). By contrast, chemogenetic activation of MS cholinergic neurons did not further increase c-Fos expression in the rACC (**Figs. 6A and D**). Thus, MS cholinergic inputs are necessary for maintaining enhanced rACC pyramidal neuronal activities in inflammatory pain.

We next examined whether inhibiting rACC neuronal activities was sufficient to induce analgesia in inflammatory pain. The majority of c-Fos-positive neurons (88.7 ± 4.9%) colocalized with pyramidal neuronal markers (**Figs. 6B and C**), and only a few with PV (Supplementary Fig. 5C, available online at http://links.lww.com/PAIN/A568). As expected, direct chemogenetic inhibition of rACC pyramidal neurons attenuated thermal hyperalgesia in inflammatory pain (7 days after CFA injection. $t = 5.9$, $P < 0.01$, after CNO vs before CNO, the paired t test, **Fig. 6E**), whereas activating these neurons did not affect pain behaviors possibly because of a ceiling effect. These findings indicate that MS cholinergic inputs maintain enhanced pyramidal neuronal activities in the rACC and in turn, thermal hyperalgesia in inflammatory pain.

3.6. Activating medial septal–vCA1 pathway relieves complete Freund adjuvant–induced inflammatory pain

Chronic pain suppresses hippocampal neuronal activities.[48,49,72] Medial septal cholinergic inputs exhibit a facilitating effect on CA1 neuronal activities,[17,24] prompting us to hypothesize that the analgesic effect of MS cholinergic activation was achieved through the MS-CA1 pathway. Considering the more significant role of the ventral hippocampus in pain modulation,[67,72] we targeted vCA1. Significant analgesia was achieved through activating the MS–vCA1 pathway (time effect: $F_{(6, 96)} = 43.25$, $P < 0.001$; group effect: $F_{(1, 96)} = 15.62$, $P < 0.01$; interaction: $F_{(6, 96)} = 3.15$, $P < 0.05$, 2-way ANOVA with the Bonferroni test, **Fig. 7A**, right). Paw withdrawal latencies did not change after aCSF microinjection (interaction: $F_{(6, 84)} = 0.62$, $P > 0.05$; time effect: $F_{(6, 84)} = 85.60$, $P < 0.001$; group effect: $F_{(1, 84)} = 0.09$, $P > 0.05$, 2-way ANOVA with the Bonferroni test, **Fig. 7A**, left). Similarly, CFA-CPA was also attenuated by activating MS–vCA1 ($t = 2.2$, $P < 0.05$, the unpaired t test, **Fig. 7B**). By contrast, inhibiting the MS–vCA1 pathway affected neither pain perception (interaction: $F_{(6, 96)} = 0.26$, $P > 0.05$; time effect: $F_{(6, 96)} = 47.02$, $P < 0.001$; group effect: $F_{(1, 96)} = 0.19$, $P > 0.05$, 2-way ANOVA with the Bonferroni test, **Fig. 7C**), nor pain affect ($t = 0.15$, $P > 0.05$, the unpaired t test, **Fig. 7D**). These results indicate that the MS–vCA1 pathway contributes to analgesia induced by MS cholinergic activation.

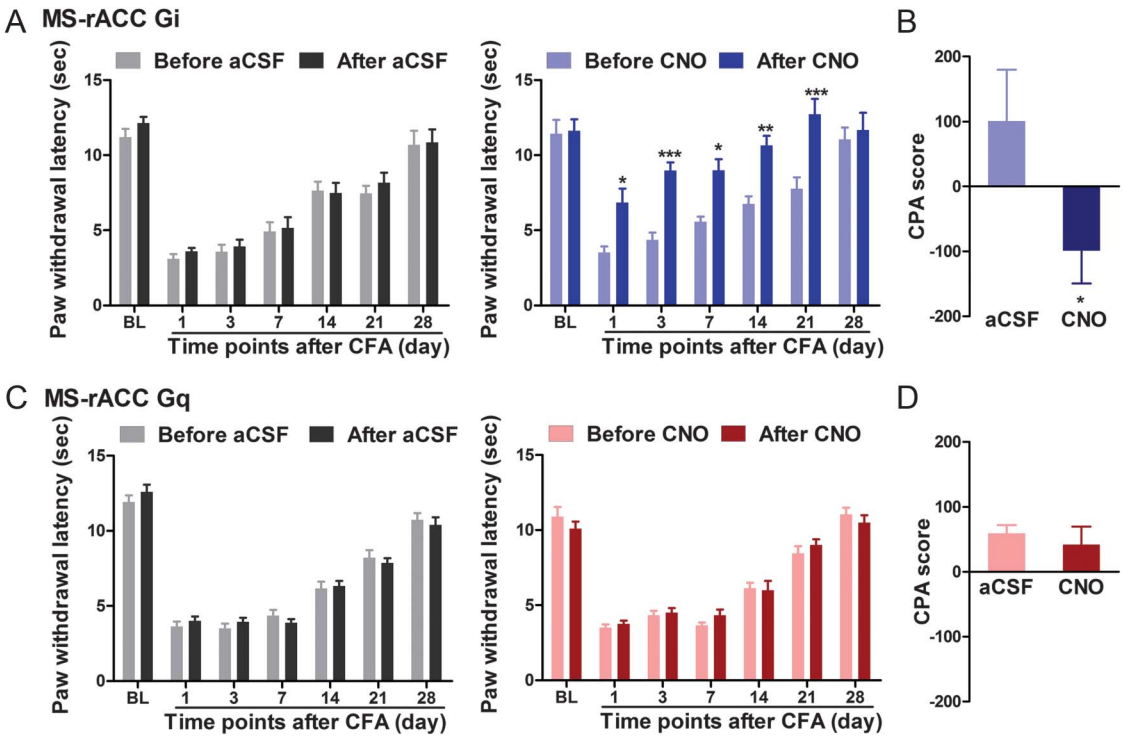

Figure 5. Inhibiting the MS–rACC pathway relieves CFA-induced inflammatory pain in mice. (A) Inhibition of the MS–rACC cholinergic pathway alleviated thermal hyperalgesia in inflammatory pain. n = 8~9 in each group. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, 2-way ANOVA with the Bonferroni test. (B) Complete Freund adjuvant–conditioned place aversion (CFA-CPA) was also attenuated by inhibiting MS–rACC cholinergic transmission. n = 8~10, *$P < 0.05$, CNO group vs aCSF group, the unpaired t test. By sharp contrast, activating the MS–rACC pathway affected neither pain perception (PWLs) (C) nor pain affect (CPA) (D). aCSF, artificial cerebral spinal fluid; ANOVA, analysis of variance; BL, baseline; CFA, complete Freund adjuvant; CNO, clozapine N-oxide; CPA, conditioned place aversion; MS, medial septum; PWL, paw withdrawal latency; rACC, rostral anterior cingulate cortex.

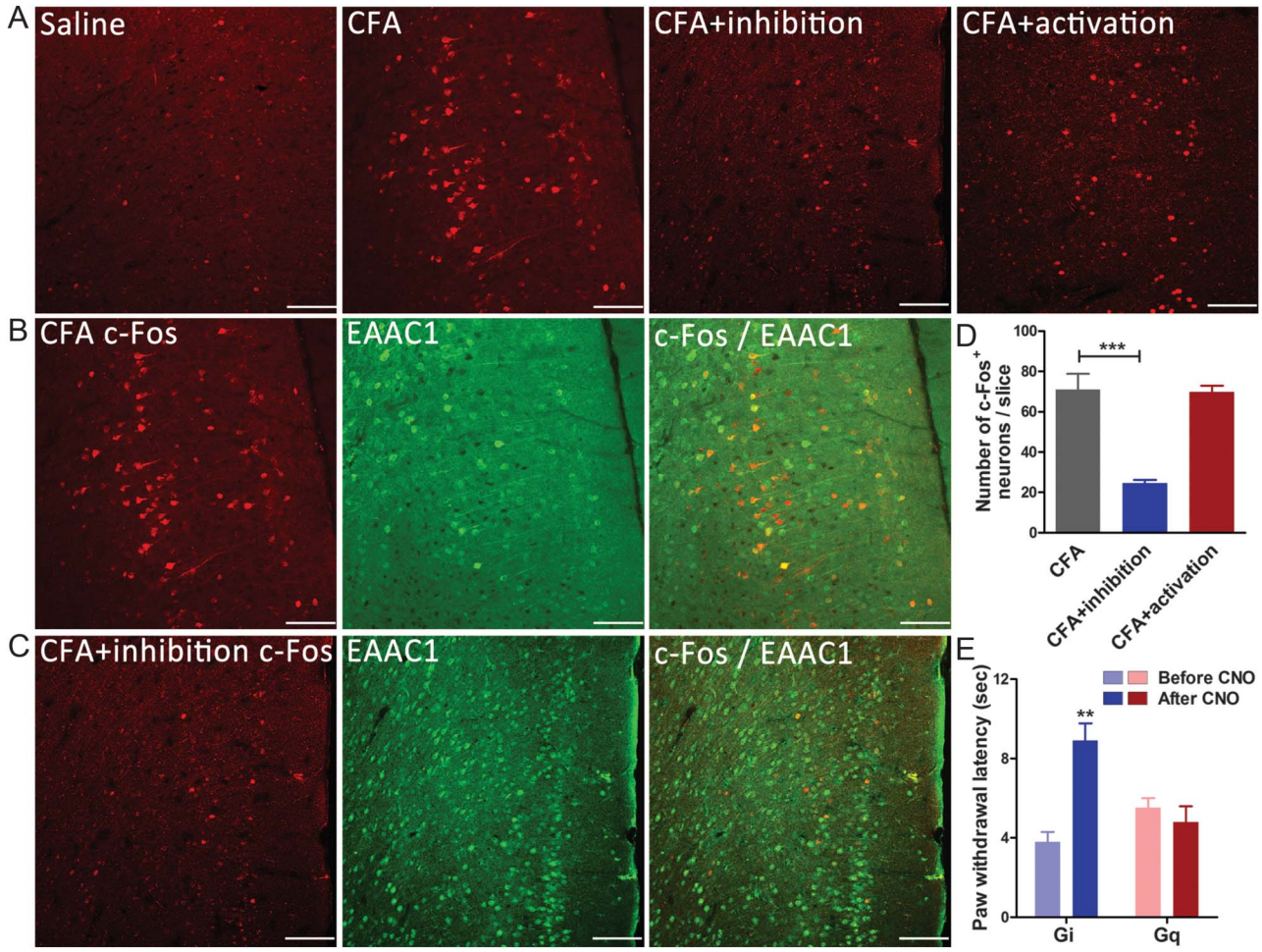

Figure 6. Medial septal cholinergic inputs maintain enhanced rACC pyramidal neuronal activities and inflammatory pain in mice. (A) Representative immunofluorescence images showing c-Fos expression in the rACC in saline, CFA, CFA + MS–rACC inhibition, and CFA + MS–rACC activation groups. (B) 88.7 ± 4.9% of c-Fos (red) expression in CFA group colocalized with EAAC1 (green). (C) Inhibition of cholinergic neurons in the MS–rACC pathway resulted in a significant decrease in both c-Fos and c-Fos/EAAC1 numbers in the rACC in inflammatory pain. Four slices were taken from each mouse, 5~6 mice in each group. Scale bar: 100 μm. (D) Quantitative analysis of (A). Compared with saline group, the number of c-Fos in CFA group significantly increased (71.0 ± 7.8 vs 24.9 ± 3.1 per slice, CFA group vs saline group, $t = 6.2$, $P < 0.001$, the unpaired t test), which was reversed with MS–rACC inhibition. ***$P < 0.001$, 1-way ANOVA. (E) Inhibiting rACC pyramidal neurons relieved thermal hyperalgesia in inflammatory pain (7 days after CFA injection), for Gi group, $t = 5.9$, $P < 0.01$, n = 6, after CNO vs before CNO, the paired t test. Activation of these neurons showed limited effect ($t = 0.81$, $P > 0.05$, n = 6, after CNO vs before CNO, the paired t test). ANOVA, analysis of variance; CFA, complete Freund adjuvant; CNO, clozapine N-oxide; MS, medial septum; rACC, rostral anterior cingulate cortex.

3.7. Medial septal cholinergic activation reverses suppressed CA1 pyramidal neuronal activities in inflammatory pain

In contrast to the rACC, we observed reduced c-Fos expression in hippocampal CA1, 6 hours after CFA injection (9.5 ± 1.1 vs 27.2 ± 2.6 per slice, CFA group vs saline group, $t = 6.7$, $P < 0.001$, the unpaired t test, **Fig. 8A**), consistent with previous reports of impaired hippocampal morphology, neuronal activities, and functions in chronic pain.[48,72] Chemogenetic activation of MS cholinergic neurons rescued the depressed neuronal activities ($F_{(2,\ 15)} = 8.6$, $P < 0.01$, 1-way ANOVA, **Fig. 8A**, Supplementary Fig. 6C, available online at http://links.lww.com/PAIN/A568), and 83.7 ± 7.7% of these activated neurons were pyramidal neurons (**Fig. 8B**).

Direct chemogenetic activation of ventral CA1 pyramidal neurons relieved CFA-induced thermal hyperalgesia (7 days after CFA injection. $t = 8.04$, $P < 0.001$, in Gq group, after CNO vs before CNO, the paired t test, **Fig. 8C**). By contrast, chemogenetic inhibition of MS cholinergic neurons showed limited effects on hippocampal activities and further inhibition of CA1 neurons failed to affect pain, both of which might be explained by a floor effect (**Figs. 8A and C**, Supplementary Fig. 6C, available online at http://links.lww.com/PAIN/A568).

These findings indicate that MS cholinergic activation recruits the hippocampus rather than the rACC under pathological pain. This pathway may constitute the forebrain basis of analgesia by cholinergic agonists, and underlie exercise, enrichment, and cognitive modulation of pain that induces cholinergic activation in the forebrain.[29,30,33]

4. Discussion

4.1. Medial septal–rostral anterior cingulate cortex cholinergic pathway maintains pathological pain

Nociceptive, cognitive, and affective processes reciprocally interact with each other during pain, and all involve central acetylcholine signaling.[50] In this study, persistent inflammatory pain induces significant cholinergic neuronal activation in the MS. Chemogenetic inhibition of MS cholinergic neurons attenuates pathological thermal hyperalgesia, mechanical allodynia, and

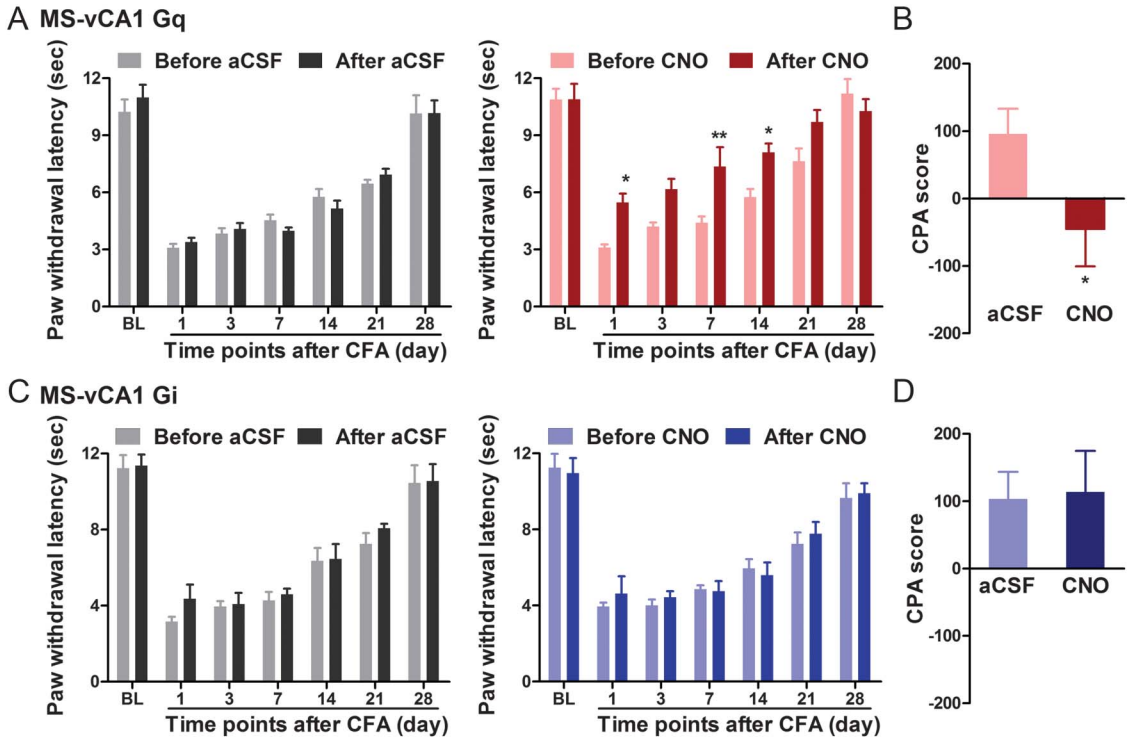

Figure 7. Activating the MS–vCA1 pathway relieves CFA-induced inflammatory pain in mice. Cholinergic activation of the MS–vCA1 pathway alleviated thermal hyperalgesia (A), n = 9 in each group. *$P < 0.05$; **$P < 0.01$, 2-way ANOVA with the Bonferroni test. No change was detected with aCSF injection (A, left), n = 8 in each group. Similarly, CFA-CPA was also attenuated by activating MS–vCA1 cholinergic transmission (B). n = 10∼12, *$P < 0.05$, CNO group vs aCSF group, the unpaired *t* test. Inhibiting the MS–vCA1 pathway affected neither pain perception (PWLs) (C) nor pain affect (CPA) (D). aCSF, artificial cerebral spinal fluid; ANOVA, analysis of variance; BL, baseline; CFA, complete Freund adjuvant; CNO, clozapine N-oxide; CPA, conditioned place aversion; MS, medial septum; PWL, paw withdrawal latency.

pain-induced negative affect, without affecting physiological pain. Responses to noxious thermal and mechanical stimuli mainly represent spinal reflections in naive animals,[1] but recruit cortical regions in subjects with pathological pain.[14]

Medial septal cholinergic neurons have an impact on the excitability of both ACC and hippocampal neurons, and are crucial to reduce attention to irrelevant stimuli.[8,53] Reexposure to novel environment evokes increased numbers of c-Fos-positive neurons in both regions, which can be attenuated by cholinergic lesions.[5] Direct nociceptive afferents from the spinal dorsal horn to the MS[4,52] might thus engage the attention system towards pain and underlie attention deficits in chronic pain.[16,21] Enhanced excitability and activities of pyramidal neurons in the ACC have been consistently reported in various pain models.[32,55,68] These neurons are also activated on pain anticipation or pain avoidance behaviors.[60,61] Activation of acetylcholine (ACh) receptors in the ACC induces robust calcium-dependent plateau potentials and persistent neuronal firing.[70] Medial septal cholinergic inhibition suppresses these neuronal hyperactivities (**Fig. 6**), consistent with previous reports of reduced ACC glucose metabolism in animals with cholinergic lesions in the MS.[35] Inhibition of pyramidal neuronal activities in the ACC is sufficient for relieving thermal hyperalgesia (**Fig. 6**) and mechanical allodynia[39] in chronic inflammatory pain. Optogenetic inhibition or local injection of GABA receptor agonists or ACh receptor antagonists in the ACC relieves pathological pain perception and affect,[15,42,54] further supporting our findings.

It is interesting to note that in CFA mice, inhibiting MS cholinergic neurons shows limited effects on hippocampal CA1 neuronal activities (**Fig. 8**), which are suppressed in persistent pain.[66] Indeed, even in naive animals, MS cholinergic lesions produce significant inhibition only in the frontal area but not in the hippocampus until very late stages.[58] Previous work has suggested that MS cholinergic lesions attenuate pain through inhibiting hippocampal theta oscillations.[63] However, our study with pathway-specific chemogenetic manipulation does not support direct contribution of the hippocampus, but the rACC instead, to pain maintenance.

4.2. Medial septal–vCA1 cholinergic pathway in pain modulation

The MS–vCA1 pathway also participates in pain modulation, but only with cholinergic activation. Pharmacological activation of the cholinergic system through different ways of administration, including subcutaneous, intrathecal, and intraventricular injection, produces consistent analgesic effects.[30,47,65] Although systematic administration might exert its effects through spinal or peripheral mechanisms, direct intracerebroventricular injection of CDP-choline indicates a clear forebrain involvement in antinociception.[30] These findings are also supported by our results that chemogenetic activation of MS cholinergic neurons relieves pain perception and affect.

In sharp contrast to the ACC, persistent pain inhibits hippocampal neurogenesis, synaptic plasticity, and pyramidal neuronal activities, and reduces hippocampal volumes,[48,49,72] through endogenous glucocorticoid and opioid mechanisms.[23] In this study, we focus on the ventral hippocampus, which is more relevant to pain perception and affect than the dorsal pole.[67,72] Indeed, our pilot work shows limited effects of chemogenetic inhibition of dorsal CA1 pyramidal neurons on pain (unpublished data). This is consistent with the fact that these neurons are

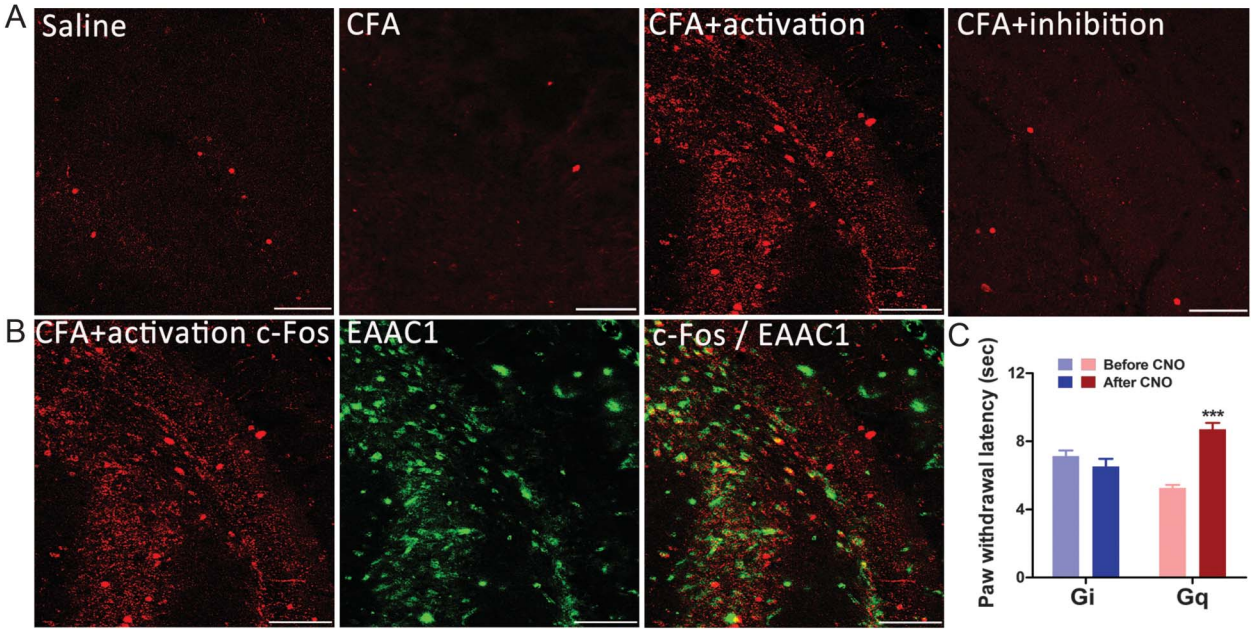

Figure 8. Activating the MS cholinergic system reverses suppressed ventral hippocampal CA1 pyramidal neuronal activities in inflammatory pain in mice. (A) Representative immunofluorescence images showing c-Fos expression in the hippocampus in saline, CFA, CFA + MS–hippocampus activation, and CFA + MS–vCA1 inhibition groups. (B) Activation of cholinergic neurons in the MS–vCA1 pathway resulted in a significant increase in both c-Fos and c-Fos/EAAC1 numbers in the mouse hippocampus in inflammatory pain. 83.7 ± 7.7% of c-Fos (red) in CFA + MS–vCA1 activation group were colocalized with EAAC1 (green). Four slices were taken from each mouse, 4~6 mice in each group. Scale bar: 100 μm. (C) Activating vCA1 pyramidal neurons relieved inflammatory pain 7 days after CFA injection (n = 10, $t = 8.04$, $^{***}P < 0.001$, in Gq group, after CNO vs before CNO, the paired t test), whereas inhibiting these neurons had no effects. CFA, complete Freund adjuvant; CNO, clozapine N-oxide; MS, medial septum.

"place cells" with very low firing rates under rest state (except for occupying their corresponding place fields). By contrast, ventral CA1 neurons receive extensive afferents from the amygdala where nociceptive neurons have been identified.[10,44] Promoting ventral hippocampal activities with environmental enrichment or local overexpression of brain-derived neurotrophic factor attenuates thermal hyperalgesia and mechanical allodynia in chronic inflammatory pain.[72] These manipulations are accompanied by increased hippocampal ACh levels, hippocampal neuronal activities, and plasticity.[9] We need to note that the effect of ACh on hippocampal electrophysiology is complex, depending on timing, ACh levels, corelease with other neurotransmitters, hippocampal subregions, and receptor subtypes activated.[26,28,51] Within the hippocampus, information flows from the dentate gyrus to CA1 through CA3. Because the dentate gyrus and CA3 do not send efferents outside the hippocampus, we focus on the CA1 subregion, where ACh is released from MS cholinergic fibers that terminate diffusely around local pyramidal neurons.[49] Postsynaptically, ACh increases the intrinsic excitability and enhances spiking responses of CA1 pyramidal neurons to excitatory afferent inputs.[17,24] In this study, chemogenetic activation of the MS–vCA1 pathway enhanced c-Fos expression in hippocampal pyramidal neurons (**Fig. 8**), in a similar manner to pain-relieving environmental enrichment.[72]

4.3. Medial septal cholinergic system in neuromodulation

Although the MS cholinergic system has long been known to be crucial for cognitive processes, more recent data indicate the involvement of this system a variety of other processes including anxiety, sleep, thermal sensation, body temperature control, and anesthesia.[31,36,41,43,46,69] Optogenetic activation of basal forebrain cholinergic neurons during nonrapid eye movement sleep is sufficient to elicit transitions to wakefulness and arousal,[31,56] whereas rats with MS cholinergic lesions show increased sensitivity to general anesthetics.[43] These functions, including pain, have a common interoceptive component. In particular, carefully controlled studies have shown that lesioning MS cholinergic neurons specifically impairs path integration, a navigation strategy based on internal senses, without affecting external landmark-based spatial orientation.[46]

The MS cholinergic system has been a focus of investigation for its contribution to hippocampal theta oscillations.[63] However, MS efferents to the neocortex lack sufficient research. Indeed, most behavioral processes involve interactions between both cortical and

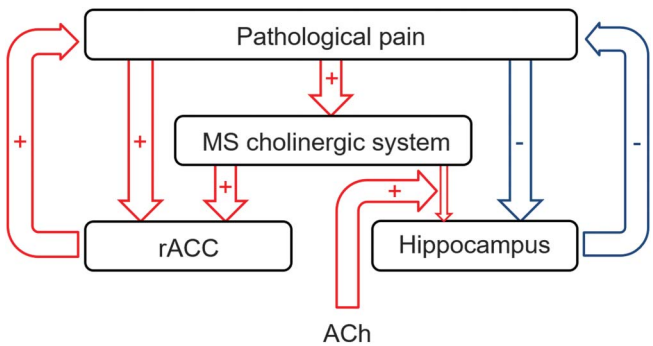

Figure 9. Proposed model of MS cholinergic modulation of pathological pain. Pain activates the MS and rACC, which in turn contribute to the maintenance of persistent pain. Inhibiting MS cholinergic neurons, MS-rACC pathway, or rACC pyramidal neurons alleviates persistent pain. Hippocampal CA1 pyramidal neuronal activities are suppressed in pain, despite excitatory projection from the MS. Activation of vCA1 pyramidal neurons with ACh receptor agonists or chemogenetics relieves pathological pain. MS, medial septum; rACC, rostral anterior cingulate cortex.

subcortical regions. A recent study[34] reveals a role of the hippocampal cholinergic system in balancing competition between memory systems that modulate learning strategy preference. Similarly, infusing the ACh ligands directly into the hippocampus or MS causes spatial memory deficits[13,22] in a similar manner to hippocampal ACh depletion.[13,22] Findings from this study also indicate the MS cholinergic system as a key modulatory factor for the maintenance of pathological pain. The balancing effects are achieved through different neural pathways, which have not been thoroughly understood in previous work.

Overall, our work confirms the central cholinergic system as a therapeutic target of pathological pain, and demonstrates that the MS cholinergic system differentially modulates chronic inflammatory pain through MS–rACC or MS–vCA1 pathways. More significantly, it represents a novel paradigm of neuromodulation: similar behavioral outcomes are achieved on opposite manipulation of the same brain region but through distinct neural pathways (**Fig. 9**).

Conflict of interest statement

The authors have no conflict of interest to declare.

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