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Role of mu-opioid receptor in nociceptive modulation in anterior cingulate cortex of rats

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Abstract

Lots of studies have demonstrated that anterior cingulate cortex plays important roles in the pain perception and pain modulation. The present study explored the role of mu-opioid receptor in nociceptive modulation in anterior cingulate cortex of rats with neuropathic pain. Neuropathic pain model was set up by chronic constriction injury of the left sciatic nerve of rats. The hindpaw withdrawal latency to thermal and mechanical stimulation, by hot plate and Randall Selitto Test respectively, was used to evaluate the rat's responses to noxious stimulation. Results showed that intra-anterior cingulate cortex injection of morphine could induce the antinociception dose-dependently. By intra-anterior cingulate cortex injection of opioid receptor antagonist, the morphine-induced antinociception could be attenuated by naloxone, as well as much significantly by the selective mu-opioid receptor antagonist β-funaltrexamine, indicating that mu-opioid receptor is involved in the morphine-induced antinociception in anterior cingulate cortex of rats with neuropathic pain. The morphine-induced antinociception was much more decreased in rats with neuropathic pain than that in normal rats, and there was a significant decrease in mu-opioid receptor messenger RNA levels in anterior cingulate cortex of rats with neuropathic pain, indicating that there may be a down-regulation in mu-opioid receptor expression in anterior cingulate cortex of rats with neuropathic pain. To further confirm the role of mu-opioid receptor in morphine-induced antinociception in anterior cingulate cortex, normal rats were received intra-anterior cingulate cortex administration of small interfering RNA targeting mu-opioid receptor and it was found that there was a down-regulation in mu-opioid receptor messenger RNA levels, as well as a down-regulation in mu-opioid receptor expression in anterior cingulate cortex tested by real-time polymerase chain reaction and western blotting. Furthermore, the morphine-induced antinociceptive effect decreased significantly in rats with small interfering RNA targeting mu-opioid receptor, which indicated that knockdown mu-opioid receptor in anterior cingulate cortex could also attenuate morphine-induced antinociceptive effect. These results strongly suggest that mu-opioid receptor plays a significant role in nociceptive modulation in anterior cingulate cortex of rats.

Keywords

Anterior cingulate cortex, antinociception, hindpaw withdrawal latency, mu-opioid receptor, morphine analgesia, neuropathic pain, sciatic nerve ligation

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Introduction

The anterior cingulate cortex (ACC) is usually recognized as a critical brain region processing the cognitive and emotional functions.[1–4] Several studies have demonstrated that ACC plays an important role in the pain perception and pain modulation. Moreover, ACC is an important area for the interpretation and evaluation of the affective and emotional components of pain.[5–13] It has been reported that ACC neurons responded to peripheral noxious stimulus in the rodents tested by

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electrophysiological methods,[14–16] and these ACC neurons were activated by acute noxious stimuli and psychological and social pain.[17,18] Neuropathic pain is one of common clinical pain.[19,20] In the brain, the role of ACC in neuropathic pain has received increasing attention.[11–13,21] Numerous studies suggest that neural plasticity in the ACC, an important brain area for neuropathic pain, is involved in the development of pain processing.[7,10–13,21–26] Previous study in our laboratory indicated that galanin-induced antinociception in ACC of rats with neuropathic pain, and both galanin and galanin receptor 2 play some role in nociceptive modulation. Furthermore, the modulative mechanism of ACC in neuropathic pain has been studied. These findings suggest that the ACC may be a key brain area involved in neuropathic pain.

Opioid peptides and opioid receptors are highly expressed in the ACC, and the endogenous opioid and opioid receptors in the ACC contribute to pain modulation.[27] There are three types of opioid receptors, mu, kappa, and delta, in the central nervous system.[28] It has been demonstrated that mu-opioid receptors (MORs) were widely distributed throughout the cingulate cortex, with increased expression of the superficial layer.[29] Opiates, especially the agents acting on MOR, serve as the most effective analgesics for the clinical patients with severe pain.[30]

Studies have reported that opioid peptides and their receptors take part in pain modulation in ACC.[27,31]Navratilova et al. demonstrate that the anti-aversive effects of morphine (e.g., reward from pain relief) are mediated by opioid circuits in the rostral ACC by using blockade of opioid receptors with intrarACC β-funaltrexamine (β-FNA).[27] However, these pharmacological results could not confirm MOR plays an important role in ACC of rats with neuropathic pain. If possible, it should knockout or knockdown MOR in ACC to confirm the pharmacological results. Thus, in the present study, firstly, two methods were used to measure the nociceptive responses in pharmacological experiments, then the changes of MOR expression in ACC of rats with neuropathic pain were observed. Furthermore, the influences of knockdown MOR in ACC by small interfering (siRNA) targeting MOR were tested by real-time Polymerase Chain Reaction (RT-PCR) and western blotting. At last summarizing the results will demonstrate the role of MOR in nociceptive modulation in ACC of normal rats and rats with neuropathic pain.

Materials and methods

Animal preparation

Sprague–Dawley (SD) rats, male, weighing 220 to 260 g, six–seven weeks, were provided by experimental Animal Center of Luye Pharmaceutical Company, Yantai, China, with certificate number 20170018. The rats were housed in the normal day/night cycle and allowed free access to food and water, and the room temperature was kept at $20 \pm 2^{\circ}C$. Before the experiment, all rats were accustomed to the test condition for three days.

All experiments relative to animals were performed according to the Guidelines of the International Association for the Study of Pain[32] and the Guidelines for the Care and Use of Laboratory Animals of Yantai University and were approved by Laboratory Animal Ethics Committee of Yantai University, with the authorization number YT-YX-1817.

To produce a neuropathic pain model

In adult male SD rats, neuropathic pain aseptic surgical procedures were established according to Bennett and Xie's model and modified as previous reports.[12,33,34] Rats were anaesthetized by injecting intraperitoneally with pentobarbital sodiums, at dosage of 50 mg/kg, and 8–10 mm of the left side of the skin was exposed. The sciatic nerve was carefully isolated with sterile glass prongs and snuggly ligated with segment of sterile 4–0. Four loose ligatures were placed around the dissected nerve at 1.0–1.5 mm intervals.

Surgical procedures and intra-ACC injection

The surgical procedure was as follows. Firstly, rats were anaesthetized by injecting intraperitoneally with pentobarbital sodiums, at dosage of 50 mg/kg, and then mounted on stereotaxic frame. For injection, the ACC, 1.6 mm anterior to Bregma; 0.7 mm lateral to midline; 2.0 mm ventral to the surface of skull, was recognized according to Paxinos and Watson[35] and a stainless steel guide cannula with 0.8 mm outer diameter was directed to the ACC and fixed to the skull by dental acrylic. Thereafter, the rats were maintained and recovered at least for three days. On the day of the following experiment, a stainless steel needle with 0.4 mm outer diameter was directly inserted into the guide cannula with 1.5 mm lying outside the tip of the cannula. There was a cut-off limit of 15 s and the operating time for every measuring the hindpaw withdrawal latency (HWL) was no more than 2 min.

Behavioral nociceptive tests

The HWLs were measured as described previously.[12,34,36] To mechanical stimulation, HWL was observed by the Randall Selitto Test (Ugo Basile, Type 7200, Italy) with a wedge-shaped pusher at a loading rate of 30 g/s being applied to the dorsal surface of the hindpaw. To noxious thermal stimulation, the entire ventral surface of the rat hindpaw was placed manually on a hot

plate (YLS-6B Intelligent Heat Panel Instrument, Jinan Yiyan Science & Technology Development Co., Ltd., Jinan, China) at the temperature of 52 ± 0.2°C. The time of hindpaw withdrawal was measured and expressed in seconds, and referred to as the HWL to mechanical stimulation or thermal stimulation. Each rat was operated with both stimulations.

The HWLs, before intra-ACC injection, were tested three times as the basal HWLs. The HWLs after drug injection were expressed as percentage changes of the basal HWL for each rat (% changes to the HWL).

Knockdown MOR by siRNA targeting MOR

Construction of the vector encoding siRNA and virus preparation for MOR-specific siRNA was designed and synthesized by Shanghai Genechem Co., Ltd. (Shanghai, China). The rat siRNA targeting MOR consisted of a 23-bp duplex oligonucleotide:[37] (sense strand: 5′-GUCAUGUAUGUGAUUGUAAGAUA-3′). For MOR meaningless sequence, a mismatch siRNA (sense strand: 5′-TTCTCCGAACGTGTCACGT-3′) was designed, containing the same nucleotide composition but was randomized and screened using a Basic Local Alignment Search Tool algorithm to exclude sequences with significant homology with any known complementary DNA (cDNA). The green fluorescent protein-reporting lentivirus encoding the siRNA targeting MOR and MOR meaningless sequence were custom-constructed into the GV493 plasmid by Shanghai GeneChem. Vector titer (viral particles per milliliter) was determined by dot-blot hybridization analysis. The titers of siRNA targeting MOR and MOR meaningless sequence were both 1×10^8 viral genomes per milliliter.

Real-time PCR for testing mRNA

The rats received overdose of 10% trichloroacetaldehyde monohydrate. The brains were removed and frozen on dry ice. Then tissues of ACC were dissected on ice and then stored at −80°C. RT-PCR was carried out as described in our published paper.[12,34] Tissues were lysed in TRIzol (CoWin Biosciences, Beijing, China), and total RNA was prepared. After reverse transcription using a first-strand cDNA synthesis kit (CoWin Biosciences, Beijing, China), the cDNA was subjected to RT-PCR assays using the 7500 Fast RT-PCR System (Applied Biosystems™, Thermo Fisher, USA). Forward and reverse primer sequences of rats MOR in present experiments were as follows: sense 5′-TCTAATGTATTGTCTGGTTTGCCGTATTG-3′ and antisense 5′-AACTCTTCATGT AAGGTGACTAGGTGCTTC-3′; rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH): sense 5′-GA CCACCCAGCCCAGCAAGG-3′ and antisense 5′-TC CCCAGGCCCCTCCTGTTG-3′. The unigene expression levels were calculated with the $2^{-\Delta\Delta CT}$ method. [38]RT-PCR data are presented as the normalized ratio of the target gene relative to the GAPDH control gene using ΔCT.

Western blotting to test the MOR expression

To investigate the expression levels of MOR, rats were randomly separated into three groups, control group (n = 3), rats received intra-ACC injection of siRNA targeting MOR (n = 3), and rats received intra-ACC injection of MOR meaningless sequence (n = 3). All experimental animals were administrated over dose of 10% trichloroacetaldehyde monohydrate. The brain tissue of ACC was removed and dissected on ice and then frozen at −80°C immediately. The brain tissue samples were homogenized in Radio Immunoprecipitation Assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing phenylmethanesulfonyl fluoride (Beyotime Institute of Biotechnology, Shanghai, China) and centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was collected and measured by bicinchoninic acid (BCA) Protein Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China). Total protein extracts (80 µg) of ACC samples were subjected to 10% Sodium Dodecyl Sulfate-polyacrylamide gel electrophoresis.[39,40] Then the proteins were transferred to polyvinylidene fluoride membranes (Millipore, MA, USA) for 1 h at 106 V. The membranes were incubated in blocking solution (5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20, TBST) for 2 h (room temperature), and sequentially in TBST (Tris-HCl, NaCl, Tween20) containing primary antibodies to the polyclonal rabbit anti-MOR antibody (1:1000; ab10275, Abcam, Cambridge, UK), or beta-actin antibody (1:1000; AA1128, Beyotime Institute of Biotechnology, Shanghai, China) overnight (4°C). The membranes were washed three times with TBST for 10 min each and then probed with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:1000; ab6721, Abcam, Cambridge, UK), HRP-conjugated goat anti-mouse secondary antibody (1:1500; A0216, Beyotime Institute of Biotechnology, Shanghai, China) for 1 h (room temperature). The membranes were washed three times with TBST for 10 min each again. The brands were visualized by enhanced chemiluminescence detection reagents (Beyotime Institute of Biotechnology, Shanghai, China) and imaged using ImageQuant LAS 4000 (GE Healthcare Bio-Sciences AB, Tokyo, Japan) automatically and quantified using Gel-Pro analyzer software.

Chemicals for intra-ACC injection

Solutions for intra-ACC administration were prepared with sterilized saline, each with a volume of 1 μl containing (1) 1, 5, or 10 μg of morphine (morphine hydrochloride; Shenyang First Pharmaceutical Factory, China); (2) 20 μg of naloxone (naloxone benzoylhydrazone; Tocris, UK); (3) 10 μg of β-FNA (β-Funaltrexamine hydrochloride; Tocris, UK); (4) siRNA targeting MOR (Shanghai Genechem Co., Ltd, Shanghai, China); (5) MOR meaningless sequence (Shanghai Genechem Co., Ltd, Shanghai, China) for control.

Statistical analysis

All the tips of the injection needle are in the ACC. Data from the experiment were expressed as mean ± S.E.M. Statistical difference between groups was determined by two-way analysis of variance (ANOVA) for repeated measurements, one-way ANOVA followed Bonferroni test or Student's t test (two-tailed). The area under the curve (AUC; 0–60 min) of antinociceptive effects was calculated. $P < 0.05$ was considered as significant difference.

Results

Intra-ACC administration of morphine-induced antinociceptive effects in rats with neuropathic pain

To set up a neuropathic pain model, eight rats underwent left sciatic nerve ligation. The HWL to noxious thermal and mechanical stimulation were measured in rats with left sciatic nerve ligation seven days after the surgery ($n = 8$) and in normal rats ($n = 8$). The results are shown in Figure 1.

As shown in Figure 1, there were significant decreases in left HWLs to thermal and mechanical stimulation (hot plate test: $P < 0.001$; Randall Selitto Test: $P < 0.001$, Figure 1(a) and (c)) in rats with neuropathic pain (hot plate test: 3.54 ± 0.09; Randall Selitto Test: 3.35 ± 0.06) than that in normal rats (hot plate test: 5.72 ± 0.09; Randall Selitto Test: 4.47 ± 0.07).

Intra-ACC injected 1 μg ($n = 8$), 5 μg ($n = 8$), or 10 μg ($n = 6$) of morphine, or 1 μl of 0.9% saline as a control ($n = 8$) in rats with neuropathic pain. The results are shown in Figure 2.

The HWLs to noxious thermal and mechanical stimulations increased significantly in rats with neuropathic pain after intra-ACC injection of 1 μg of morphine (hot plate test: left, $P < 0.001$; right, $P < 0.001$, Figure 2(a) and (b); Randall Selitto Test: left, $P < 0.001$; right, $P < 0.001$, Figure 2(c) and (d)), 5 μg of morphine (hot plate test: left, $P < 0.001$; right, $P < 0.001$, Figure 2(a) and (b); Randall Selitto Test: left, $P < 0.001$; right, $P < 0.001$, Figure 2(c) and (d)), or 10 μg of morphine (hot plate test: left, $P < 0.001$; right, $P < 0.001$, Figure 2 (a) and (b); Randall Selitto Test: left, $P < 0.001$; right, $P < 0.001$, Figure 2(c) and (d)) compared with the saline group in rat with neuropathic pain. The results demonstrate that intra-ACC injection of morphine-induced significant antinociceptive effects in rats with neuropathic pain.

Opioid receptor antagonists decreased the morphine-induced antinociception in ACC of rats with neuropathic pain

In order to know whether opioid receptor mediates morphine-induced antinociception in ACC of rats with

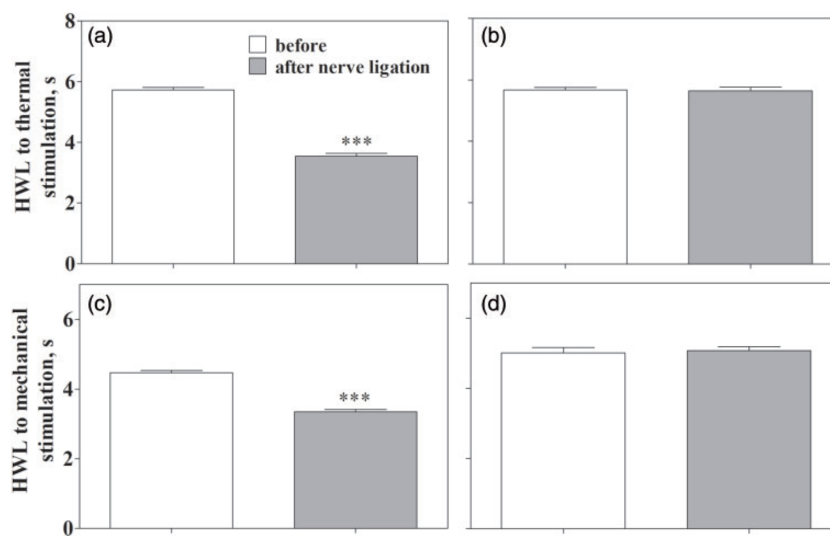

Figure 1. Basal HWLs before and after nerve ligation. (a and b) HWLs to thermal stimulation. (c and d) HWLs to mechanical stimulation. Student's t-test (two tails), ***$P < 0.001$. HWL: hindpaw withdrawal latency.

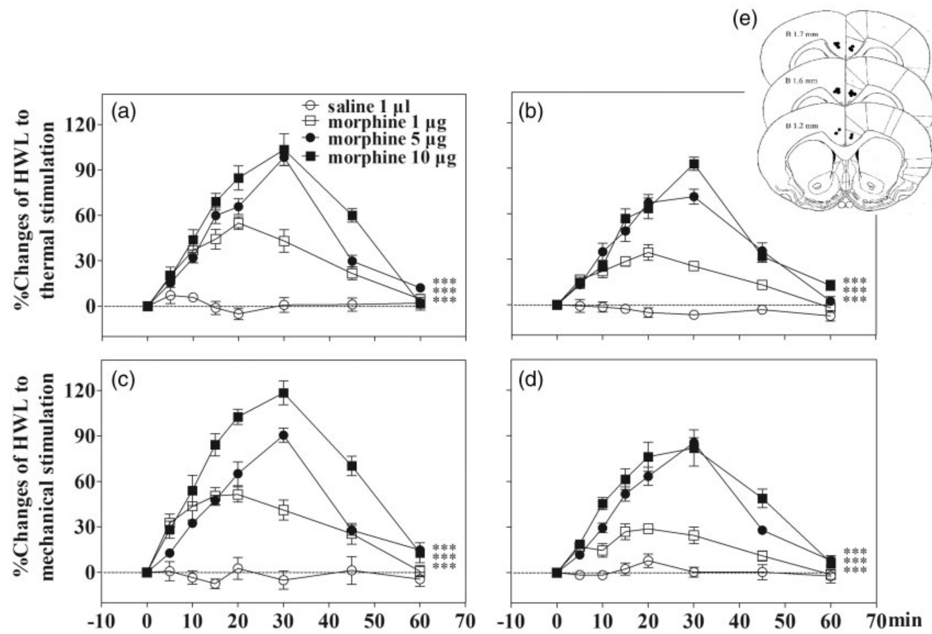

Figure 2. Antinociceptive effects induced by intra-ACC administration of morphine in rats with neuropathic pain. (a and b) The HWLs to thermal stimulation increased significantly after intra-ACC injection of morphine. (c and d) The HWLs to mechanical stimulation increased significantly after intra-ACC injection of morphine. (e) The cannula implantation sites. Data are presented as mean ± S.E.M. Two-way ANOVA, ***$P < 0.001$. ACC: anterior cingulate cortex; HWL: hindpaw withdrawal latency.

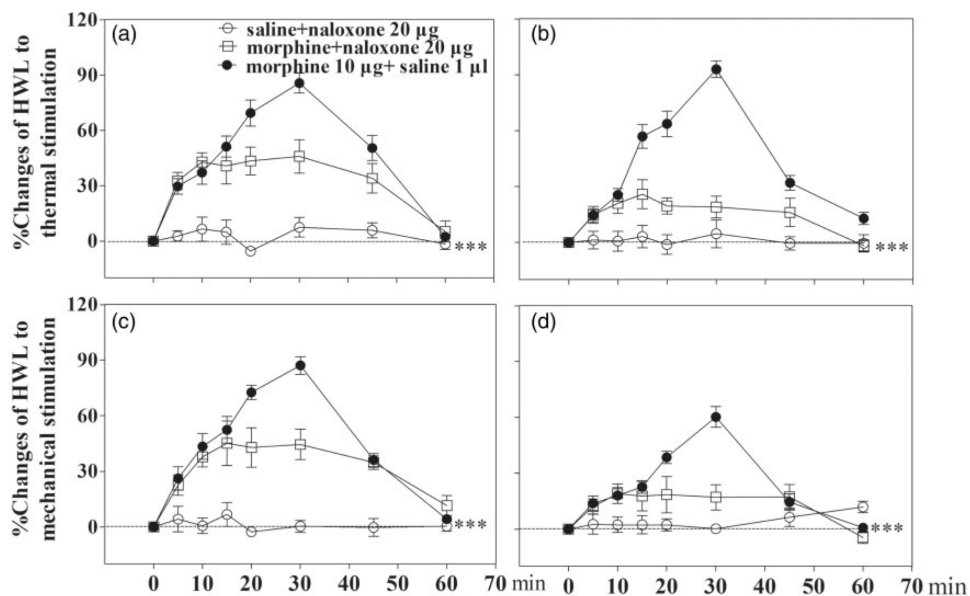

Figure 3. Opioid receptor mediated the morphine-induced antinociception in ACC of rats with neuropathic pain.The morphine-induced increases in HWLs were attenuated by intra-ACC injection of opioid-receptor antagonist naloxone tested by hot-plate test (a and b) and Randal Selitto Test (c and d). Data are presented as mean ± S.E.M. Two-way ANOVA, ***$P < 0.001$. ACC: anterior cingulate cortex; HWL: hindpaw withdrawal latency.

neuropathic pain, two groups of rats with neuropathic pain received intra-ACC injection of 10 μg of morphine, followed 5 min later by intra-ACC injection of 20 μg ($n = 8$) of naloxone, or 1 μl of 0.9% saline (control, $n = 6$). The results are shown in Figure 3(a) to (d). After intra-ACC injection of 10 μg of morphine, the HWL to noxious thermal and mechanical stimulation increased markedly in rats with neuropathic pain.

The morphine-induced increases in HWLs were significantly attenuated after intra-ACC injection of 20 μg of naloxone (Left hot plate test: $P < 0.001$, 21% decreased, Figure 3(a); Right hot plate test: $P < 0.001$, 59% decreased, Figure 3(b); Left Randall Selitto Test: $P < 0.001$, 17% decreased, Figure 3(c); Right Randall Selitto Test: $P < 0.001$, 35% decreased, Figure 3(d)) compared with the control group, and those percentages were calculated by the formula $(AUC_{morphine+saline} - AUC_{morphine+naloxone})/AUC_{morphine+saline}$.

Another group of rats with neuropathic pain (n = 6) received intra-ACC injection of 1 μl of 0.9% saline, followed 5 min later by intra-ACC injection of naloxone, there was no marked influences on the HWLs to thermal and mechanical stimulation after intra-ACC administration of naloxone in rats with neuropathic pain, as shown in Figure 3. The results indicate that opioid receptors may be involved in morphine-induced antinociceptive effects in rats with neuropathic pain.

To determine the involvement of MOR in morphine-induced antinociception in ACC of rats with neuropathic pain, two groups of rats with neuropathic pain received intra-ACC injection of 10 μg of morphine, 5 min later, followed by intra-ACC injection of 10 μg (n = 6) of the selective MOR antagonist β-FNA, or 1 μl of 0.9% saline (n = 6). The results are shown in Figure 4 (a) to (d).

After intra-ACC injection of 10 μg of morphine, the HWLs to thermal and mechanical stimulation increased markedly in rats with neuropathic pain. The morphine-induced increases in HWLs were significantly attenuated after intra-ACC injection of 10 μg of β-FNA (Left hot plate test: $P < 0.001$, 37% decreased, Figure 4(a); Right hot plate test: $P < 0.001$, 80% decreased, Figure 4(b); Left Randall Selitto Test: $P < 0.001$, 44% decreased, Figure 4(c); Right Randall Selitto Test: $P < 0.001$, 61% decreased, Figure 4(d)) compared with the control group, and those percentages were calculated by the formula $(AUC_{morphine+saline} - AUC_{morphine+\beta\text{-}FNA})/AUC_{morphine+saline}$.

Another group of rats with neuropathic pain (n = 6) received intra-ACC injection of 1 μl of 0.9% saline, followed 5 min later by intra-ACC injection of 10 μg of β-FNA, there were no marked influences on the HWLs to thermal and mechanical stimulation.

These results indicate that MOR is involved in the morphine-induced nociceptive modulation in the ACC in rats with neuropathic pain.

Influences of neuropathic pain on both morphine-induced antinociceptive effects and MOR mRNA levels

Figure 5(a) and (b) showed that the morphine-induced increases in left HWL to noxious thermal and mechanical stimulation in normal rats and rats with neuropathic pain. There are significant decreases in HWL to noxious thermal and mechanical stimulation in rats with neuropathic pain compared with normal rats (hot plate test: $P < 0.001$, Figure 5(a); Randall Selitto Test: $P < 0.001$, Figure 5(b)).

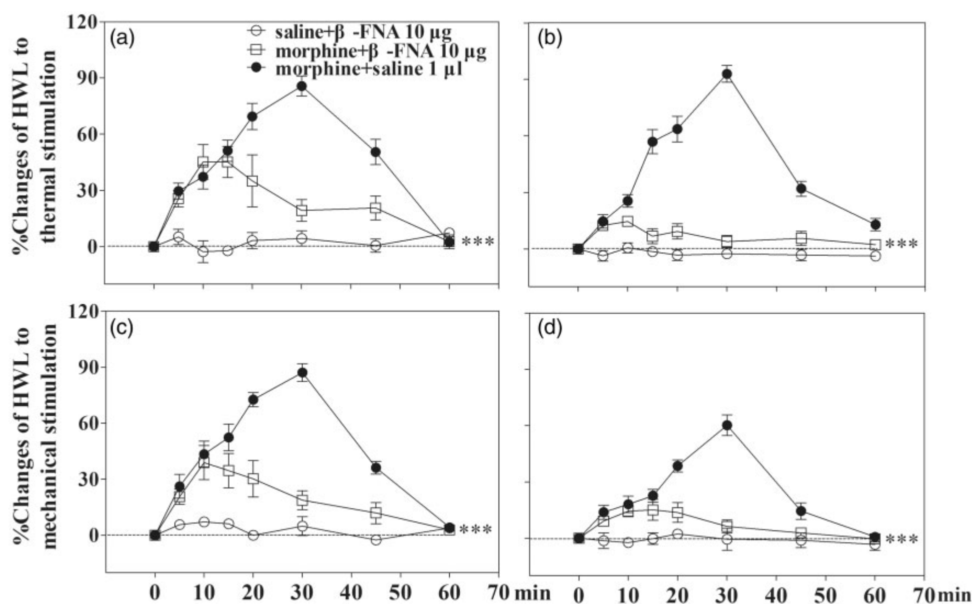

Figure 4. MOR mediated the morphine-induced antinociception in ACC of rats with neuropathic pain. The morphine-induced increases in HWLs were attenuated by intra-ACC injection of MOR antagonist β-FNA tested by hot-plate test (a and b) and Randal Selitto Test (c and d). Data are presented as mean ± S.E.M. Two-way ANOVA, $^{***}P < 0.001$. ACC: anterior cingulate cortex; HWL: hindpaw withdrawal latency; MOR: mu-opioid receptor.

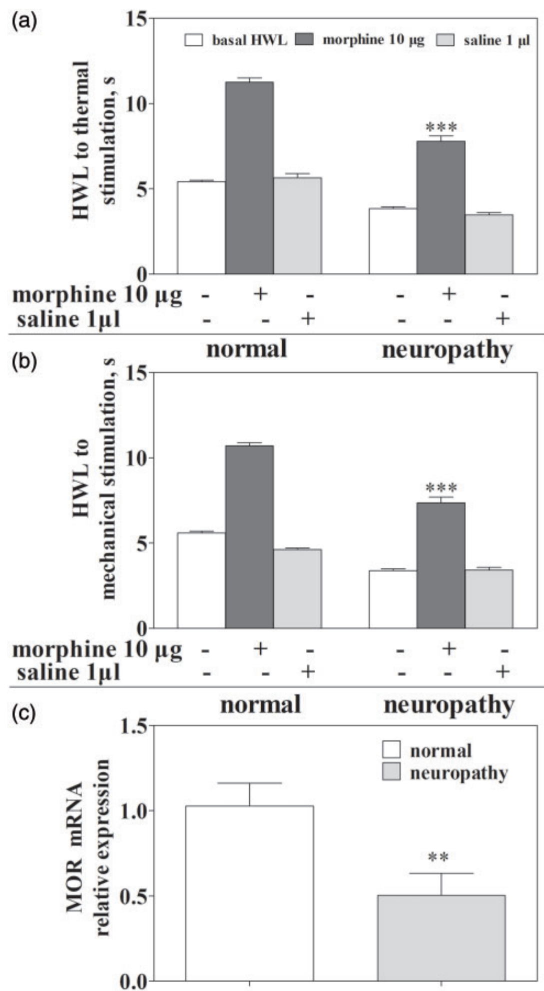

Figure 5. The morphine-induced antinociception and MOR mRNA levels in normal rats and rats with neuropathic pain. (a and b) A comparison of the morphine-induced antinociception in normal rats and rats with neuropathic pain. The MOR mRNA levels in ACC of normal rats and in rats with neuropathic pain tested by RT-PCR (c). Student's t-test (two tails), **P < 0.01, ***P < 0.001. ACC: anterior cingulate cortex; HWL: hindpaw withdrawal latency; MOR: mu-opioid receptor.

As we found that the morphine-induced antinociception was decreased in rats with neuropathic pain than that in normal rats, we further check the influence of neuropathic pain on MOR expression in ACC. The mRNA levels of MOR in ACC of normal rats (n = 3) and rats with neuropathic pain (n = 3) were tested by RT-PCR.

The results are shown in Figure 5(c). There is a significant lower mRNA level of MOR (P < 0.01, Figure 5(c)) in ACC of rats with neuropathic pain (0.50 ± 0.13) than that in normal rats (1.03 ± 0.13). The results demonstrate that there may be a down-regulation in MOR expression in ACC of rats with neuropathic pain.

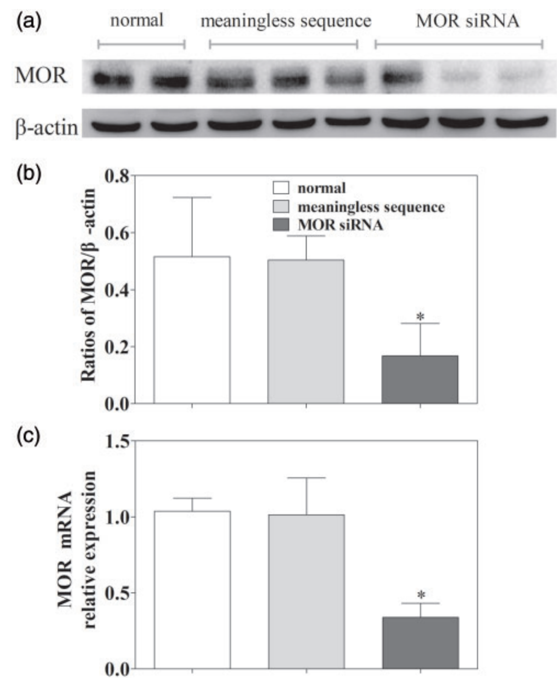

Figure 6. Influence of intra-ACC administration of the siRNA targeting MOR on MOR expression in ACC. (a and b) Results from western blotting test. (c) Results from RT-PCR test. Student's t-test (two tails), *P < 0.05. MOR: mu-opioid receptor.

Influence of knockdown MOR in ACC on both MOR expression and morphine-induced antinociceptive effects

To further confirm the role of MOR in morphine-induced antinociception in ACC, the MOR was knockdown by siRNA targeting MOR. Three groups of normal rats received intra-ACC injection of siRNA targeting MOR (n = 6), intra-ACC injection of MOR meaningless sequence (n = 6), or saline (n = 6), respectively. After three days, all experimental rats received injection of over dose of 10% trichloroacetaldehyde monohydrate and the brain was removed immediately. The MOR expression and the mRNA levels of MOR in ACC were measured by western blotting and RT-PCR, respectively. The results are shown in Figure 6.

Figure 6(a) and (b) showed the MOR expression in ACC of normal rats (0.52 ± 0.21) and in rats received intra-ACC injection of the siRNA targeting MOR (0.17 ± 0.11), or MOR meaningless sequence (0.50 ± 0.08). There is a significant decrease in the expression of MOR in ACC of rats with injection of the siRNA targeting MOR than that in rats with injection of MOR meaningless sequence (P < 0.05, Figure 6(b)) tested by western blotting, and there is no significant change in the expression of MOR in ACC of rats with intra-ACC injection of MOR meaningless sequence compared to normal rats (P = 0.95, Figure 6(b);

two-tailed Student's t test). The results demonstrate that the expression of MOR decreased in ACC of rats with injection of the siRNA targeting MOR.

The MOR mRNA levels in ACC of normal rats (1.04 ± 0.09) and in rats with intra-ACC injection of the siRNA targeting MOR (0.34 ± 0.09) or MOR meaningless sequence (1.01 ± 0.24) were tested by RT-PCR and are shown in Figure 6(c). As shown in Figure 6(c), there is a significant decrease in the mRNA level of MOR ($P < 0.05$, two-tailed Student's t test, Figure 6(c)) in ACC of rats with intra-ACC injection of the siRNA targeting MOR than that in rats with intra-ACC injection of the MOR meaningless sequence, while there are no marked change in the mRNA level of MOR ($P = 0.93$, two-tailed Student's t test, Figure 6(c)) in ACC of rats with intra-ACC injection of MOR meaningless sequence compared to normal rats. The results indicate that after intra-ACC administration of siRNA targeting MOR, the mRNA level of MOR decreased significantly by interfering mRNA.

Further, the experiment was performed to check the influence of knockdown MOR on the morphine-induced antinociception. Five groups of rats: (1) intra-ACC administration of 10 µg of morphine in intact rats ($n = 7$); (2) intra-ACC administration of 1 µl of saline in intact rats ($n = 7$); (3) intra-ACC administration of 10 µg of morphine in rats with three days after intra-ACC injection of siRNA targeting MOR ($n = 8$); (4) intra-ACC administration of 10 µg of morphine in rats with six days after intra-ACC injection of siRNA targeting MOR ($n = 6$); (5) intra-ACC administration of 10 µg of morphine in rats with nine days after intra-ACC injection of siRNA targeting MOR ($n = 8$). The results are shown in Figure 7.

The HWL to thermal and mechanical stimulation increased significantly after morphine injection (hot plate test: 81.01 ± 6.22; Randall Selitto Test: 74.88 ± 7), the morphine-induced increases in HWLs to noxious thermal and mechanical stimulation are significant lower in rats with three days after intra-ACC injection of siRNA targeting MOR (hot plate test: $P < 0.001$, 20.03 ± 2.36, Figure 7(a); Randall Selitto Test: $P < 0.001$, 29.73 ± 3.55, Figure 7(b); one-way ANOVA followed Bonferroni test) and six days after intra-ACC injection of siRNA targeting MOR (hot plate test: $P < 0.001$, 28.54 ± 5.00, Figure 7(a); Randall Selitto Test: $P < 0.001$, 27.41 ± 2.1, Figure 7(b); one-way ANOVA followed Bonferroni test), but not in rats with nine days after intra-ACC injection of siRNA targeting MOR (hot plate test: $P = 1.0$, 79.23 ± 5.11,

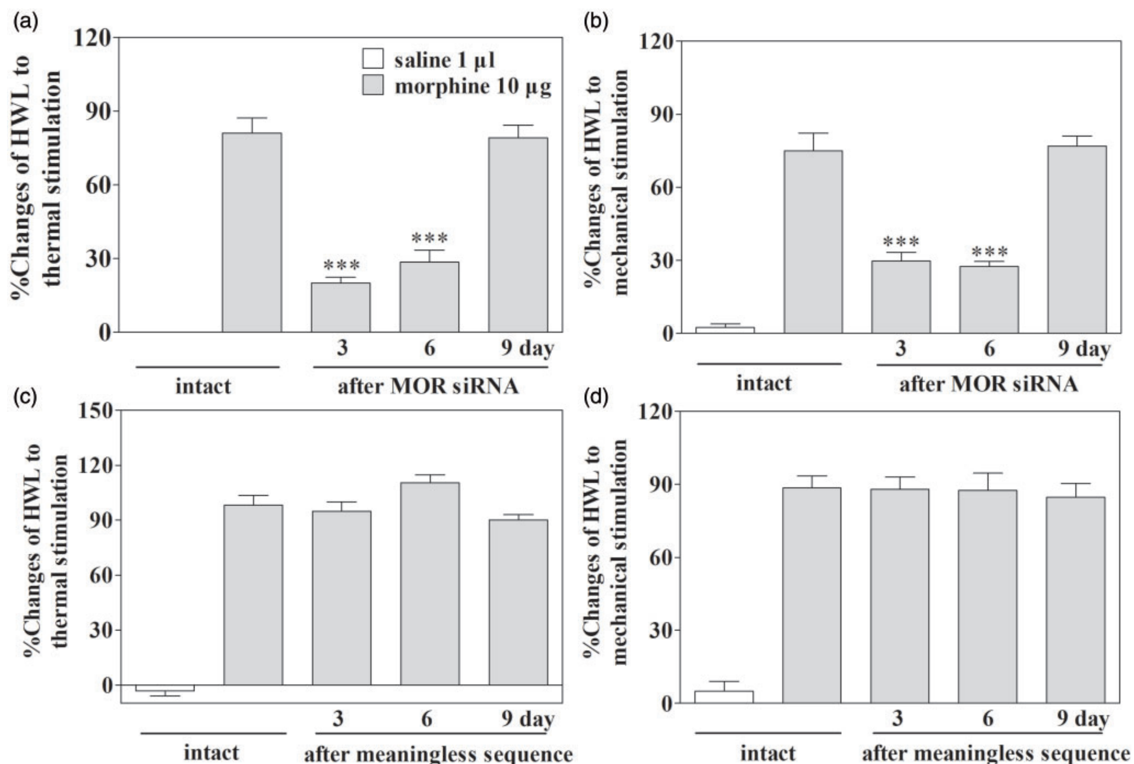

Figure 7. Influence of down-regulation of MOR in ACC on the morphine-induced antinociception in rats. (a and b) Representation of the morphine-induced antinociceptive effects in rats with intra-ACC siRNA targeting MOR. (c and d) Representation of the morphine-induced antinociceptive effects in rats with intra-ACC MOR meaningless sequence. One-way ANOVA followed Bonferroni test. ***$P < 0.001$. MOR: mu-opioid receptor.

Figure 7(a); Randall Selitto Test: P = 1.0, 77.00 ± 4.02, Figure 7(b); one-way ANOVA followed Bonferroni test). The results strongly support our above results from western blotting and RT-PCR.

Another five groups of rats received (1) intra-ACC administration of 10 μg of morphine in intact rats (n = 7); (2) intra-ACC administration of 1 μl of saline in intact rats (n = 7); (3) intra-ACC administration of 10 μg of morphine in rats with three days after intra-ACC injection of MOR meaningless sequence (n = 6); (4) intra-ACC administration of 10 μg of morphine in rats with six days after intra-ACC injection of MOR meaningless sequence (n = 6); (5) intra-ACC administration of 10 μg of morphine in rats with nine days after intra-ACC injection of MOR meaningless sequence 6 (n = 6). The results are shown in Figure 7(c) and (d), the morphine-induced increases in HWLs showed no significant changes in rats with three days after intra-ACC administration of MOR meaningless sequence (hot plate test: P = 1.0, 94.82 ± 5.16, Figure 7(c); Randall Selitto Test: P = 1.0, 87.89 ± 5.09, Figure 7(d); one-way ANOVA followed Bonferroni test), six days after intra-ACC injection of MOR meaningless sequence (hot plate test: P = 0.41, 110.4 ± 4.25, Figure 7(c); Randall Selitto Test: P = 1.0, 87.44 ± 7.09, Figure 7(d); one-way ANOVA followed Bonferroni test), and nine-days after intra-ACC injection of MOR meaningless sequence (hot plate test: P = 1.0, 90.18 ± 2.77, Figure 7 (c); Randall Selitto Test: P = 1.0, 84.44 ± 7.09, Figure 7 (d); one-way ANOVA followed Bonferroni test) compared to intact rats with morphine injection (hot plate test: 98.22 ± 5.26; Randall Selitto Test: 88.45 ± 4.88).

These results strongly suggest that it is MOR to mediate morphine-induced antinociceptive effects in ACC.

Discussion

Recently, there are many evidences show that ACC is of particular importance for the perception and evaluation of the unpleasantness of pain, which is an integral part of the limbic system and is central for processing the emotional aspects of pain.[4,13,41] Based on accumulating evidence from both human beings and animals, ACC is important for pain-related perception and thus is likely a target for pain relief therapy.[5,19] Currently, opiates, especially the agents acting on MOR, serve as the most effective analgesics for the clinical patients with severe pain.[30]

Neuropathic pain is a chronic pain disease caused by primary or secondary damage to the nervous system or dysfunction. Studies in recent years have found that there is a close relationship between the ACC and neuropathic pain.[7,10–12,23–25] Neuropathic pain model was set up by chronic constriction injury of the left sciatic nerve of rats. The left HWL to thermal and mechanical stimulation, by hot plate and Randall Selitto Test respectively, were used to evaluate the antinociception.

The present study showed that the intra-ACC administration of morphine-induced antinociceptive effects in a dose-dependent manner in rats with neuropathic pain. In order to know whether opioid receptor mediates morphine-induced antinociception in ACC of rats with neuropathic pain, the rats with neuropathic pain were administered with opioid receptor antagonist naloxone. The morphine-induced antinociception could be attenuated by followed intra-ACC injection of opioid receptor antagonist naloxone, indicating that opioid receptor involves in the morphine-induced antinociception in ACC of rats with neuropathic pain. There exist three major opioid receptor subtypes, mu, kappa, and delta, in the central nervous system. It is generally recognized that opiates, especially the agents acting on MOR, serve as the most effective analgesics for the clinical patients with severe pain, but which of three major opioid receptors mediate the morphine-induced antinociception in ACC need to be confirmed.

To determine the involvement of MOR in morphine-induced antinociception in ACC of rats with neuropathic pain, the rats with neuropathic pain received the selective MOR antagonist β-FNA. The morphine-induced antinociception was significantly attenuated by followed intra-ACC injection of selective MOR antagonist β-FNA, indicating that MOR playing an important role in the morphine-induced antinociception in ACC of rats with neuropathic pain. The effect of β-FNA, attenuating morphine-induced antinociception, is more significantly than naloxone in rats with neuropathic pain. Navratilova et al. have demonstrated that intra-ACC injection of the MOR antagonist β-FNA inhibited intravenous morphine treatment on mechanical allodynia tested by von Frey filaments.[27] However, our first step was injected morphine into the ACC, and the second step was to observe the effect of β-FNA blocking MOR. As we found that the morphine-induced antinociception was decreased in rats with neuropathic pain than that in normal rats, we further check the influence of neuropathic pain on MOR expression in ACC. Interestingly, there was a significant decrease in MOR mRNA levels in ACC of rats with neuropathic pain. As the mRNA levels of MOR decreased after nerve ligation, we speculated that the expression of MOR was down-regulated. These results indicate that MOR plays a main role in nociceptive modulation in ACC of rats with neuropathic pain.

In order to further confirm the involvement of MOR in nociceptive modulation in ACC, the present study designed a lentiviral vector expressing a siRNA targeting

MOR and administered the siRNA targeting MOR to ACC to down-regulate the MOR expression. The results showed that there was a down-regulation in MOR mRNA levels as well as a down-regulation in MOR expression in ACC tested by RT-PCR and western blotting compared to intra-ACC of MOR meaningless sequence group. Interestingly, the morphine-induced antinociceptive effects decreased significantly in rats with siRNA targeting MOR tested by hot-plate and Randall Selitto Test, an effect lasted for six days. These results indicate that MOR plays an important role in nociceptive modulation in ACC of rats.

In the present study, we demonstrated that morphine-induced antinociception in ACC of rats with neuropathic pain and found that MOR plays a key role in morphine-induced antinociceptive effects in ACC. Recently, Zhang et al. found that galanin-induced antinociception in ACC of normal rats and rats with neuropathic pain.[12] Is there an interaction between galanin system and opioid system in ACC of nociceptive modulation? Previous studies have demonstrated that there may be an interaction between opioids and galanin in the brain. Wang et al. showed that intra-Periaqueductal gray injection of galanin-induced antinociception, and the galanin-induced antinociceptive effects were blocked by naloxone, indicating a possible interaction between galanin and opioid in nociceptive modulation in Periaqueductal gray.[42]

Furthermore, Sun et al. found that intra-ARC (arcuate nucleus) injection of galanin-induced antinociception, and the galanin-induced antinociceptive effects were attenuated by intra-ARC administration of the selective MOR antagonist β-FNA in a dose-dependent manner, indicating that MOR was involved in the galanin-induced antinociception.[43] These results strongly indicate that there may be interactions between galanin and the opioid system in the ARC in pain processing.[43] Our further study will focus the interactions between the opioid system and the galanin system in the ACC in pain modulation.

Authors' contributions

Linlin Wang completed the whole experiments. Kesai Hou performed part of the behavioral test, Hongbo Wang edited the manuscript, Fenghua Fu and Long-Chuan Yu designed the experiments and edited the manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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