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Research report

Repeated peripheral electrical stimulations suppress both morphine-induced CPP and reinstatement of extinguished CPP in rats: accelerated expression of PPE and PPD mRNA in NAc implicated

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Abstract

Previous studies have shown that peripheral electrical stimulation (PES) can suppress morphine-induced conditioned place preference (CPP) and the reinstatement of extinguished CPP in the rat. The present study was performed to elucidate if preproenkephalin (PPE) and preprodynorphin (PPD) mRNAs in the nucleus accumbens (NAc) play a role in this event. Rats were trained with morphine for 4 days to establish CPP paradigm. They were then given 15-min test once a day for eight consecutive days for extinction trial. Twenty-four hours after the 8th session of extinction trials, rats were given peripheral electrical stimulation (PES) at 2 or 100 Hz once a day for 3 days, then a morphine-priming injection at a dose of 1, 2, or 4 mg/kg to reinstate the extinguished CPP. At the end of the experiment, PPE and PPD mRNA levels in the nucleus acccumbens (NAc) were determined by the semiquantitative RT-PCR technique. The results showed that PES at 2- and 100-Hz administered 30 min a day for 3 days suppressed both the expression of morphine-induced CPP and the reinstatement of extinguished CPP. PES at 2 Hz increased preproenkephalin (PPE) mRNA levels, whereas PES of 100 Hz that of preprodynorphin (PPD) mRNA levels in the NAc. These findings suggest that enkephalin and dynorphin in NAc may play important roles in the mechanisms underlying the inhibitory effect of PES on the expression and reinstatement of morphine-induced CPP in rats.
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Theme: Neural basis of behavior
Topic: Drugs of abuse: opioids and others

Keywords: Conditioned place preference (CPP); Peripheral electric stimulation (PES); Reinstatement; Preproenkephalin (PPE); Preprodynorphin (PPD)

1. Introduction

In human drug addicts, re-exposure to a drug often induces drug-seeking behaviour and precipitates relapse even after long-term periods of abstinence [7,19]. It has been made clear that administration of opiates increases the craving for opioids in drug-free addicts, and may reinstate drug-seeking behaviour after prolonged periods of extinction in opiate-experienced animals [1,27]. Conditioned place preference (CPP) paradigm has been used widely to study the rewarding effects of various drugs of abuse [34], since it involves the drug-associated conditioned cue, which may be responsible for relapse in drug free former addicts. This property makes the CPP paradigm a useful tool for testing medications or other approaches for their effects of anti-craving and anti-relapse to drugs of abuse [2,34].

Substitution treatments are popularly used for alleviation of opiate addiction worldwide, although most of the substitution agents themselves possess some side effects such as abuse potential. In contrast, peripheral electrical stimulation (PES), developed on the basis of acupuncture,

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has been shown to be successful for the treatment of drug abuse [15,39]. Our previous work [18] has demonstrated that 2- or 100-Hz PES could suppress the craving for opiates in heroin addicts and morphine-induced CPP in rats [30]. However, the mechanisms underlying the inhibitory effects of PES on craving for opiates are still obscure and need further investigation.

PES has been shown to facilitate the biosynthesis of endogenous opioid peptides. For example, an earlier study of Guo et al. [11] reported that 100 Hz electrical stimulation could accelerate the expression of mRNAs encoding preproenkephalin (PPE) and preprodynorphin (PPD) in brain, which might account for the cumulative therapeutic effect observed in the treatment of chronic pain or other disorders with PES. However, whether the levels of PPE and PPD mRNAs in brain are involved in the effect of 2- or 100-Hz PES on opiate dependence remains unknown. In the present study, we aimed to explore whether multiple sessions of mild PES with low or high frequency could inhibit the expression and reinstatement of morphine-induced CPP. In addition, we observed the levels of PPE and PPD mRNAs in the nucleus accumbens (NAc) of rats in response to these treatments.

2. Materials and methods

2.1. Animals

All experiments were performed on male Sprague–Dawley rats, obtained from the Peking center of experimental animals, weighing 180–220 g at the beginning of the experiment. Animals were housed 4 per cage in a 12:12 h light/dark cycle (lights on at 07:00 h) with food and water available at all times. The room temperature was maintained at 24±1 °C and relative humidity at 50%. Animals were conditioned and tested during the light phase of the cycle. They were handled daily during the first week after arrival. All experimental procedures were approved by the Animal use Committee of Peking University Health Science Center.

2.2. Apparatus

Conditioning was conducted in black colored rectangular PVC boxes (71.5×36.5×30 cm), containing three chambers separated by guillotine doors. The two large black colored conditioning chambers (A and C, 24×35 cm) were separated by a small gray colored center choice chamber B (15.5×19.5 cm). Chamber A has 4 light-emitting diodes (LEDs) forming a square on the walls and a stainless steel mesh floor (1.3×1.3 cm^2), and chamber C has 4 LEDs forming a triangle on the wall and a stainless-steel rod floor (1.3 cm apart), whereas chamber B has a plain floor. Fifteen photobeams were placed across chambers with 4.75 cm apart. Through a computer interface, the time spent for the rat in each chamber was recorded by means of infrared beam crossings.

2.3. Place preference paradigm

The methods of CPP have been described in details previously [29]. Briefly, animals received a single pre-conditioning test in which they were placed in the center choice chamber with the guillotine doors removed to allow access to the entire apparatus for 15 min. The amount of time spent in each chamber was monitored and used to assess natural preferences. The next day, rats were assigned to receive saline or morphine (4 mg/kg) paired with one of the two conditioning environments in a counterbalanced manner (the 'unbiased' procedure). Each animal received four saline and four morphine pairings on alternating days, and was placed into the assigned chamber for 45 min. The center choice chamber was never used during conditioning and was blocked by guillotine doors.

2.4. Extinction by repeated testing and reinstatement

After conditioning and following the initial CPP test, rats were given 15-min tests once a day for 11 days. No injections were given during this extinction period. The day following the last extinction trial, all rats received a priming injection of morphine (1.0, 2.0 and 4.0 mg/kg, i.p.) and were placed in the center choice chamber with access to the entire apparatus for 15 min.

2.5. Peripheral electric stimulation

Rats were kept in specially designed holders, with their hind legs and tails exposed [16]. Two stainless 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 100 Hz (0.2 ms pulse width) or 2 Hz (0.6 ms pulse width). The intensity of the stimulation was increased stepwise from 0.5 to 1 mA and 1.5 mA, with each step lasting for 10 min. The PES was given 24 h after the last conditioning test.

2.6. Tissue dissection

Rats were decapitated immediately after the expression of morphine CPP. The brains were removed and the nuclei accumbens were rapidly dissected out. Tissue samples were stored at −80 °C until analysis.

2.7. RT-PCR analysis of PPE and PPD mRNA expression

The relative levels of mRNAs encoding the PPE and PPD gene were measured in brain samples of extracted RNA

using the semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) technique. Inter-sample variability was controlled by standardization of assay conditions. Fixed amounts of RNA were used in each reaction, and reproducibility was routinely monitored. 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 and PPD [18] were synthesized by the Sangon company (China), primers for GAPDH cDNA were obtained from Maximbio (San Francisco, CA, USA) (Table 1). The predicted sizes of amplified products were 532 bp for GAPDH cDNA, 402 bp for PPE cDNA and 250 bp for PPD cDNA.

Total RNA was extracted from NAc by Trizol reagent (Invitrogen Corporation, Carlsbad, CA). Approximately 2 μg total RNA was used for cDNA synthesis by reverse transcription with 200 U M-MLV reverse transcriptase (Invitrogen Corporation, Carlsbad, CA) 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 Corporation, Carlsbad, CA) 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, 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, 1 min at 72 °C). After amplification, the products were separated on a 1.5% agarose gel in the presence of ethidium bromide and visualized under u.v. light.

2.8. Statistical analysis

Data from the CPP test were expressed as a preference ratio scores [3]. A preference ratio for each rat was calculated by dividing the duration spent in the drug-paired compartment by the duration spent in both conditioning compartments. Data were processed by commercially available software GraphPad Prism 3.0. Results are presented as mean±S.E.M. Comparisons between means of groups were analyzed with one-way analysis of variance (ANOVA) followed by Student–Newman–Keul's test. The accepted level of statistical significance is $p<0.05$.

Table 1
Primers for amplification of cDNA for PPE, PPD and GAPDH

Primers for amplification of cDNA for PPE	
Sense primer	5′-TAG CCA AGA AGT ATG GAG GG-3′
Antisense primer	5′-TCT GAT AGT CCA TCC ACC AC-3′
Primers for amplification of cDNA for PPD	
Sense primer	5′-GAG GAC TTG AGA AAA CAG GCC-3′
Antisense primer	5′-GGT ATT GGG GTT CTC CTG GGA-3′
Primers for amplification of cDNA for GAPDH	
Sense primer	5′-GGG TGG TGC CAA AAG GGT C-3′
Antisense primer	5′-GGA GTT GCT GTT GAA GTC ACA-3

3. Results

3.1. Experiment 1: The effects of PES at different frequencies on morphine-induced CPP and expression of PPE and PPD mRNAs in the NAc

3.1.1. Morphine-induced CPP

The pre-conditioning test showed that animals spent almost equal amount of time in the two end chambers (A: 317±7.39 s, C: 318±6.52 s) and less time in the small center choice chamber (B: 264±8.72 s). There were no significant differences in the time spent in the two end chambers ($P>0.05$). Thus, the test boxes were considered unbiased in terms of chamber preferences of untreated rats.

A total of 72 rats were evenly and randomly distributed into six groups. Five of the six groups were trained with morphine 4 mg/kg for 4 days as described by Shi et al [30] for CPP, one group was given same volume of normal saline (NS) as control. As shown in Fig. 1A, all groups trained with morphine presented significantly higher CPP scores ($P<0.01$) than the control group, with no apparent difference between the five morphine treated groups.

3.1.2. Effects of repeated treatment at 2- or 100-Hz PES on morphine-induced CPP

Twenty four hours after the CPP expression, 48 rats were evenly and randomly distributed into four groups of 12 each. Group 1 received no treatment serving as blank control, group 2 was merely restrained in the holder for 30 min, serving as control for restrain stress, and group 3 and group 4 were given 2 Hz and 100 Hz PES, respectively once a day for three days. All groups were tested again for their CPP expression 24 h after the treatment. It was shown in Fig. 1B that both 2-and 100-Hz PES significantly decreased the expression of the morphine-induced CPP.

3.1.3. The effect of 2- or 100-Hz PES on PPE mRNA expression in the NAc of rats exhibited CPP to morphine

Rats were decapitated immediately after the CPP trial. The brains were removed and the NAc was rapidly dissected out. The results depicted in Fig. 2 showed that PPE mRNAs in the NAc was at a low basal level in NS control group. The PPE mRNAs level tended to decrease during the expression of morphine-induced CPP, although no significant differences were found ($p>0.05$) between the CPP and NS group. Compared with the simple CPP group or restrain+CPP group, PPE levels were increased significantly in rats subject to 2 Hz PES ($p<0.05$).

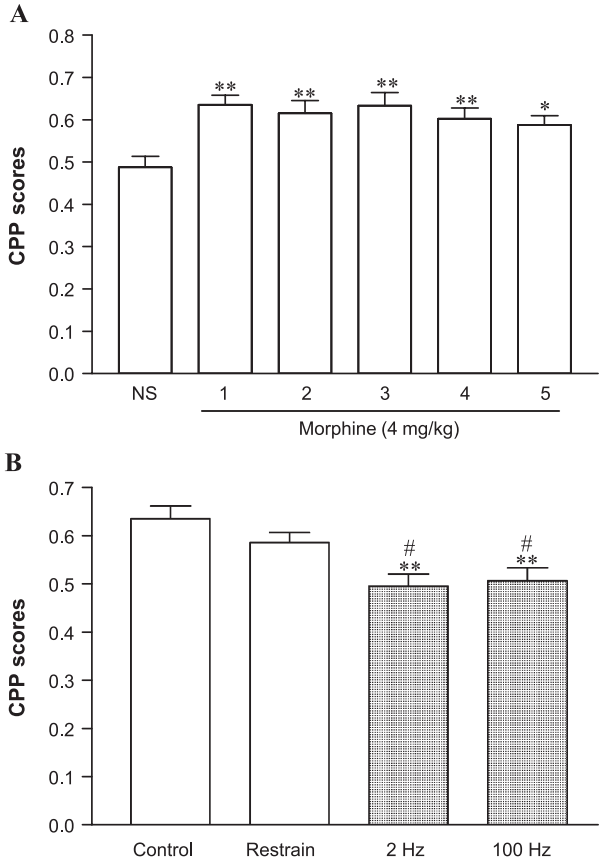

Fig. 1. Establishment of morphine-induced CPP (n=10–12). *p<0.05, **p<0.01 compared with saline control group (A). Effects of 2-Hz or 100-Hz PES on morphine-induced CPP in rats (n=9–11). **P<0.01, compared with control group. $^{\#}p$<0.05, compared with restrain group (B).

3.1.4. The effect of 2- or 100-Hz PES on PPD mRNA expression in the NAc of rats showing CPP to morphine

We examined changes in PPD mRNA levels in the NAc following PES. As shown in Fig. 3, baseline levels of PPD mRNAs was relatively high. There were no apparent differences in PPD mRNA levels in the NAc between morphine CPP group and NS control group. However the PPD mRNA level was significantly higher in 100 Hz PES group but not in the 2 Hz group compared with that of simple CPP or the restrain group (p<0.05).

3.2. Experiment 2. The effects of 2-or 100-Hz PES on morphine priming-induced reinstatement of extinguished CPP in rats

3.2.1. Extinction of morphine-induced place preference

Seventy-two rats were randomly assigned into six groups of twelve. Four of the groups were trained with morphine (4 mg/kg) for 4 days in the CPP paradigm, while the other two groups were given the same procedure with saline instead of morphine as the control. After conditioning and following the initial CPP test, rats were given 15-min tests once a day for 11 days. It can be seen in Fig. 4 that the place preference for morphine-pairing chamber gradually diminished over days and did not differ from that of NS groups by day 7 (p>0.05). These results showed that morphine-induced CPP disappeared after seven consecutive days of extinction trials.

3.2.2. Reinstatement of the extinguished place preference with a priming injection of morphine

Twenty four hours following the final extinction trial, rats were given morphine priming at doses of 1, 2 or 4 mg/kg and tested for CPP. The results are shown in Fig. 5. Rats receiving morphine priming showed a significantly stronger preference than those administered with saline (P<0.05 vs. NS control groups), with no apparent dose-effect relationship. A dose of 2 mg/kg was thus used in subsequent studies. The results indicate that the extinguished CPP could be reinstated by a priming injection of a small dose of morphine.

3.2.3. Effects of repeated treatment with 2- or 100-Hz PES on the reinstatement of extinguished CPP

Fifty-six rats were randomly distributed into five groups, with 10–12 in each group. Twenty-four hours following the establishment of the CPP, the rats were given extinction trials for 7 days. As described previously, morphine CPP

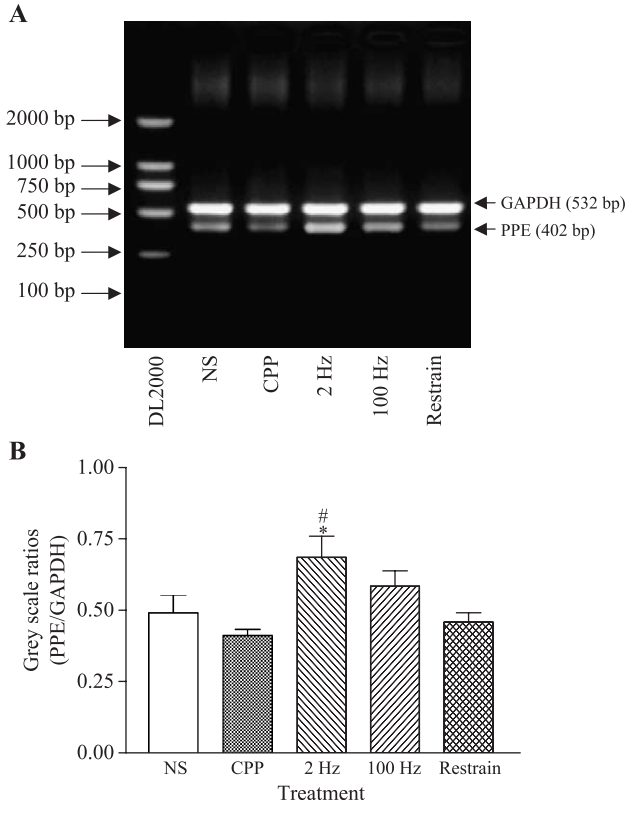

Fig. 2. Effect of 2 Hz and 100 Hz PES on the expression of preproenkephalin (PPE) mRNA in NAc of CPP rats (n=4). CPP rats received 2-Hz and 100-Hz PES once a day for 3 days, followed by CPP test. The mRNA products were run on an agarose gel in the presence of ethidium bromide and visualized under UV light (A). The grey ratios of PPD to GAPDH from four independent experiments were calculated (B). *p<0.05 compared with CPP control group. $^{\#}p$<0.05 compared with restrain group.

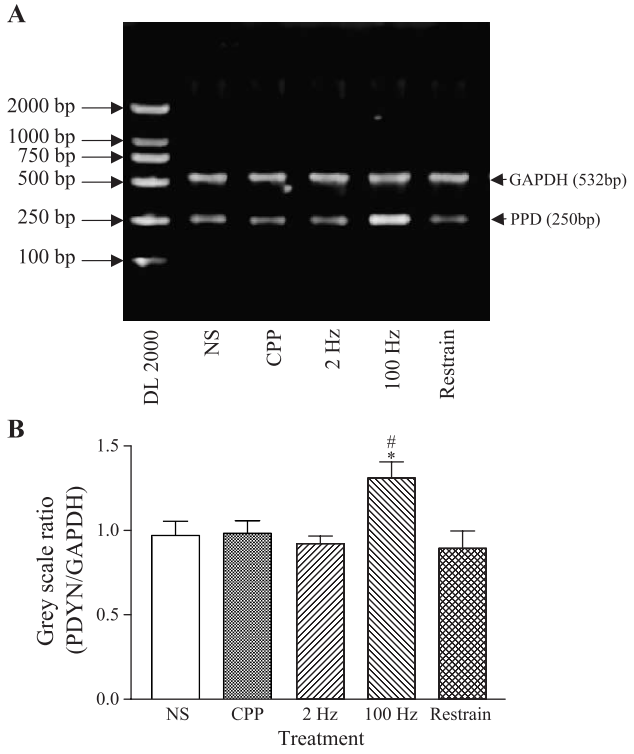

Fig. 3. Effect of 2 Hz and 100 Hz PES on the expression of preprodynorphin (PPD) mRNA in NAc of CPP rats (n=4). CPP rats received 2-Hz and 100-Hz EA for 3 days, followed by CPP test. The mRNA products were run on an agarose gel in the presence of ethidium bromide and visualized under UV light (A). The grey ratios of PPD to GAPDH from four independent experiments were calculated (B). *p<0.05 compared with CPP group. $^{\#}p$<0.05 compared with restrain+group.

disappeared by day 7. From days 8 to 10, three groups received restrain, 2-, or 100 Hz-PES treatments respectively once a day for 3 days, the other two groups received no special treatment serving as control. On day 11, one of the control groups received saline injection, and all the other groups were administered with morphine at 2 mg/kg (i.p.) 15 min before the testing for CPP. Fig. 6 showed that 2 mg/kg morphine priming but not NS priming produced a reinstate-

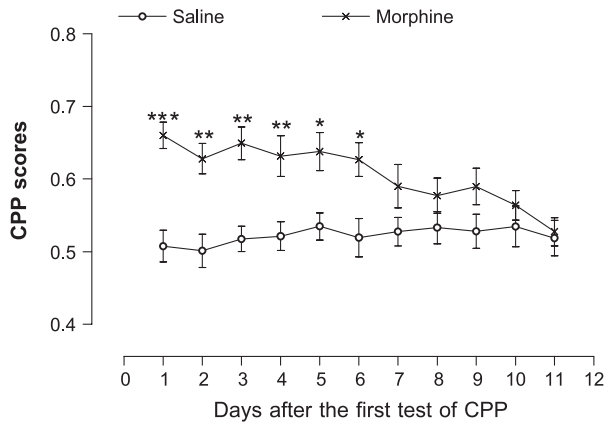

Fig. 4. The extinction of morphine-induced CPP induced by daily test (n=9–12). *p<0.05, **p<0.01, ***p<0.001 compared with their corresponding saline control groups.

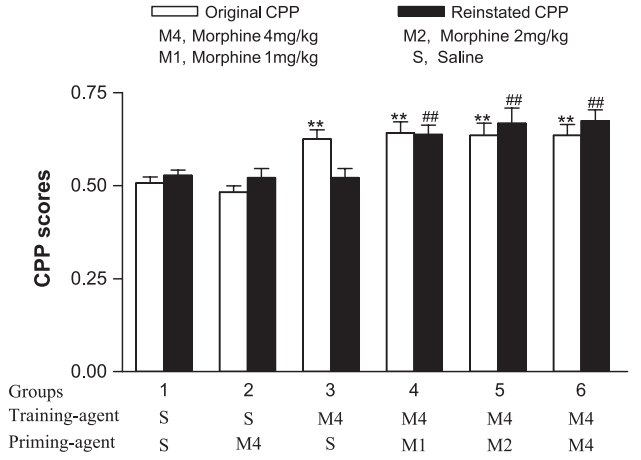

Fig. 5. The effect of morphine priming on the reinstatement of extinguished place preference to morphine (n=10–12). **p<0.01, compared between the saline-trained and 4 mg/kg morphine trained groups $^{\#\#}p$<0.01, compared with their corresponding initial CPP.

ment of CPP. This reinstatement was suppressed by PES of either 2 or 100 Hz (P<0.01 compared with their initial CPP groups or morphine priming group), but not by restrain.

3.2.4. Effects of 2- or100-Hz PES on PPE mRNA expression in the NAc as related to the effect of PES on the reinstatement of extinguished CPP

Rats were decapitated immediately after the CPP trial when morphine priming-induced reinstatement of distinguished CPP was blocked by PES. A representative result is shown in Fig. 7A, depicting ethidium bromide stained RT-PCR products of PPD and GAPDH mRNAs in the NAc. The results of semiquantitative analysis of these bands are shown in Fig. 7B. Morphine priming-induced CPP did not exert any effect on PPE mRNA levels in the NAc (p>0.05). A significant increase in PPE mRNA level was found in rats subject to 2 Hz PES, but not 100 Hz PES or restrain. The results suggest that PES at 2 Hz facilitates the biosynthesis

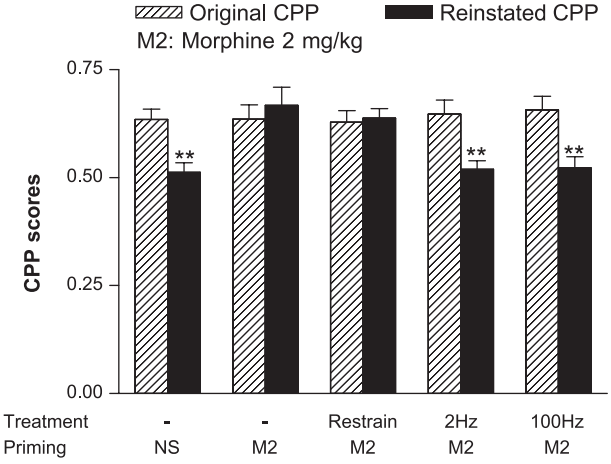

Fig. 6. Effect of PES on morphine-priming-induced CPP reinstatement (n=12). **p<0.01 compared with corresponding initial CPP or the restrain group.

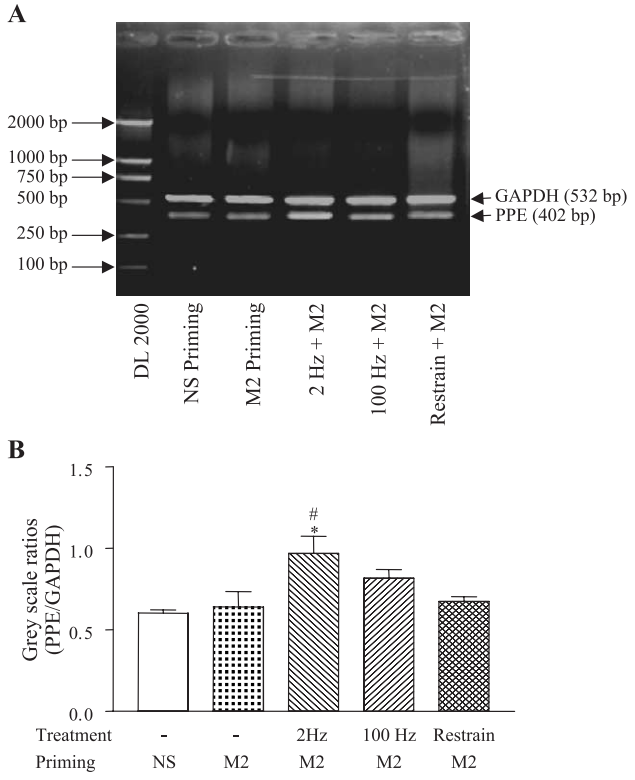

Fig. 7. Effect of 2 Hz and 100 Hz PES on the expression of preproenkephalin (PPE) mRNA in NAc of CPP reinstatement rats (n=4). After amplification, the mRNA products were run on an agarose gel in the presence of ethidium bromide and visualized under UV light (A). The grey ratios of PPE to GAPDH from four independent experiments were calculated (B). *p<0.05 compared with the M2 priming group. $^{\#}p$<0.05 compared with restrain+M2 group.

of dynorphin in the NAc, which may contribute to the suppression of reinstatement of morphine CPP.

3.2.5. Effects of 2- or 100-Hz PES on PPD mRNA expression in the NAc as related to the effect of PES on the reinstatement of extinguished CPP

The design of experiment in this study was same as the mentioned above except that PPD mRNAs were measured in stead of PPE mRNAs. The results are shown in Fig. 8. Significant increase in PPD mRNA levels was found in rats treated with 100 Hz PES, but not 2 Hz PES or restrain. The results indicate that PES at 100 Hz facilitates the biosynthesis of dynorphin, which may play important role in the mechanisms underlying the inhibitory effect of 100 Hz PES on reinstatement of morphine-priming-induced CPP.

4. Discussion

4.1. Three sessions of 2 Hz PES at a low intensity produce the same inhibitory effect on morphine CPP as that of single session PES at a high intensity

Previous studies in our laboratory clearly indicate that morphine-induced CPP can be suppressed not only by PES at 2 Hz [36] but also by PES at 100 Hz. [30]. The intensity of electrical stimulation could be set at a higher level (1–3 mA) or a lower level (0.5–1.5 mA). Using two-compartment CPP model, Wang et al. [36] reported that one session of 2 Hz PES at an increasing intensity of 1–2–3 mA for a total of 30 min could effectively suppress morphine CPP in the rat. In the present study, using 3-compartment CPP paradigm, we have chosen to use 2- or 100-Hz PES at a lower intensity (0.5–1.0–1.5 mA) in order to eliminate the possible stress induced by electrical stimulation [30]. Results showed that one session of low intensity PES was not enough to reduce morphine CPP (data not shown), whereas three consecutive daily sessions of PES could significantly suppress the expression of morphine CPP, suggesting a cumulative therapeutic effect.

Considerable evidence has been obtained in our laboratory to show that PES of identified frequencies could mobilize different kinds of endogenous opioid peptides, acting on their corresponding receptors to induce analgesia [14]. For example, PES of 2 Hz could increase the release of enkephalin in brain to interact with μ- and δ-receptors, while PES of 100 Hz could increase the release of dynorphin to interact with κ-receptors. It was also demonstrated [12] that

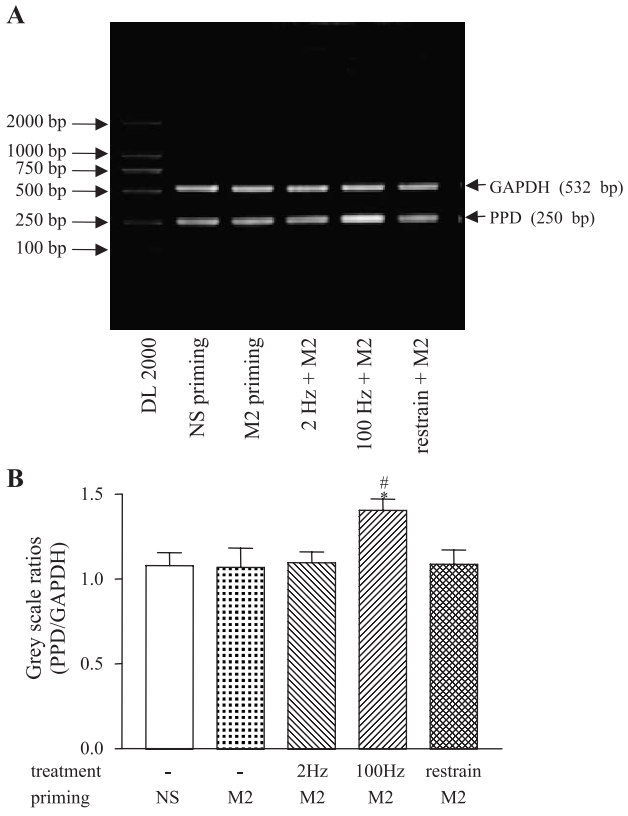

Fig. 8. Effect of 2 Hz and 100 Hz PES on the expression of preprodynorphin (PPD) mRNA in NAc of CPP reinstatement rats (n=4). The mRNA products were run on an agarose gel in the presence of ethidium bromide and visualized under UV light (A). The grey ratios of PPD to GAPDH from three independent experiments were calculated (B). *p<0.05 compared with the M2 (morphine 2 mg/kg) priming. $^{\#}p$<0.05 compared with restrain+M2 group.

a single session (30 min) of 2 Hz and 100 Hz PES at a higher intensity (1–3 mA) could accelerate the expression of mRNAs encoding preproenkephalin and preprodynorphin, respectively, in the brain. This may explain why the effects produced by weak PES can be additive or cummulative when it is administered repeatedly to match that produced by strong PES.

It seems less likely that the effects of multiple PES in suppressing CPP is due to its interference with locomotor activity, since the motor activities of the rat was not hindered by the electrical stimulation (Fig. 9).

4.2. Both the release and biosynthesis of enkephalin seem to be accelerated by multiple low intensity low frequency PES

It was reported that heroin appeares to have a more pronounced effect than cocaine on dynorphin, enkephalin and substance P levels in the caudate striatum and septum when the rat is experiencing self-administration of drug [5]. Recently, Nieto et al. [22] using in vivo microdialysis in freely moving rats observed an increase in enkephalin levels in the NAc of rats when they were placed in the drug-associated compartment. These results suggest that there is an increase in release of brain enkephalin during reward expectation (craving), as previously suggested for dopamine [10,26]. In the present study, the absence of an alteration in the transcription of PPE gene was observed during the expression of morphine CPP, suggesting an increase in the release, but not the biosynthesis of enkephalin during morphine CPP expression, at least in that specific time frame (24 h after the CPP test).

Nieto et al. [22] also found a decrease in the extracellular level of enkephalin when the CPP rats (24 h after the last conditioning training) were placed in the saline-paired compartment, which might be related to an aversive effect. It was also reported [6] that reduction in brain dopamine (DA) concentration is present at the post-conditioning period (72 h after final drug dose), which suggests that place preference conditioning may, in part, result from negative motivational

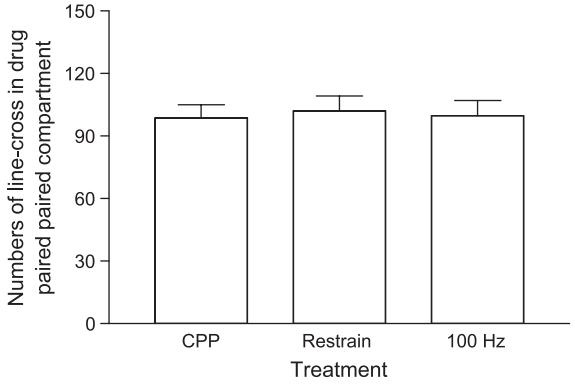

Fig. 9. Locomotor activity recorded during the CPP test in a period of 15 min (n=11). The data of locomotion were expressed as numbers of line-crosses in the drug-paired compartment (mean±S.E.M.). No significant difference (p>0.05) was found among the three groups.

or aversive effects. In the present study, PES was administered 48 h after the abstinence of the drug when the rats might experience aversive effects and the concentration of enkephalin in brain might be very low. During this period of time, multiple treatments of 2 Hz PES produced a significant increase in PPE mRNAs level in the NAc of rats, which may result in an accelerated biosynthesis and release of enkephalins, and this may account for abolishment of aversive effects and suppression of craving for opiates. On the other hand, the extent of the release of enkephalin triggered by a single treatment of low intensity PES may not be strong enough to abolish the expression of morphine CPP.

4.3. Multiple treatments with low intensity 2 Hz PES can suppress the reinstatement of morphine-priming-induced CPP

Relapse is a major characteristic of drug addiction and remains the primary problem in treating drug abuse [23]. It is extremely important to establish an animal model mimicking the relapse to drug in humans. Over the past few decades, some laboratories have been using the reinstatement model to study factors that underlie relapse to heroin and cocaine seeking induced by exposure to self-administered drug, drug cues, and stressors [28,29]. Most recently, several laboratories have developed a conditioned place preference reinstatement model [4,21,24,37]. CPP technique has a number of advantages over the self-administration procedure as an animal model of relapse, which may serve as an alternative to the traditional intravenous self-administration method [20].

In the present study, we established an animal model of reinstating a CPP by priming injection of morphine. Daily extinctive trials accelerated the extinction of CPP, which is in agreement with the findings reported by Parker et al. [24]. In our study, an extinguished CPP can be reinstated following a priming injection of morphine. This result is also in line with the hypothesis suggested by Mueller et al. [20,21] that the incentive salience and attractiveness of drug-related stimuli are renewed by the presence of the drug.

Drug-induced reinstatement of drug craving seems to result from the rewarding properties of drugs of abuse. Robinson and Berridge [25] found that rewarding effects may increase progressively as a result of repeated drug-taking and that this so-called incentive sensitization plays a crucial role in the persistence of drug craving. Reinstatement of CPP appeared to be highly sensitized by priming injection. Following a 11-day period of extinction, morphine-induced CPP could be reactivated by a single injection of morphine at a dose as low as 1 mg/kg, which indicates that the sensitizing properties of addictive drugs play a significant role in drug-seeking behavior that persists long after discontinuation of drug use.

Wang et al. [37,38] reported that a single session of 2 Hz PES but not 100 Hz PES could inhibit the reinstatement of morphine-induced CPP in rats. In the present study,

however, multiple sessions (three consecutive days) of both 2- and 100-Hz PES at low intensity can attenuate the reinstatement of extinguished morphine CPP. These results suggest that the parameters of the electrical stimulation (frequency and intensity) and the design of treatment protocols (single and multiple sessions) play important role in determining the effectiveness of the PES treatment.

4.4. The involvement of PPE mRNAs in the suppressive effect of multiple 2 Hz PES on the reinstatement of morphine-priming-induced CPP

There are very few reports dealing with the possible linkage between enkephalin and renewed drug-seeking behavior. Results obtained in the present study showed that no significant changes were found in PPE mRNA levels in the NAc of rats during the reinstatement of CPP. However, an increase in PPE mRNAs was evident when reinstatement of CPP was eliminated by multiple treatments of 2 Hz PES. Using heroin self-administration model, Shaham et al. [28] reported that chronic occupation of the opioid receptors with heroin given via Alzet osmotic minipumps attenuated heroin-induced reinstatement. In contrast, 24 h after the removal of the minipump, animals showed reinstated heroin-seeking behaviour by heroin priming injection. In our laboratory, Wang et al. [38] found that the reinstatement of morphine CPP could be abolished by a single session of higher intensity 2 Hz PES in a naloxone-reversible manner, which indicates the involvement of endogenous opioid peptides interacting with μ- and δ-opioid receptors. A hypothesis could thus be put forward that three consecutive sessions of low intensity 2 Hz PES could stimulate the biosynthesis and release of enkephalin, which in turn interacts with μ- and δ-opioid receptors, thus eliminating the reinstatement of morphine priming induced CPP. It is probably the increase in biosynthesis of opioid peptides that kept PES still effective even 24 h after the termination of the treatment.

4.5. Treatments of multiple 100 Hz PES increased brain PPD mRNA levels and suppressed the expression of morphine CPP

Results of the present study showed that an increased level of PPD mRNAs was found in the NAc during the inhibition of morphine CPP produced by 100 Hz PES, suggesting an increased synthesis of dynorphin in the NAc. This is in line with our previous findings that 100 Hz PES could increase the abundance of preprodynorphin mRNAs in rat brain [13]. It has been shown that activation of mesolimbic DA system is critically linked to the expression of morphine-induced place preference in mice [8]. Activation of presynaptic κ-opioid receptor located in dopaminergic nerve terminals in the NAc is known to suppress the release of DA [17]. Activation of κ-opioid receptor is also known to abolish morphine-induced CPP and the increase in DA metabolites produced by morphine [8]. Our present study also revealed a significant decrease in DA and its metabolites in the NAc during the inhibition of morphine CPP by 100 Hz PES (data to be published). In relevance with this, Uneklabh et al. [35] have shown that intravenous injections of dynorphin at 180 or 60 μg/kg three times a day for 6 days produced a significant reduction of craving in heroin addicts after detoxification. In short, results mentioned above suggest that inhibition of morphine-induced CPP produced by three daily PES of 100 Hz may be mediated by sustained activation of κ-opioid receptor, which in turn reduces the release of DA in the NAc. However, in the current study, we only explored the biogenesis of opioid peptides at the transcription level. Further studies are needed to identify the release of neuropeptides (enkephalin and dynorphin) in the NAc of rats using the same experimental setup.

4.6. Involvement of PPD gene expression in the inhibitory effect of multiple 100 Hz PES on morphine priming-induced reinstatement of extinguished CPP

It is speculated that behavioral sensitization plays an important role in the development of addiction, especially in the high rate of relapse seen in drug addicts even after very long periods of abstinence [9]. Spanagel et al. [31–33] found in their studies that endogenous κ-opioid systems play an important role in morphine-induced sensitization, and that manipulations of these systems can markedly influence both its behavioral (CPP) and neurochemical (i.e., within the mesolimbic dopaminergic system) expression. In the present study, we observed that three consecutive sessions of 100 Hz PES could significantly augment the level of PPD mRNAs in the NAc. Using HPLC technique we have also found that the brain levels of DA and its metabolites declined during the suppression of reinstated CPP (data to be published) by 100 Hz PES. It is speculated that an increased release of dynorphin in the NAc as triggered by 100 Hz PES would activate presynaptic κ opioid receptor in the NAc, leading to a decrease in the release of DA in the NAc and the abolishment of sensitized dopaminergic activity. These would naturally be followed by an attenuation of reinstatement of CPP. Since the whole procedure involves the modulation of biosynthesis of both dynorphin and dopamine systems, it is reasonable that the effects of multiple PES demonstrated a slow onset and slow decay (long after effects). However, in order to clarify this point, further studies should be performed to monitor the release of dynorphin in NAc during the whole time course of PES treatments.

5. Conclusions

(1) Morphine induced conditioned place preference (CPP) can be taken as an animal model of craving for opiates (model A). Morphine induced CPP can be extinguished by daily testing without reinforcement. The

extinguished CPP can in turn be reinstated by priming injection of morphine, which can be taken as a model for relapse of drug-taking behaviour (model B).

(2) Both model A and B can be suppressed by repeated administration of 2 Hz or 100 Hz peripheral electrical stimulation (PES). This effect of 2 Hz versus 100 Hz PES may be related with their augmentatory effect on the expression of mRNA encoding preproenkephalin versus preprodynorphin, respectively.

(3) Enkephalin may ameliorate morphine craving via its interaction with μ- and δ-opioid receptors in the NAc. Dynorphin, on the contrary, may interact with kappa opioid receptors located on dopaminergic nerve terminals to block the release of dopamine, thus eliminates craving and drug seeking behaviour.

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