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Morphine-induced conditioned place preference in rats is inhibited by electroacupuncture at 2 Hz: Role of enkephalin in the nucleus accumbens

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ABSTRACT

Our previous studies have demonstrated that morphine-induced conditioned place preference (CPP) can be inhibited by 2 Hz electroacupuncture (EA). This inhibition can be blocked by either the opioid receptor antagonist naloxone (i.p.) or lesion in the nucleus accumbens (NAc), providing evidence that endogenous opioid system in the NAc mediates the effects of EA. Here we report that 1) A single session of 2 Hz EA produced a significant increase of the content of enkephalin in the NAc of morphine-induced CPP rats, and this effect was stronger in three consecutive sessions of EA; 2) Intracerebroventricular injection of the μ-opioid receptor antagonist CTAP or δ-opioid receptor antagonist NTI, but not κ-opioid receptor antagonist nor-BNI, dose-dependently reversed the inhibitory effects of 2 Hz EA on the expression of morphine-induced CPP; 3) Three consecutive sessions of 2 Hz EA up-regulated the mRNA level of pre-proenkephalin in the NAc of morphine-induced CPP rats. The results suggest that the inhibitory effects of 2 Hz EA on the expression of the morphine CPP is mediated by μ- and δ-, but not κ-opioid receptor, possibly *via* accelerating both the release and synthesis of enkephalin in the NAc. These findings support the possibility of using 2 Hz EA for the treatment of opiate addiction.

1. Introduction

Opioids, including opiates and endogenous opioids, have attracted an increasing research interest, primarily because the former produce not only an analgesic effect but also a psychologically reinforcing effect which can result in their abuse (Muranyi and Radak, 2008), and the latter has been known as one kind of the central neurotransmitters mainly implicating in the nociception, the pain management, and the reinforcement induced by opiates in mammalian animals. Opioids exert their actions through three types of opioid receptors: μ-, δ- and κ-receptors. Interestingly, different endogenous opioid ligands show distinct affinities for the three types of receptors (Mansour et al., 1995). Met-enkephalin shows a higher affinity to δ-opioid receptor, with *Ki* value (nM) being 0.45 to δ-receptor, 1.80 to μ-receptor and 47.44 to κ-receptor respectively. Dynorphin $A_{(1-13)}$ shows a higher affinity to κ-receptor, with 0.23, 4.14 and 5.56 to κ-, μ- and δ-receptor respectively. In rodent animals, the reinforcing properties of μ-receptor agonists have been demonstrated by their induction of CPP. Both the selective μ-opioid receptor agonist DAMGO and the selective δ-opioid receptor agonist DPDPE can produce a dose-related CPP in rats (Suzuki et al., 1991). By contrast, κ-receptor agonists generally lack the positive reinforcing effects (Mucha and Herz, 1985), moreover the selective κ-receptor agonist, U50, 488H can abolish the reinforcing effects of morphine in the CPP paradigm (Bolanos et al., 1996).

CPP, as a commonly used model, can represent the reinforcing effect of a drug with abused potential. Based on modern and traditional theoretical formulations of Pavlovian conditioning, the CPP paradigm reflects a preference for a context due to the contiguous association between the context and the drug stimulus (Bardo and Bevins, 2000), and considered to be close to the addictive situations of humans in reflecting the craving for a drug induced by a cue associated with the drug (van Ree et al., 1999).

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Drug-seeking behavior has long been linked to craving from the perspective of conditioned positive reinforcement, where previously neutral stimuli linked to the positive reinforcing or rewarding effects of the addictive drugs acquire motivational significance (Shaham et al., 1994).

Acupuncture therapy, including electroacupuncture (EA), has been accepted world widely for various medical purposes (Ulett et al., 1998). It is developed on the basis of traditional Chinese acupuncture and has been shown to be able to exert various effects in modulating central nervous system functions. EA can efficaciously relieve pain perception (Han et al., 1999; Wang et al., 2005; Xing et al., 2007), as well as suppress the withdrawal syndrome and the craving for opioids in heroin addicts (Wu et al., 1999; Wang et al., 2000a). Simultaneously, we reported that the expression of morphine-induced CPP could be inhibited by a single session of 2 Hz EA, and this effect could be blocked by administration of the opioid receptor antagonist naloxone (i.p.) or lesion of the NAc (Wang et al., 2002). Additionally, Chen et al. (2005) found that the effect of three consecutive sessions of 2 Hz EA was better than that of a single session of the EA for inhibiting morphine-induced CPP, in which the EA was shown to increase the level of preproenkephalin (PPE) mRNA (Guo et al., 1996; Shi et al., 2004). In the present study, we would like to ask 3 questions: 1) Would 2 Hz EA accelerate the release of enkephalin in NAc (direct evidence for the immediate effect); 2) Could the suppressive effect of 2 Hz EA on morphine-induced CPP be blocked by δ- or μ-opioid receptor antagonist (indirect evidence); and 3) Would the effects of three consecutive sessions of EA be stronger than that of a single session on releasing enkephalin and up-regulating PPE gene transcription (evidence for a cumulative effect).

2. Materials and methods

2.1. Subjects

Male Sprague–Dawley rats used in this study, weighing 250–300 g at the beginning of the experiment, were obtained from Experimental Animal Center, Peking University. Four animals per cage were housed in a thermo-regulated room (22 ± 1 °C) with a 12/12 h dark–light cycle (lights on at 07:00 a.m.). Food and water were available *ad libitum*. The experimental procedures were approved by the Committee on Animal Care and Use of Peking University.

2.2. Conditioned place preference

2.2.1. Apparatus

Place conditioning was conducted in a three-compartment apparatus with an unbiased design. The apparatus was a black rectangular PVC box ($75 \times 22 \times 30$ cm), divided into two equal-sized outer sections ($30 \times 22 \times 30$ cm) joined by a small central compartment ($10 \times 22 \times 30$ cm) accessed through guillotine doors. The two end chambers were distinguished from each other in two ways. One had a group of 4 lights arranged in a square pattern on the end wall and a stainless-steel mesh floor (1.3×1.3 cm^2), whereas the other had the 4 lights arranged in a triangle form on the wall and a rod floor (1.3 cm apart). The center chamber had gray walls and a smooth floor. Fifteen infrared beams spaced 5 cm apart monitored the motion of the rat. The infrared sensors communicate to a computer (at 10 Hz) through an interface. All experimental events were controlled and recorded automatically by the computer and the interface located in the same room. The computer also provided continuous white noise to mask external sounds.

2.2.2. Procedure

The methods of CPP have been described in detail previously (Liang et al., 2006). Briefly, rats were tested on day 1 before any treatment to establish pre-conditioning responses and any possible box bias. Testing involved placing individual animals in the small central compartments and allowing them to freely explore the entire apparatus for 15 min. Over the next 8 sessions (2 sessions per day) subjects received a double-alternating sequence of differential conditioning. In the morning, rats were injected with saline (2 ml/kg) and immediately placed in the assigned "non-drug" compartment for 45 min. In the afternoon (6 h later), rats were injected with morphine at the dose of 4 mg/kg and placed in the assigned 'drug' compartment for the same time period. The schedule was counterbalanced in the next day. The post-conditioning testing was carried out on day 6. As in the pre-conditioning phase, the guillotine doors were raised and the animals were allowed free access to all compartments for 15 min. The amount of time spent in each compartment was recorded to assess individual preference.

2.3. Electroacupuncture

Rats were acclimated to a treatment box ($50 \times 30 \times 25$ cm) for 1 h prior to EA treatment. Animals were gently restrained by hand. Two stainless-steel needles of 0.3 mm diameter were inserted into each hind leg, one in the acupoint ST36 (5 mm lateral to the anterior tubercle of the tibia), and the other in SP6 (3 mm proximal to the medial malleolus, at the posterior border of the tibia). Constant current square-wave electrical stimulation produced by a programmed pulse generator (HANS LH-800, produced by Peking University of Astronautics and Aeronautics Aviation) was given *via* the two needles for a total of 30 min. The frequency of stimulation used was 2 Hz (0.6 ms pulse width). The intensity of the stimulation was increased stepwise from 0.5 to 1 and 1.5 mA, with each step lasting for 10 min. This was a very low intensity stimulation treatment, with no obvious struggle or painful behavior other than the light dithering of hind legs along with the frequency of electric stimulations.

2.4. Experimental procedures

2.4.1. Experiment 1

218 rats were divided into three sub-experiments, and each sub-experiment included seven groups to receive different treatments (Table 1).

After habituating with the housing environment, the rats in Sub-experiment 1 were implanted unilaterally with stainless-steel cannula guides for push-pull perfusion. The rats were given 2 Hz EA for one trial or three trials after CPP expression, and the dialysis samples were collected every 30 min before, during and after EA, respectively, over a total period of 90 min. The levels of immunoreactive met-enkephalin or dynorphin $A_{(1-13)}$ in the perfusate and the NAc samples in Sub-experiment 2 were detected by radioimmunoassay (RIA). The relative levels of mRNAs encoding the preproenkephalin (PPE) and preprodynorphin (PPD) gene were measured in NAc from Sub-experiment 3 using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) technique (Haberny and Carr, 2005). The methods used in Experiment 1 were described below.

2.4.1.1. Push-pull perfusion (PPP). The superiority of microdialysis was hindered by its low efficacy in collecting larger molecular substances including larger molecular peptides (such as dynorphin) and proteins by the limit of the fiber pore size (Myers et al., 1998). Therefore we chose to use thin-caliber, slow-flow rate push-pull perfusion in the present study.

2.4.1.2. Surgical procedures. The animals were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). The scalp was incised and retracted, and the head position was adjusted to place bregma and lambda in the same horizontal plane. Small burr holes (1 mm in diameter) were drilled on the skull for unilateral placement of stainless-steel push-pull guide cannula (22 gauge, ID 0.58 mm, OD 0.90 mm) into the NAc shell (1.7 mm anterior to bregma, 1.0 mm lateral to midline and 7.2 mm ventral to the surface of the skull). Cannula was chronically fixed with dental acrylic cement onto the skull, serving as a guide for the accurate insertion of the push-pull connector-internal cannula (28-gauge, extending 0.5 mm below the tip of the guide cannula). The lower end of the guide cannula was placed 0.5 mm above the brain region that would be reached by the tip of the push-pull cannula in the process of perfusing. To prevent clogging, a push-pull dummy cannula (OD 0.56 mm) was placed in the guide cannula until the animals received perfusion treatment. All animals recovered for 5 d after surgery. All materials of push-pull cannula systems were purchased from Plastics One Inc. of America.

2.4.1.3. Perfusion procedures. The animals with implanted cannulae were placed in transparent plastic cages in a quiet, isolated room 1 h before the beginning of the session and were freely moving throughout the perfusion. Subsequently, the animals were subjected to a single perfusion session as described in the protocol. The protective mandrel was removed from the guide and the perfusing cannula slowly inserted and screwed down. The push end of the cannula was connected through PE50 tube to the pump. Artificial cerebrospinal fluid (127 mM NaCl, 3.73 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 20 mM $NaHCO_3$, 0.1% BSA, 30 μg/ml bacitracin) was administered through the push-pull cannula at a flow rate of 15 μl/min. Perfusate fractions (450 μl) were collected every 30 min over a total period of 90 min after 30 min of washout. The perfusates were immediately frozen on dry ice and lyophilized.

2.4.1.4. Radioimmunoassay (RIA). Brains were removed and placed into a matrix with 1-mm division, cooled on ice. Slices were rapidly sectioned and collected, and identified structures were dissected based on the stereotaxic rat brain atlas (Paxinos and Watson, 1997). Bilateral punches (2 mm diameter) were taken for NAc (+2.5 to +0.5), from coronal sections between bregma +2.7 to bregma +0.7. Brain tissue samples were homogenized with 0.8 μl 0.1 N HCl. The homogenate was centrifuged at 4000 rpm for 20 min and the suspension was neutralized with 0.8 μl 0.1 N NaOH,

Table 1
Experiment design.

Experiment	Grouping	Treatment
Sub-experiment 1: To test endogenous release of met-enkephalin, dynorphin $A_{(1-13)}$ in the NAc	1) Sal CPP	Collecting 30-min perfusate in NAc 24 h after CPP test.
	2) Mor CPP control	Serving as control of 3).
	3) Mor CPP + EA × 1	24 h after morphine-CPP test, collecting 90-min perfusate sample in NAc, 30-min before, during and after EA × 1, respectively.
	4) Mor CPP control	Serving as control of 5).
	5) Mor CPP + EA × 3	EA treatment 24 h after morphine-CPP test, once daily for 3 d, collecting perfusate sample in NAc for 90 min: 30-min before, during and after the last EA, respectively.
	6) Mor CPP control	Serving as control of 7).
	7) Mor CPP + EA × 3	EA treatment 24 h after morphine-CPP test, once daily for 3 d, collecting 30-min perfusate sample in NAc, 24 h after the last EA.
Sub-experiment 2: To evaluate tissue content of met-enkephalin, dynorphin $A_{(1-13)}$ in the NAc	1) Sal CPP	Isolating NAc tissue 24 h after CPP test.
	2) Mor CPP control	Serving as control of 3).
	3) Mor CPP + EA × 1	24 h after morphine-CPP test, isolating NAc tissue 30 min after EA × 1.
	4) Mor CPP control	Serving as control of 5).
	5) Mor CPP + EA × 3	EA treatment 24 h after morphine-CPP test, once daily for 3 d, isolating NAc tissue 30 min after the last EA.
	6) Mor CPP control	As corresponding control of 7).
	7) Mor CPP + EA × 3	EA treatment 24 h after morphine-CPP test, once daily for 3 d, isolating NAc tissue 24 h after the last EA.
Sub-experiment 3: To assess mRNA level of PPE and PPD in the NAc	1) Sal CPP	Isolating NAc tissue 24 h after CPP test.
	2) Mor CPP control	Serving as control of 3).
	3) Mor CPP + EA × 1	24 h after morphine-CPP test, isolating NAc tissue 30 min after EA × 1.
	4) Mor CPP control	Serving as control of 5).
	5) Mor CPP + EA × 3	EA treatment 24 h after morphine-CPP test, once daily for 3 d, isolating NAc tissue 30 min after the last EA.
	6) Mor CPP control	As corresponding control of 7).
	7) Mor CPP + EA × 3	EA treatment 24 h after morphine-CPP test, once daily for 3 d, isolating NAc tissue 24 h after the last EA.

then stored at −80 °C until assayed. The residue of the lyophilized perfusate was reconstituted with 150 μl RIA buffer at use.

The levels of immunoreactive met-enkephalin or dynorphin $A_{(1-13)}$ in the perfusate and the brain samples were detected by RIA. ^{125}I-met-enkephalin RIA and ^{125}I-dynorphin $A_{(1-13)}$ RIA kits were supplied by the Department of Neurobiology of the Second Military Medical University, Shanghai, China. The dynorphin $A_{(1-13)}$ antiserum (Peninsula) showed no detectable cross-reactivity with β-endorphin or met-enkephalin. Similarly, the met-enkephalin antiserum (Peninsula) showed no detectable cross-reactivity with β-endorphin or dynorphin $A_{(1-13)}$. 100 μl standards (or samples) and 100 μl met-enkephalin antiserum (1:10,000) or dynorphin $A_{(1-13)}$ antiserum (1:10,000) were added into the polyethylene test tubes, and incubated for 24 h at 4 °C after sufficient mixture. ^{125}I-met-enkephalin (20,000 c.p.m./100 μl, Amersham, U.S.A.) or ^{125}I-dynorphin $A_{(1-13)}$ (20,000 c.p.m./100 μl, Amersham, U.S.A.) was then added and incubated for another 24 h at 4 °C. Free labeled peptide was separated using assay buffer (pH 7.4, 0.15 M NaCl, 0.1 M PB, 0.1% Triton X-100, 0.1% BSA, 0.3% lysozyme). The reaction mixtures were centrifuged for 20 min at 4000 rpm, 4 °C. The residues were counted using micro-γ-counter (Beckman, U.S.A.).

2.4.1.5. RT-PCR analysis. NAc tissues were isolated and collected according to the method described in segment 'RIA'. And then, tissue samples were immediately frozen in liquid nitrogen and kept at −80 °C until use.

The relative levels of mRNAs encoding the preproenkephalin (PPE) and preprodynorphin (PPD) gene were measured in brain samples of extracted RNA using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) technique (Haberny and Carr, 2005). Total RNA was isolated from NAc using Trizol reagent (Invitrogen Corporation, Carlsbad, CA). The RNA was extracted from Trizol with chloroform, centrifuged at 12,000 rpm for 15 min, precipitated from the aqueous layer with isopropanol, washed with 75% ethanol and air dried. The pellet was then re-solubilized in Milli Q water. The RNA concentration was determined spectrophotometrically by measuring the absorbance at 260 nm and 280 nm.

Fixed amounts of RNA were used in each reaction, and reproducibility was routinely monitored. Approximately 2 μg total RNA was used for cDNA synthesis by reverse transcription with 200 U M-MLV reverse transcriptase (Invitrogen Corporation) in an RT buffer in the presence of 0.5 mM dNTPs, 30 U RNase inhibitor, and 0.5 μg oligodT as primers. The thermal cycler was programmed for 60 min at 42 °C and 5 min at 95 °C.

A 4-μl aliquot of cDNA synthesized in the RT reaction was used for PCR amplification in the presence of 1 U Taq DNA polymerase (Invitrogen) in Taq buffer, 0.2 mM each of dNTPs and 1 μM of each primer. The PPE was amplified for 29 cycles using a three-step program (45 s at 94 °C, 30 s at 58 °C, and 1 min at 72 °C). PPD were amplified for 31 cycles using a three-step program (45 s at 94 °C, 45 s at 56 °C, and 1 min at 72 °C). The house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified in parallel tubes to control for RNA quantity and extraction efficiency. Primers for amplification of cDNA for PPE, PPD and GAPDH were synthesized by the Sangon Company (China). Primers for amplification of cDNA for PPE: the sense primer, 5′-TAG CCA AGA AGT ATG GAG GG-3′; the antisense primer, 5′-TCT GAT AGT CCA TCC ACC AC-3′. Those of PPD were 5′-GAG GAC TTG AGA AAA CAG GCC-3′ and 5′-GGT ATT GGG GTT CTC CTG GGA-3′, respectively. Primers for amplification of cDNA for GAPDH were 5′-GGG TGG TGC CAA AAG GGT C-3′ and 5′-GGA GTT GCT GTT GAA GTC ACA-3′, respectively. The predicted sizes of amplified products were 532 bp for GAPDH cDNA, 402 bp for PPE cDNA and 250 bp for PPD cDNA. After amplification, the products were separated on a 1.5% agarose gel in the presence of ethidium bromide and visualized under U.V. light.

2.4.2. Experiment 2

The schedule in experimental 2 was illustrated in Fig. 3A. After the post-conditioning test, the rats received 3 consecutive daily administrations of normal saline or opioid antagonists (i.c.v.), respectively 15 min before EA treatment. All rats were tested for their CPP expression 24 h after the termination of the last EA session. The descriptions of surgery and i.c.v. infusion of drugs are as follows.

2.4.2.1. Surgery and infusion perfusions. The animals were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and positioned in a Kopf stereotaxic instrument. The 24-gauge stainless-steel guide cannulae were placed unilaterally in the lateral ventricles according to the atlas of Paxinos and Watson (1997), 0.8 mm posterior to bregma, 1.6 mm lateral to midline and 4 mm ventral to the surface of the skull. Cannulae were secured to the skull by jewelers' screws with dental acrylic. To prevent clogging, the stainless-steel stylets (27-gauge) were placed in the guide cannulae until the animals were given the i.c.v. injection. All animals were allowed 5 d for recovering from surgery.

For drug infusion, the animals were gently restrained by hand; the stylets were removed from the guide cannulae and replaced by 27-gauge injection needles. For i.c.v. injection, the tip was extended for 0.5 mm to reach the ventricle. Each i.c.v. injection unit was connected by polyethylene tubing to a 10-μl Hamilton syringe. The lateral ventricles were infused with 10 μl solution (10 μl/rat) over a 2-min period. The injection needles were left in place for an additional 60 s to allow diffusion, and then the stylets were reinserted into the guide cannulae.

2.5. Drugs

Morphine hydrochloride (the First Pharmaceutical Factory of Shenyang, Shenyang, China) was dissolved in 0.9% saline to the final concentration of 2 mg/ml. Opioid receptor antagonists used in the present experiment included H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP), a selective μ-opioid receptor antagonist, naltrindole hydrochloride (NTI), a selective δ-opioid receptor antagonist, and nor-binaltorphimine dihydrochloride (nor-BNI), a selective κ-opioid receptor antagonist. All drugs were purchased from Sigma Company.

2.6. Histology

At the end of the experiments, the rats were sacrificed by decapitation, after which the location of the injection site was confirmed. Data from rats with inexact injection sites were discarded.

2.7. Data analysis

In the CPP experiments, the preference scores were expressed as a ratio of the time spent in the drug-paired compartment to the total time spent in both outer compartments. The data were processed by commercially available software Graph Pad Prism 4.0. Results were analyzed with one-way ANOVA followed by Newman–Keuls test and two-way ANOVA or repeated measures ANOVA followed by Bonferroni *post hoc*-test. In all tests, the criterion for statistical significance was $P < 0.05$.

3. Results

3.1. EA increased release of met-enkephalin and level of PPE mRNA in the NAc of morphine-induced CPP rats

Results were shown as the percentage change from baseline levels. The baseline levels of met-enkephalin and dynorphin $A_{(1-13)}$ were from the perfusate samples of saline CPP rats. The concentration of met-enkephalin immunoreactivity (ir) in the NAc shell perfusate of the saline CPP rats was 129.4 ± 3.7 pg/ml. Data were analyzed using repeated measures ANOVA, followed by Bonferroni *post hoc*-test.

Fig. 1A shows that 2 Hz EA × 1 significantly increased met-enkephalin-ir in the NAc from the morphine-induced CPP rats during and after EA [$F_{(1,15)} = 5.174$, $P = 0.038$], and the treatment × time correlation was also significant [$F_{(2,30)} = 4.925$, $P = 0.014$], suggesting that EA treatments increased the met-enkephalin release. A significant increase of met-enkephalin-ir could be found 'during' (39.23%) and 'after' (44.17%) EA, compared to the control group at the corresponding time point ($P < 0.05$). In contrast, 2 Hz EA × 3 increased the levels of met-enkephalin in NAc shell, enhancing 101.7% baseline during the period of the third EA stimulation ($P < 0.01$), and an overshoot 180.4% baseline after 2 Hz EA × 3 ($P < 0.01$), compared to the control group at the corresponding time point (Fig. 1B). The treatment × time interaction was highly significant [$F_{(2,32)} = 5.833$, $P = 0.007$]. This augmentation of met-enkephalin release could not be found 24 h after the termination of EA treatments ($P > 0.05$, data not shown).

NAc tissue was isolated according to the schedule of Experiment 2 shown in Table 1 and the concentration of met-enkephalin in the NAc tissue was detected using RIA (Fig. 1C). Using one-way ANOVA followed by Newman–Keuls test, we analyzed the differences of met-enkephalin concentration in NAc among saline CPP (24 h after CPP test) and three time points after morphine CPP (i.e. three control groups of EA treatments, corresponding 24 h, 72 h and 96 h after morphine-CPP test respectively). Due to independent group designed, repeated measure test was not used. And we considered concentration of met-enkephalin should maintain a steady level after saline CPP test, so just one saline CPP group was designed, being a common reference. As results shown in Fig. 1C, there was a significant difference of met-enkephalin content in NAc among the groups [$F_{(3,23)} = 3.83$, $P = 0.0256$], and the concentration of met-enkephalin in the NAc of rats from morphine CPP 24 h after CPP expression was found significantly decreased to that from saline CPP ($P < 0.05$). By two-way ANOVA of all groups of morphine CPP (three control groups and three EA groups), we found a significant effect of EA Treatment [$F_{(1,30)} = 6.98$, $P = 0.013$], although no significant interaction of Treatment × Time [$F_{(2,30)} = 1.87$, $P = 0.1716$] was observed. EA treatment for one trial increased met-enkephalin content in the NAc after CPP test, approaching the saline control level. Bonferroni post-test showed that EA for three trials produced a further increase of the tissue content of met-enkephalin in the NAc 30 min after the last EA, as compared with the paired control rats receiving no EA treatment ($P < 0.05$).

The relative levels of mRNAs encoding the PPE gene were measured in the NAc samples using semi-quantitative RT-PCR (Fig. 1D). Similarly, one-way ANOVA followed by Newman–Keuls test was used, analyzing the differences of PPE mRNA levels among the different time points of morphine CPP and saline CPP. Results shown in Fig. 1D indicate that there was a significant difference of PPE mRNA levels among the groups [$F_{(3,15)} = 5.252$, $P = 0.0152$], and the PPE mRNA level in the NAc of the morphine-induced CPP rats 24 h after CPP test was apparently higher compared to that of the saline control rats ($P < 0.05$). This high expression of PPE in the NAc returned to the control level 72 h after the morphine-CPP session. By two-way ANOVA of all groups of morphine CPP, a significant effect of EA Treatment [$F_{(1,18)} = 5.16$, $P = 0.0356$] and interaction of Treatment × Time [$F_{(2,18)} = 4.787$, $P = 0.0036$] were found, suggesting that EA treatments significantly up-regulated the expression of PPE mRNA in the NAc. Bonferroni post-test showed that the PPE mRNA level in the NAc 24 h after the last trial of three EA treatments was significantly higher than that of the control group ($P < 0.01$).

Fig. 2A–D show the effects of 2 Hz EA on release of dynorphin $A_{(1-13)}$, concentration of dynorphin $A_{(1-13)}$ and PPD mRNA levels in the NAc, respectively. In obvious contrast to the enkephalin data shown in Fig. 1, no significant changes in dynorphin were seen following the 2 Hz EA treatments, even though multiple trials of EA were administered.

At the end of the experiments of perfusion, the rats were sacrificed by decapitation, after which the location of the injection site was confirmed. Data from 6 rats with inexact injection sites were discarded in 72 rats.

3.2. Selective μ- or δ-opioid receptor antagonists dose-dependently reversed the inhibitory effects of 2 Hz EA on morphine-induced CPP expression

Previous studies have shown that multiple 2 Hz EA treatments were more effective than single trial EA in suppressing morphine-induced CPP (Chen et al., 2005), and the effect of 2 Hz EA could be blocked by naloxone at 1 mg/kg i.p. (Wang et al., 2000a). To test the roles played by three different kinds of opioid receptors in the efficacy of EA, 48 rats were conditioned with morphine at 4 mg/kg. The CPP score of post-conditioning test (0.6227 ± 0.0113) was significantly higher than that of pre-conditioning test (0.4933 ± 0.0074, $P < 0.001$). After the post-conditioning test, the rats were randomly divided into four groups, receiving 3 consecutive daily administrations of normal saline (10 μl, i.c.v.) or 0.25, 0.5, 1.0 μg of CTAP (i.c.v.), respectively 15 min before EA treatment. All rats were tested for their CPP expression 24 h after the termination of the last EA session. The experimental schedule is illustrated in Fig. 3A. Fig. 3B shows the reversing effect of CTAP administered prior to EA. Repeated measures ANOVA revealed significant effects of Time [$F_{(1,44)} = 13.004$, $P = 0.001$] and almost significant interaction of Treatment × Time [$F_{(3,44)} = 2.714$, $P = 0.056$], although no significant effect of Treatment [$F_{(3,44)} = 0.932$, $P = 0.433$] was found. Bonferroni post-test showed that three trials of EA stimulation markedly blocked the expression of morphine CPP in rats ($P < 0.05$,

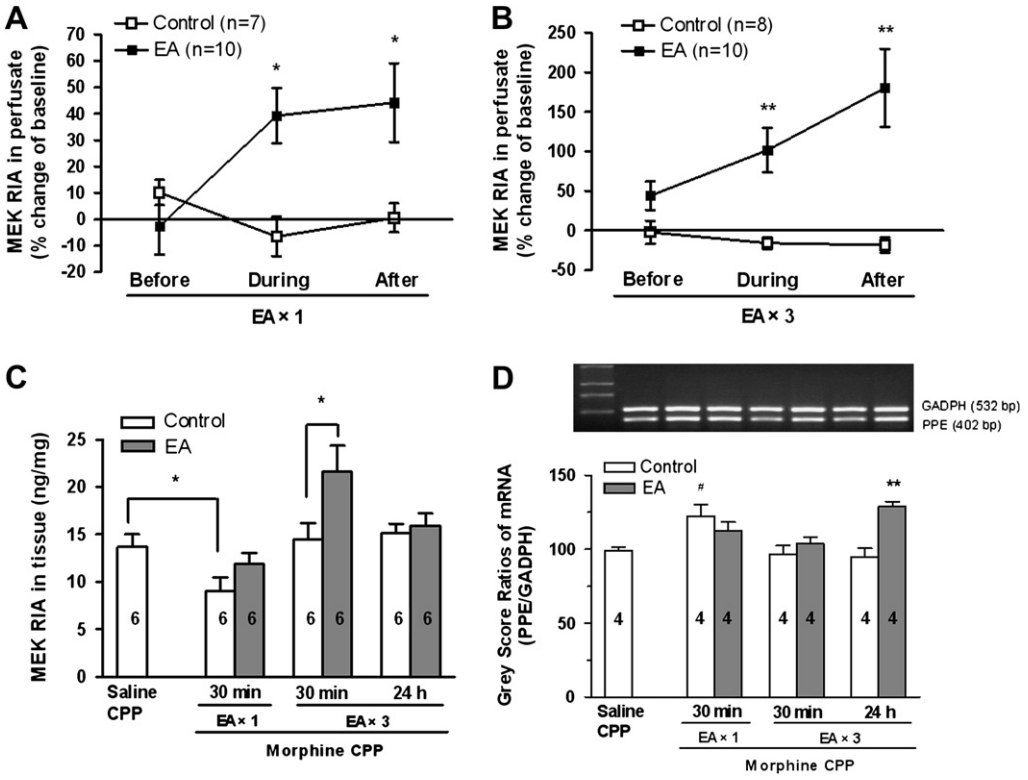

Fig. 1. Effects of 2 Hz EA on met-enkephalin release (A and B, $n = 8$–10), content (C, $n = 6$), and PPE mRNA level (D, $n = 4$) in the NAc from the morphine-CPP rats. (A) 24 h after morphine-CPP test, collecting 90-min perfusate sample in NAc, 30-min before, during and after EA × 1, respectively. (B) EA was given 24 h after the CPP test, once daily for 3 d, collecting perfusate sample in NAc for 90 min: 30-min before, during and after the last EA, respectively. Data were analyzed using repeated measures ANOVA followed by Bonferroni *post hoc*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, compared to the control group at the corresponding time point. (C) Content of met-enkephalin was detected in the total NAc tissue. (D) mRNA products of PPE were run on an agarose gel in the presence of ethidium bromide and visualized under UV light. Representative results of RT-PCR are shown at the top of the panel. $\#P < 0.05$, compared to the saline CPP group. $^{**}P < 0.01$, compared with the corresponding control group.

post-EA test *vs.* pre-EA test). The effect of 2 Hz EA on expression of morphine-induced CPP was reversed by CTAP (0.5 and 1.0 μg, but not by 0.25 μg/10 μl, i.c.v.) given 15 min prior to the EA ($P < 0.01$). Thus, the suppressive effect of 2 Hz EA on morphine-induced CPP could be antagonized by CTAP in a dose-dependent manner.

Using the same protocol, experiments were performed with NTI or nor-BNI. Similarly, Fig. 3C shows the reversing effect of NTI administered prior to EA. Repeated measures ANOVA revealed significant effects of Time [$F_{(1,43)} = 7.721$, $P = 0.008$] and the interaction of Treatment × Time [$F_{(3,43)} = 3.771$, $P = 0.017$], although no significant effect of Treatment [$F_{(3,43)} = 1.883$, $P = 0.147$] was found, Bonferroni post-test showed that the inhibition of CPP by EA of 2 Hz could be antagonized dose-dependently by 0.5 and 5 μg ($P > 0.05$), but not by 0.05 μg of NTI ($P < 0.05$).

The result of i.c.v. injection of nor-BNI prior to EA was shown in Fig. 3D. Repeated measures ANOVA showed no significant effects of the nor-BNI Treatment [$F_{(1,38)} = 0.156$, $P = 0.925$] and the interaction of Treatment × Time [$F_{(3,38)} = 0.090$, $P = 0.965$], although a significant effect of the Time (pre-EA test *vs.* post-EA test) [$F_{(3,38)} = 49.164$, $P < 0.001$] was observed. Bonferroni post-test showed that, nor-BNI administered at the dose from 2.5 to 10 μg, did not block the effect of EA on place preference to morphine-paired chambers ($P < 0.01$, pre-EA test *vs.* post-EA test).

4. Discussions

In the present study, we demonstrated that 2 Hz EA increased the release, and the synthesis of met-enkephalin, but not dynorphin $A_{(1-13)}$ in the NAc of morphine-induced CPP rats. We also confirmed that the inhibitory effects of 2 Hz EA on the expression of morphine-induced CPP could be reversed by selective antagonists of μ- or δ-, but not κ-opioid receptor.

4.1. Involvement of met-enkephalin in the expression of morphine craving

Evidence has shown that the endogenous opioid system contributes to the acute and long-term effects of opiate and other addictive drugs (van Ree et al., 1999). Nieto and his colleagues analyzed the extracellular met-enkephalin in NAc when the morphine-trained rats were exposed to a training-specific context (Nieto et al., 2002). They found that met-enkephalin outflow in the NAc was modified by the induction of positive place conditioning. When rats were placed in morphine-paired environment, the transient increase in met-enkephalin efflux in the NAc could be observed using microdialysis technique. Due to the association of the compartment with morphine-induced euphoria, this may reflect an anticipation of reward. Thus, the enkephalin released by the local enkephalinergic neurons in the NAc may serve as a neural substrate for reward expectation. In the present study, indirect results were observed. Fig. 1C and D show that morphine-CPP expression led to a significant decrease in the content of met-enkephalin and a marked increase in PPE mRNA level, compared to the saline control group, in the NAc 24 h after CPP test. Together, these results suggest that reward-related cues may increase the release of met-enkephalin in the NAc, which was subsequently replenished by newly translated PPE mRNA, but there was still a lack in the total concentration of met-enkephalin in the NAc. This reflected a dynamic change of enkephalin activity in reward-related brain areas during

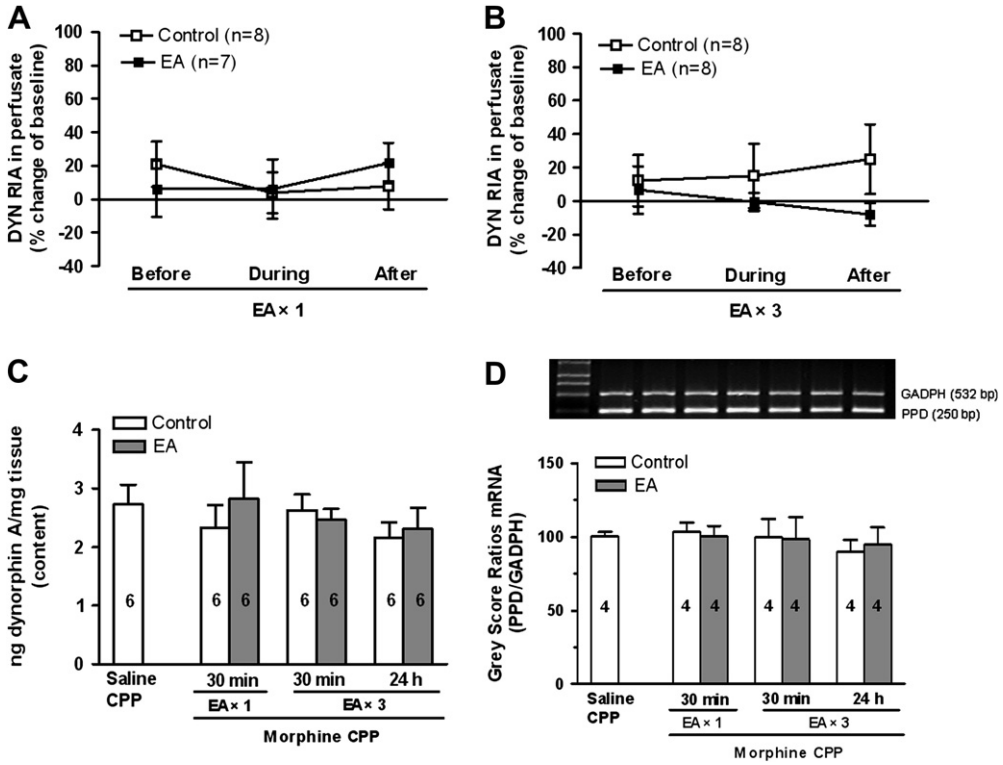

Fig. 2. Effects of 2 Hz EA on dynorphin $A_{(1-13)}$ release (A and B, $n = 8$), content (C, $n = 6$), and PPD mRNA expression (D, $n = 4$) in the NAc from the morphine-CPP rats. (A) EA was given 24 h after the CPP expression, collecting 90-min perfusate sample in NAc, 30-min before, during and after EA × 1, respectively. (B) EA was administered after the CPP expression, once daily for 3 d, collecting perfusate sample in NAc for 90 min: 30-min before, during and after the last EA, respectively. Data were analyzed using repeated measures ANOVA. No significant difference was observed. (C) Content of dynorphin $A_{(1-13)}$ was detected in the total NAc tissue. (D) mRNA products of PPD were run on an agarose gel in the presence of ethidium bromide and visualized under UV light. Representative results of RT-PCR are showed at the top of the panel.

and after re-exposure to the environmental cues conditioned by morphine. Although systemic acute morphine administration was reported to induce an increase in enkephalin release (Olive et al., 1995), it is not likely that these changes were induced purely by the pharmacological intervention, because conditionings by morphine at 5 mg/kg had not influenced the extracellular level and the gene expression of enkephalin in the NAc 24 h after the last morphine-conditioning (Nieto et al., 2002; Shi et al., 2003).

Our previous study revealed that activation of opioid receptors by exogenous or endogenous opioids could suppress morphine-CPP expression (Liang et al., 2006) in rats. In contrast, the opioid antagonist, naloxone enhanced the degree of preference to the morphine-paired compartment (Neisewander et al., 1990). Similar findings were obtained in our laboratory (data not shown). It could be hypothesized that the antagonism of opioid receptors may lead to an increased motivation toward the drug-contextual environment. On the other hand, a saturation of the opioid receptors in mesolimbic areas by exogenous opiates or endogenously released opioid peptides may result in a reduction of the drug seeking or craving.

4.2. Involvement of met-enkephalin and its receptors in the inhibitory effect of 2 Hz EA on morphine-induced conditioned reward

Our previous studies have shown that frequency of the electrical stimulation plays a crucial role in determining the effectiveness of EA treatment. Low frequency (2 Hz) is more effective than high frequency (100 Hz) EA in suppressing the expression and the reinstatement of morphine-induced CPP (Chen et al., 2005; Shi et al., 2003, 2004; Wang et al., 2000a,b). There are two possible mechanisms underlying this inhibitory effect of 2 Hz EA on morphine-induced CPP: 1) EA treatment impairs the function of associative learning and memory; 2) EA stimulation modulates the reward-related neural system, e.g. the mesolimbic area, and reduces CPP expression.

To address the first possibility, experiments were performed to assess whether 2 Hz EA would affect the learning and memory on Morris water maze (MWM) tasks (Chen et al., 2005). The results showed that EA not only produces no deteriorating effect on learning and memory, rather, it causes a promotion of memory consolidation. Emphasis was put on the second question whether 2 Hz EA can increase the activity of enkephalin system. In the present study, direct evidence was obtained at 3 levels: release, content and gene transcription.

Fig. 1A and B indicate that a single trial of 2 Hz EA increased the content of met-enkephalin in the NAc perfusate by approximately 40% and 3 consecutive trials increased it by more than 100%. Since the enzymatic degradation of enkephalin has been largely inhibited by the peptidase inhibitor in the perfusion fluid, this increase in peptide levels in perfusate may represent an immediate increase of enkephalin release, which disappears after 24 h. This increase in release was not only time-dependent, but also neuropeptide-dependent, since 2 Hz did not increase dynorphin release, even after 3 trials of EA stimulation (Fig. 2A and B).

The content of enkephalin immunoreactivity in brain tissue is a balanced result between producing and releasing of endogenous enkephalin. Morphine-conditioning *per se* resulted in a significant decrease in enkephalin content in the NAc, which may reflect an accelerated release of the preexisting peptide content. While a single episode of EA was barely enough to bring the content to the normal level, 3 consecutive episodes of EA did increase the content to a significantly higher level than baseline, suggesting that the rate of *de novo* synthesis or that of precursor processing overweighed

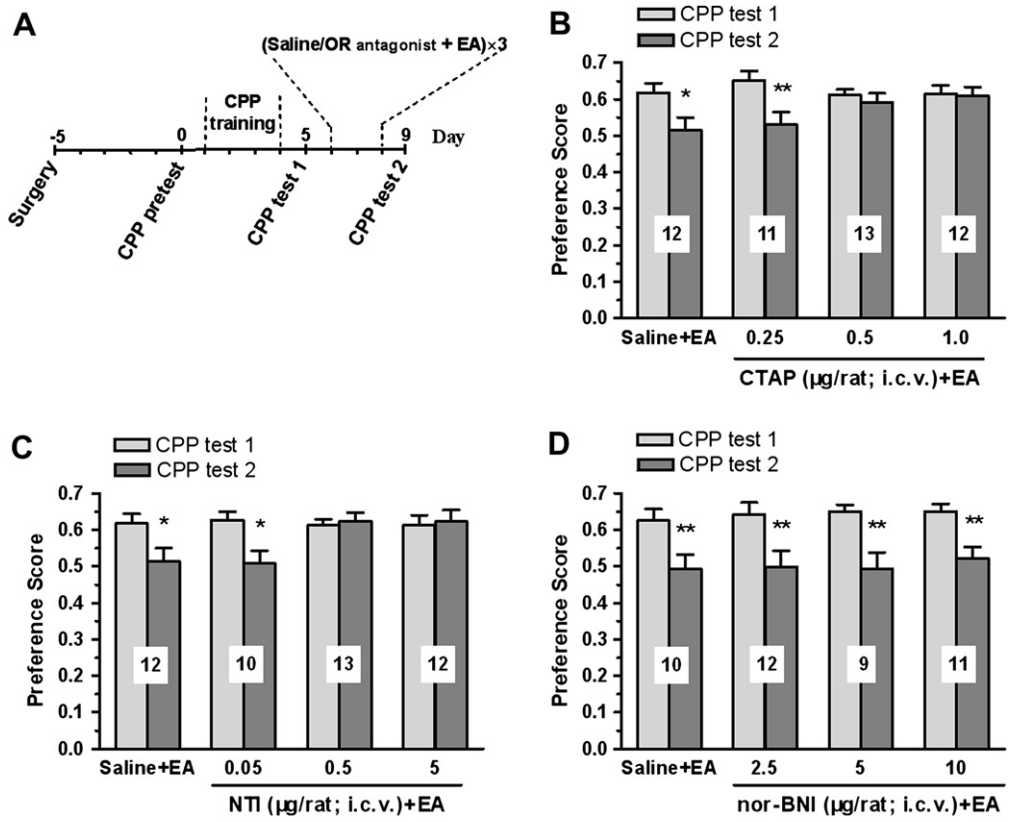

Fig. 3. Effects of i.c.v. injection of selective opioid receptor antagonists on 2 Hz EA inhibition of morphine-induced CPP expression (n = 9–13). Values are expressed as mean (±S.E.M.) of the CPP score. A outlines the experiment procedure. B, C and D panels, the first pair of columns show the data of animals receiving vehicle (10 μl/rat, i.c.v.), and the other three pairs of columns show the data of animals receiving CTAP (0.25, 0.5, 1.0 μg/10 μl, i.c.v.), NTI (0.05, 0.5, 5.0 μg/10 μl, i.c.v.) or nor-BNI (2.5, 5, 10 μg/10 μl, i.c.v.) 15 min before each EA. Blank and striped columns represented data from pre- and post-EA test, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, post- *vs.* pre-EA test (repeated measures ANOVA, Bonferroni *post hoc*-test).

that of the increased release. An increased transcription of the mRNA encoding preproenkephalin after 3 trails of EA (Fig. 1D) seems to support this notion. It should be mentioned that all these changes were observed only in enkephalin system, but not in the dynorphin system (Fig. 2). Similar observations have been reported based on EA treatments conducted in naïve animals (Guo et al., 1996) and rats dependent to morphine (Shi et al., 2004).

Different kinds of endogenous opioid ligands show some preference for different receptors: enkephalins for μ- and δ-opioid receptors and dynorphins for κ subtype (Raynor et al., 1994). Interestingly, activation of μ- and δ-opioid receptors by relevant agonists can produce euphoria in humans and function as positive reinforcers in animals, whereas agonists to κ-opioid receptors induce dysphoria and aversive effects (Narita et al., 2001; Bodnar and Hadjimarkou, 2002). For example, both systemic and intra-VTA/NAc injection of μ-receptors agonists induced self-administration in animals (McBride et al., 1999). Similarly in the CPP animal model, morphine or the selective μ-, δ-receptors agonists, administered in VTA or NAc, can induce the CPP development (Phillips and LePiane, 1980; Olds, 1982; van der et al., 1982; Mucha and Herz, 1986; Bals-Kubik et al., 1993). In the present study, it was found that the inhibitory effects of 2 Hz EA on morphine CPP could be reversed by the μ- or δ-receptor antagonists, but not by the κ-receptor antagonists. Previous researches have confirmed that animals receiving no EA could still express CPP at the corresponding time point, and the opioid receptors antagonists alone without EA did not affect morphine CPP (Shi et al., 2003). Furthermore, 2 Hz EA itself can induce CPP, and naloxone at the dose of 2 mg/kg, sufficient to block the μ- and δ-opioid receptors (Iwamoto, 1985), block the EA-induced CPP (Xia et al., 2008). Hence, it is likely that the endogenous enkephalin and their interaction with μ- and δ-receptors might be involved in the rewarding effect of 2 Hz EA. The positive reinforcement induced by repeated 2 Hz EA stimulation might suppress the expression of morphine-induced CPP.

In conclusion, the present study provides direct evidence that 2 Hz EA could play a role to weaken morphine-induced reward memory, *via* modulating the endogenous opioid system in the mesolimbic system. The low frequent EA may exert its effect through the release of enkephalin and activation of μ- and δ-receptors in reward-related brain areas to suppress the craving and the motivation of drug seeking in morphine-induced CPP rats. These results provide a promising therapeutic approach of using low frequency EA for the treatment of opiate addiction.

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