



Multiple 100 Hz electroacupuncture treatments produced cumulative effect on the suppression of morphine withdrawal syndrome: Central preprodynorphin mRNA and p-CREB implicated

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ABSTRACT

Alleviating opiate withdrawal syndrome in addicts is a critical precondition to break away from drug and further to prevent reuse. Electroacupuncture (EA) was claimed to be effective for alleviating withdrawal syndrome, but the optimal protocol remained unclear. In the present study we found that (1) 100 Hz EA administered 12–24 h after the last morphine injection suppressed the withdrawal syndrome in rats, multiple sessions of EA were more effective than single session, with the after-effect lasting for at least 7 days. (2) A down-regulation of preprodynorphin (PPD) mRNA level was observed in spinal cord, PAG and hypothalamus 60 h after the last morphine injection, which could be reversed by multiple sessions, but not a single session of EA. (3) Accompanied with the decrease of PPD mRNA level, there was an up-regulation of p-CREB in the three CNS regions, which was abolished by 100 Hz EA treatment. The findings suggest that down-regulation of p-CREB and acceleration of dynorphin synthesis in spinal cord, PAG and hypothalamus may be implicated in the cumulative effect of multiple 100 Hz EA treatment for opioid detoxification.

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1. Introduction

Drug dependence is characterized by compulsive drug-seeking and drug-taking behaviors, which is powered by the desire of escape from the extremely aversive opiate withdrawal symptoms at the abstinence of drug supply. Our previous work has demonstrated that 100 Hz transcutaneous electrical acupoint stimulation (TEAS) could ameliorate withdrawal syndrome in heroin addicts [24] and in rats dependent on morphine [25]. In the further clinical detoxification studies we observed that multiple sessions of TEAS within a day are more effective in relieving withdrawal syndrome of heroin addicts compared with single session of TEAS. Our earlier studies showed that release of dynorphin in the spinal cord may play important roles in the effect of 100 Hz EA for suppressing morphine withdrawal syndrome via interacting with κ -opioid receptor [12,44,47]. Besides spinal cord, periaqueductal gray (PAG) and hypothalamus are sensitive structures involved in morphine withdrawal [4,5,9,10,29,34]. The content of endogenous opioid

peptides in PAG and hypothalamus was reported to fluctuate during chronic morphine treatment and withdrawal [30,36]. And local injection of opioid receptor antagonist precipitated somatic signs of opiate withdrawal [26]. It would be interesting to explore the relation between the behavioral expression of withdrawal and the dynamic changes of dynorphin release and its replenishment in the CNS.

A series of evidence showed that preprodynorphin (PPD) and preproenkephalin (PPE) are products of CRE-containing genes, and their transcription is mediated by transcription factor-cAMP response element-binding protein (CREB) [8,11,14,16,38]. The activity of CREB can be regulated by phosphorylation of Serine 133. Chronic morphine treatment and its withdrawal have been shown to up-regulate the level of phosphorylated CREB protein and affect the transcription of downstream molecules [6,14].

In the present study we investigated the change of PPD- and PPE-mRNA level in the spinal cord, PAG and hypothalamus during morphine withdrawal, and observed whether these changes could be reversed by multiple sessions of 100 Hz EA treatment. And if so, whether PPD and PPE synthesis in the central regions contribute to the cumulative and sustained efficacy of 100 Hz EA, and whether the phosphorylation of CREB is implicated in the process of EA regulation of PPD and PPE synthesis.

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2. Material and methods

2.1. Morphine-dependent animal model

Male Sprague–Dawley rats weighing 200–270 g were housed in a 12:12 h light/dark cycle and free to food and water ad libitum. Morphine dependence was induced by subcutaneous injections of morphine twice daily at 8:00 and 20:00 for ten days with increasing dose (10, 20, 40, 80, 120 mg/kg), with each dose remained for two days. Rats in the control group received equivalent volume of saline twice per day.

2.2. EA

The EA began at 12 h after the last injection of morphine, that is, at 8:00 next day. The morphine-dependent rats were randomly divided into four groups: the four times EA group which was given EA at 8:00, 9:30, 18:00 and 19:30; the twice EA group, given EA at 8:00 and 19:30; the once EA group, given EA at 19:30; and the non-EA group without giving electrical stimulation. For the administration of 100 Hz EA, the rat was individually restrained in a plastic cylinder holder with the tail and the hind legs protruding. Two pairs of stainless-steel needles were inserted in each leg at the acupoint Zusanli (ST36, 5 mm lateral to the anterior tubercle of the tibia) and Sanyinjiao (SP6, 3 mm proximal to the medial malleolus, 1 mm posterior to the tibia). Square waves generated from a Han's Acupoint Nerve Stimulator (HANS, manufactured at the Beijing University of Aviation and Astronautics) were applied to the needles inserted in each leg. The pulse width was 0.2 ms and frequency 100 Hz. The intensity of the stimulation was increased stepwise from 1 mA to 2 mA and 3 mA, and each intensity lasted for 10 min. Rats of the control group were treated with needles placed in situ without connecting to the electronic stimulator. The tail flick latency (TFL) was assessed before and at the end of 30 min EA stimulation.

2.3. Scoring of the morphine withdrawal signs

At the end of 30 min EA, that is, 24 h after the last injection of morphine rats were individually placed in plastic cages for the assessment of 5 spontaneous withdrawal signs for 30 min: rearing, grooming, wet-dog shakes, teeth-chatting and penile-licking [32].

After these tests, half of the rats were extinguished for sampling of the brain and spinal cord, and half were fed for six more days. They were then given an injection of naloxone (1 mg/kg, i.p.), and withdrawal signs were scored immediately for 45 min, followed by brain and cord sampling.

2.4. Nociceptive test

The nociceptive threshold of the rats was measured by TFL elicited by radiant heat. The rat was partially restrained in plastic holders with its tail and hind legs protruding naturally. Focused light from a projection bulb (8–12 v, adjustable) was applied through an aperture of 6 mm diameter to the junction between the middle and the lower 1/3 of its tail, and the TFL was recorded by an automatic electronic timer to the accuracy of 0.1 s. At the beginning of the experiment, TFL was assessed three times at 5-min interval and the mean value from the first three assessments was taken as the basal pain threshold, usually within the range of 4–6 s. An elevation over 15 s was taken as a cut-off limit to avoid unnecessary skin damage.

2.5. Intracranial surgical procedures

Stereotaxic surgery was conducted under pentobarbital sodium (40 mg/kg, i.p.) anesthesia. Animals were implanted bilaterally with

stainless-steel guide cannulae aimed to give access to the PAG (AP – 7.3 mm from bregma, Lat \pm 1.2 mm, Vert 4.0 mm from dura) [42].

Intracerebral infusions were made bilaterally 36 h after the last morphine injection. Rats were hand-held while injection needles were placed into the surgically implanted guide cannulae. The injection needles protruded 2.0 mm beneath the tip of the guide cannulae and terminated in the PAG (6.0 mm from dura). The injection needles were attached to syringes (10 μ l) by PE20 tubing filled with Rp-cAMPS (Sigma) or saline solution. The dose infused bilaterally was 40 nmol/0.5 μ l delivered over a 2 min period. After the infusion a further 2 min was allowed to elapse before the injection needles were removed.

2.6. Tissue dissection

Rats were decapitated immediately, 36 h and 6 days after the last EA respectively. The brain and spinal cord were removed, and PAG, hypothalamus and L 3–5 were rapidly dissected on the ice and drop into the liquid nitrogen. The rats that experienced intracerebral infusions were decapitated 6 h after drug treatment. Tissue samples were stored at –80 °C until analysis.

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

The relative levels of PPE and PPD mRNAs were measured by reverse transcriptase polymerase chain reaction (RT-PCR) technique. The analysis was performed as described previously [43]. Total RNA was extracted from L 3–5, PAG and hypothalamus in Trizol reagent (Invitrogen Corporation, Carlsbad, CA), then was quantified spectrophotometrically. cDNA was synthesized using 1 μ g RNA in a 25 μ l reaction including 200 U M-MLV reverse transcriptase (Invitrogen), 0.5 mM dNTPs, 30 U RNase inhibitor, and 0.5 μ g oligodT_{12–18} primers. Duplicate aliquots of cDNA (2 μ l) were used to amplify the fragments by PCR with 1 U Taq DNA polymerase (Invitrogen), 0.2 mM each of dNTPs and 1 μ M of each primer in a 20 μ l reaction. To amplify PPE, these “standard” PCRs were incubated for 3 min at 94 °C, and then cycled 29 times for 45 s at 94 °C, 30 s at 58 °C and 1 min at 72 °C. And to PPD, these “standard” PCRs were cycled 29 times for 45 s at 94 °C, 30 s at 56 °C and 1 min at 72 °C. Afterwards, the products were resolved in ethidium bromide-stained 1.5% agarose gels and visualized under u.v. light.

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 were synthesized by the Sangon Company (China), primers for GAPDH cDNA were obtained from Maximbio (San Francisco, CA, USA), the sequences of primer are shown in 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.

2.8. Western blotting

The nucleus protein was extracted by nucleus-cytosol-membrane preparation kit (Appligen Inc., Peking, China). The tissue were homogenized in ice-cold cytosol extraction reagent (CER and phosphatase inhibitor), and then centrifuged at 4 °C, 800 \times g for 5 min. The precipitation was resuspended in 500 μ l membrane extraction reagent (NER) and centrifuged at 4 °C, 4000 \times g for 5 min. The supernatant was discarded and the precipitation was washed in 500 μ l NER again. The condition was as above. The precipitation was resuspended in proper volume of suspension buffer. Protein concentration was measured with the BCA assay (BCA protein assay kit, Pierce Inc., USA). 200 μ g of protein was loaded in 10% polyacry-

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

Gene	Gene forward primer (sense)	Gene reverse primer (antisense)
PPE	5'-TAG CCA AGA AGT ATG GAG GG-3'	5'-TCT GAT AGT CCA TCC ACC AC-3'
PPD	5'-GAG GAC TTG AGA AAA CAG GCC-3'	5'-GGT ATT GGG GTT CTC CTG GGA-3'
GAPDH	5'-GGG TGG TGC CAA AAG GGT C-3'	5'-GGA GTT GCT GTT GAA GTC ACA-3'

lamide gels and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Inc., Massachusetts, USA). Membranes were incubated in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBST) containing 5% nonfat milk for 1 h at room temperature with agitation to block nonspecific binding. The membrane was incubated with primary antibody diluted in TBS (CREB, p-CREB), 1:500 (Cell signaling Inc., USA) containing 5% nonfat dried milk overnight at 4 °C. The membrane was washed three times (5 min each) with PBS-T and incubated with anti-rabbit conjugated with horseradish peroxidase secondary antibody (Zhongshan Biotechnology, Beijing, China) at a dilution of 1:2000 for 1 h at room temperature. Bands were visualized using the ECL system (Appligen Inc.). The bands on the autoradiogram were quantified with the TotalLab 2.01 Analysis System (Phoretix, UK), and the optical density of each p-CREB band was corrected by that of the corresponding CREB band.

2.9. Statistical analysis

The scores of spontaneous and naloxone-precipitated withdrawal signs were analyzed by non-parameter test (Kruskal–Wallis ANOVA test), and Neuman–Kuels test was used for post-test. The RT-PCR and Western blotting results were analyzed by one way ANOVA followed with Bonferroni post-test. Statistical significance was set at $p < 0.05$. Results were expressed as mean \pm SE.

Timeline of experimental procedures (see Fig. 1).

3. Results

3.1. Effects of 100 Hz EA on withdrawal syndrome score and tail flick latency in morphine withdrawal rats

Rats that withdrawn from repeated administration of morphine for 24 h and 60 h displayed significant spontaneous withdrawal syndrome including rearing, grooming, wet-dog shakes, teeth-chattering and penile-licking signs, which disappeared 7 days later. While challenged with 1 mg/kg naloxone (i.p.), the morphine treated rats showed obvious precipitated withdrawal syndrome, including rearing and teeth-chattering compared with control group. During 12 h of morphine abstinence, the rats were randomly divided into four groups, and received restraint (without EA), single-, twice- and four-times of 100 Hz EA, respectively.

Fig. 2 shows suppressive effect of 100 EA on the morphine withdrawal syndrome at 24 h (A), 60 h (B) and 7 days (C) after the last morphine injection. At 24 h, the four-time EA treated rats showed less rearing, grooming, wet-dog shakes and penile licking compared with non-EA rats. The twice EA group showed less

rearing and grooming signs, and single EA had only a moderate decrease of rearing. At 60 h, the four-time EA group showed a significant suppression of rearing, wet-dog shakes and teeth-chattering behaviors, whereas the twice EA and single EA group showed only a decrease of rearing signs. Regarding the naloxone-precipitated withdrawal syndrome observed 7 days after morphine abstinence, the four-time EA group still showed significant suppression of the 3 withdrawal signs (rearing, wet-dog shakes and penile licking), and twice EA group had 2 of them (wet-dog shakes and penile licking).

To determine if the morphine treated rats were tolerant to repeated EA administered within 12 h, we tested the efficacy of EA-induced analgesic effect, as assessed by the TFL. The basal TFL of morphine dependent rats (2–3 h after the last morphine injection) was significantly higher than the baseline level before morphine treatment (Fig. 3A). It then decreased to lower than the baseline level 12 and 24 h after the morphine abstinence. 100 Hz EA increased the TFL 24 h after morphine abstinence. The effect of four times EA was greater than twice EA, and the twice EA was greater than the single EA treatment (Fig. 3B). The results suggest that repeated EA remained effective in producing an analgesic effect.

3.2. Effects of multiple 100 Hz EA on the level of PPD mRNA and PPE mRNA in the spinal cord, PAG and hypothalamus

A down-regulation of PPD mRNA was observed by RT-PCR in spinal cord, PAG and hypothalamus 60 h after the last morphine injection. This effect could be reversed by 100 Hz EA, and the multiple EA was more effective than the single EA (Fig. 4A). PPD mRNA level returned to the control level 7 days after the morphine abstinence, and 100 Hz EA could not further change its level (Fig. 4B).

The level of PPE mRNA was not significantly influenced both 60 h and 7 days after the morphine abstinence in spinal cord, PAG and hypothalamus, which was not affected by 100 Hz EA (Fig. 4C and D).

3.3. Effects of multiple 100 Hz EA on the activity of CREB in the spinal cord, PAG and hypothalamus

Phosphorylated CREB in the spinal cord, PAG and hypothalamus of morphine withdrawal rats was up-regulated 60 h after morphine abstinence (all $p < 0.05$). This elevation was suppressed by multiple sessions, but not single session of 100 Hz EA. The four-time EA was more effective than the twice-EA (Fig. 5A and B).

Seven days after the last morphine injection, rats were decapitated immediately after the scoring of naloxone-precipitated morphine-withdrawal syndrome for 45 min. Fig. 5C shows that the p-CREB of morphine treated rats was still higher than the saline

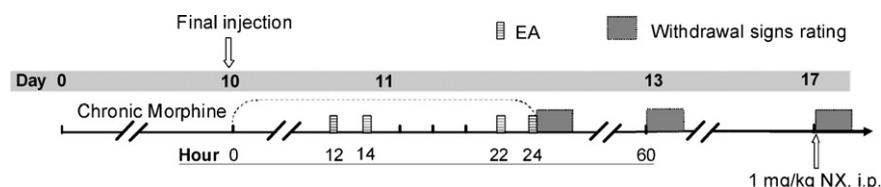


Fig. 1. Timeline of experimental procedures. Chronic morphine: 20–120 mg/kg, s.c., twice per day; EA: 100 Hz, 1–2–3 mA, 30 min/session, Zusanli and Sanyinjiao; 24 h and 60 h withdrawal from morphine: observation of spontaneous withdrawal syndrome (30 min); 7 d withdrawal from morphine: observation of naloxone-precipitated withdrawal syndrome (45 min).

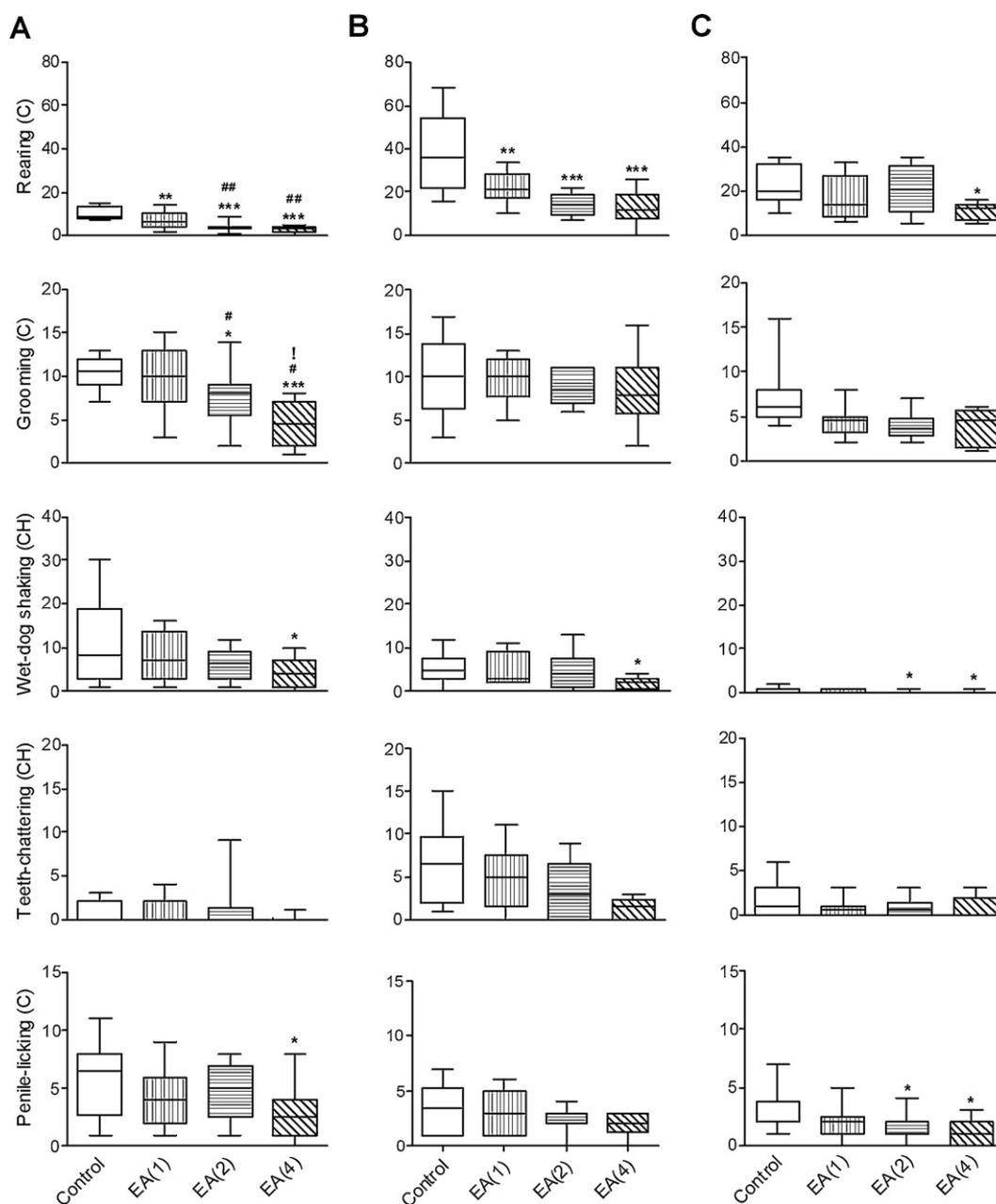


Fig. 2. Effect of 100 Hz EA on the withdrawal score in morphine-treated rats. A. 24 h after the last morphine injection ($n = 13-15$) [rearing: $F(3, 43) = 18.69, p < 0.001$; grooming: $F(3, 51) = 12.61, p < 0.001$; wet-dog shakes: $F(3, 54) = 4.024, p < 0.05$; and penile licking: $F(3, 52) = 3.727, p < 0.05$]. (B) 60 h after the last morphine injection ($n = 10-13$) [rearing: $F(3, 46) = 13.36, p < 0.001$; wet-dog shakes: $F(3, 45) = 3.013, p < 0.05$; and teeth-chattering: $F(3, 49) = 5.912, p < 0.01$]. (C) 7 d after the last morphine injection ($n = 9-12$) [rearing: $F(3, 36) = 3.24, p < 0.05$; wet-dog shakes: $F(3, 39) = 3.579, p < 0.05$; and penile licking: $F(3, 39) = 3.224, p < 0.05$]. Data were expressed as median values and analyzed by the non-parameter ANOVA followed by Newman-keulz post-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, versus NS group. # $p < 0.05$, ## $p < 0.01$, versus EA (1) group. ! $p < 0.05$, versus EA (2) group.

control group in the spinal cord and PAG, but not the hypothalamus. Four times EA decreased the p-CREB significantly in the spinal cord and PAG.

3.4. The effect of intra-PAG injection of Rp-cAMP on the PPD mRNA level of the PAG

To verify dynorphin was one of the downstream molecules of CREB, we injected Rp-cAMPS into PAG which was a highly specific PKA inhibitor and had been shown to inhibit the phosphorylation of CREB [38–49]. Result showed that Rp-cAMPS increased PPD mRNA 6 h after intra-PAG Rp-cAMPS injections (Fig. 6).

4. Discussion

4.1. How frequent should 100 Hz EA be used for detoxification of morphine dependence

In morphine dependent rats, abstinence of morphine produced marked withdrawal syndrome. 100 Hz EA was administered 30 min per session, for a total of 1, 2 and 4 sessions. One session of EA for 30 min produced only a marginal therapeutic effect, shown as a moderate reduction of the rearing. Two sessions of EA separated by 11.5 h showed a more marked therapeutic effect as shown in a significant reduction of both rearing and grooming, suggesting that the after-effect of the first session can remain for 11.5 h to add

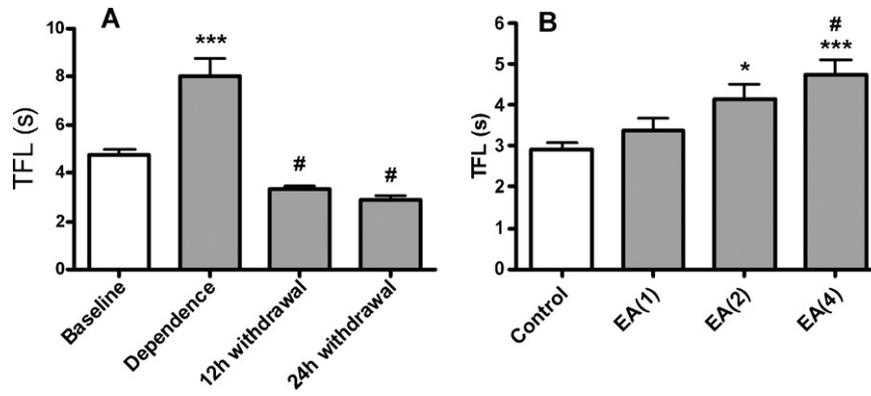


Fig. 3. Changes of tail flick latency (A) and the effect of EA analgesia [$F(3, 65) = 31.74, p < 0.001$] (B) in rats subject to morphine dependence and withdrawal [$F(3, 62) = 7.051, p < 0.001$]. Data were analyzed with ANOVA followed by Bonferroni post-test. $p < 0.05$, $*** p < 0.001$, versus NS group. # $p < 0.05$, versus EA (1) group.

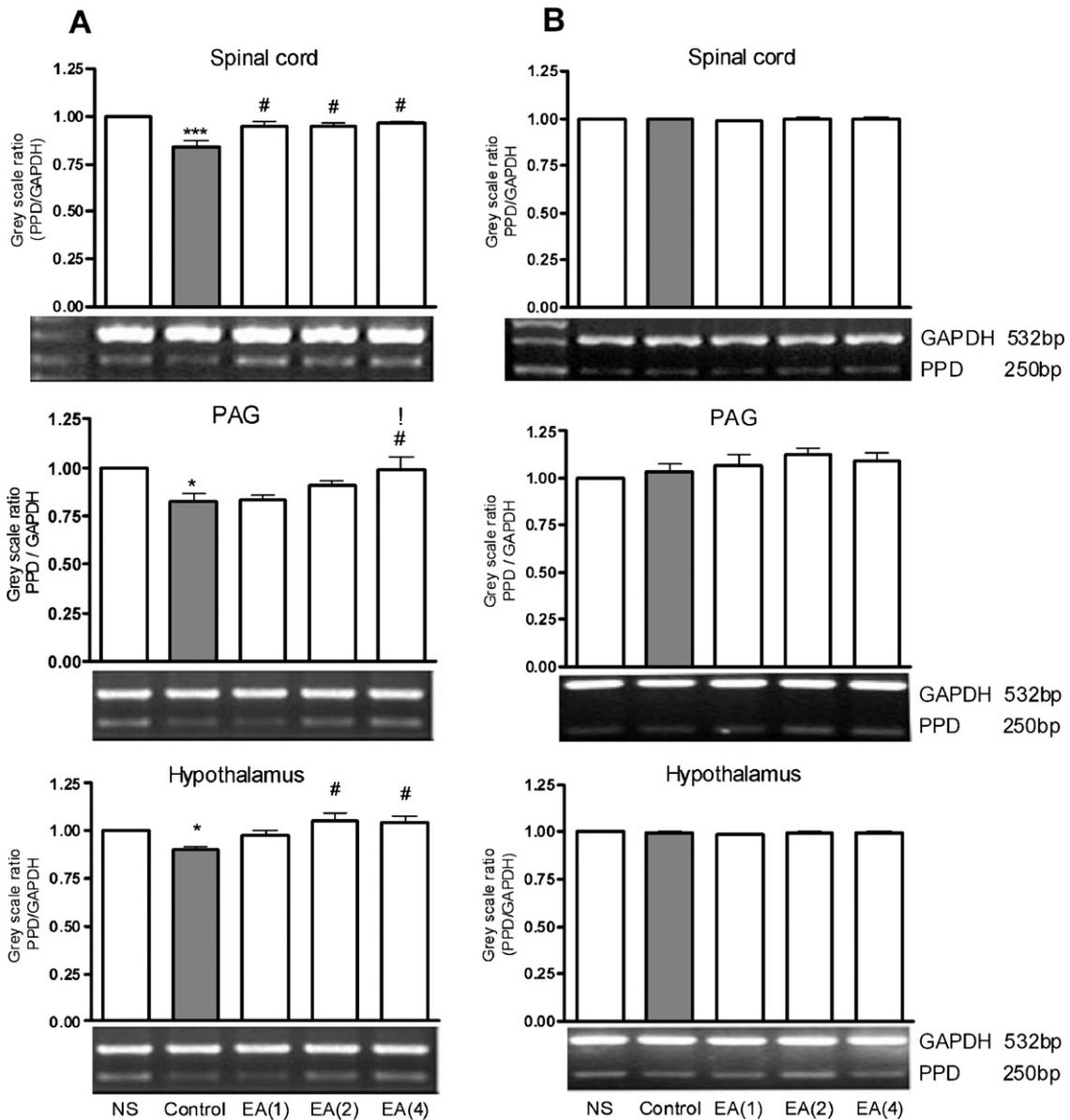


Fig. 4. Effect of 100 Hz EA on the level of PPD mRNA (A and B) and PPE mRNA (C and D) in spinal cord, PAG and hypothalamus 60 h (A and C) and 7 d (B and D) after the last morphine injection ($n = 4-5$). NS: saline treated group; Control: morphine treated but no EA group; EA (1): morphine treated + single EA group; EA (2): morphine treated + twice EA group; EA (4): morphine treated + quartic EA group. One way ANOVA followed by Bonferroni post-test [spinal cord: $F(4, 15) = 10.16, p < 0.01$; PAG: $F(4, 15) = 5.965, p < 0.01$; hypothalamus: $F(4, 18) = 4.027, p < 0.05$]. $p < 0.05$, $*** p < 0.001$, versus NS group; $^{\#} p < 0.05$, versus control group; $! p < 0.05$, versus EA(1) group.

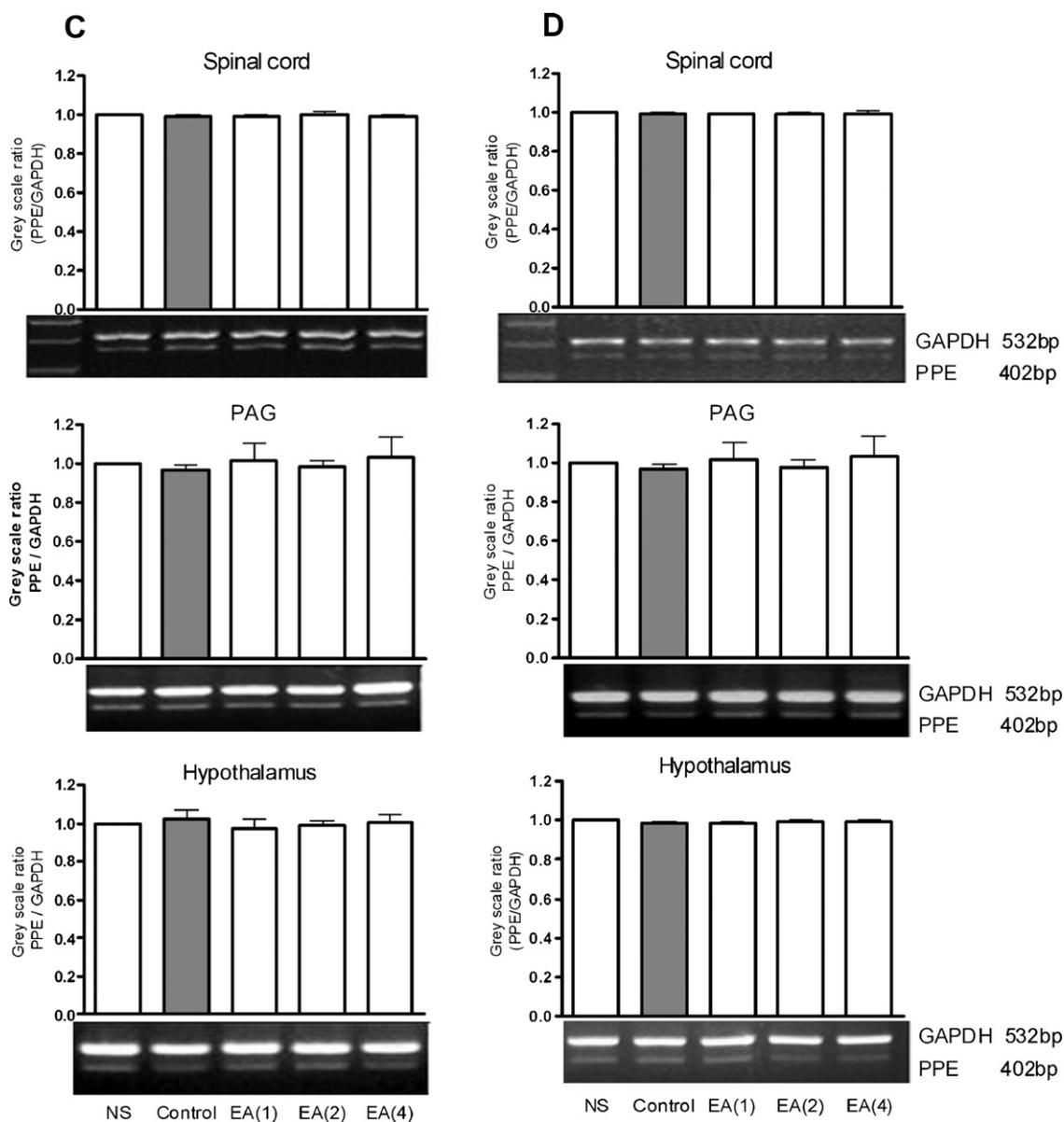


Fig. 4. (Continued).

up with that of the second session. A most significant effect was observed in the 4-session group, where 4 of the 5 withdrawal signs were significantly reduced. The results suggest that the cumulative effect produced by 4 sessions of EA were significantly better than those of one or two sessions.

It was also interesting to ask how long the effect of EA treatment(s) would last. Since all EA treatments were administered within 24 h of morphine abstinence, we tested the after effect 60 h (2.5 days) and 7 days after the drug abstinence. Results shown in Fig. 2B suggested that the therapeutic effect of EA on spontaneous withdrawal syndrome can last for at least 48 h. After that, the spontaneous withdrawal signs gradually faded and became hard to test. In order to assess the effect of EA longer than 2–3 days, one has to use naloxone to precipitate withdrawal syndrome. Fig. 2C shows that even in the 7th day, the therapeutic effect produced by 4 sessions of EA was still evident in 3 of the 5 indices. So the conclusion is that the therapeutic effects of 4 sessions of EA administered 12–24 h after drug abstinence can last for 2–7 days.

Pain and drug abuse are closely related phenomena. During the period of morphine abstinence, we also measured the changes of tail flick latency (TFL). The TFL remained high ($TFL = 8.20 \pm 3.18$ s

2–3 h after the morphine abstinence (Fig. 3A), representing the after-effect of the analgesic effect of the last injection of morphine. Twelve and 24 h after the morphine abstinence, the TFL decreased to 3.37 ± 0.63 s and 2.9 ± 0.76 s respectively (Fig. 3A), which were significantly lower than the baseline level, representing a sensitization of the CNS.

Twenty-four hours after the morphine abstinence, all 4 groups of rats were given a session of EA, and the change of TFL was monitored. Fig. 3B shows that the effect of EA analgesia was highest in the 4 times EA group, and least in the single EA group. The increase of analgesic effect induced by multiple EA treatments (Fig. 3B) seems to well match with the decrease of withdrawal syndrome (Fig. 2). In other words, for the best result of detoxification, EA should be used as many as 4 times a day in the first day of abstinence, without the worry of development of tolerance.

4.2. A possible link to opioid mechanism

Morphine is known to have high affinity for mu opioid receptor, and moderate affinity for kappa and delta receptor. However, a high and repeated dose of morphine may not only saturate the

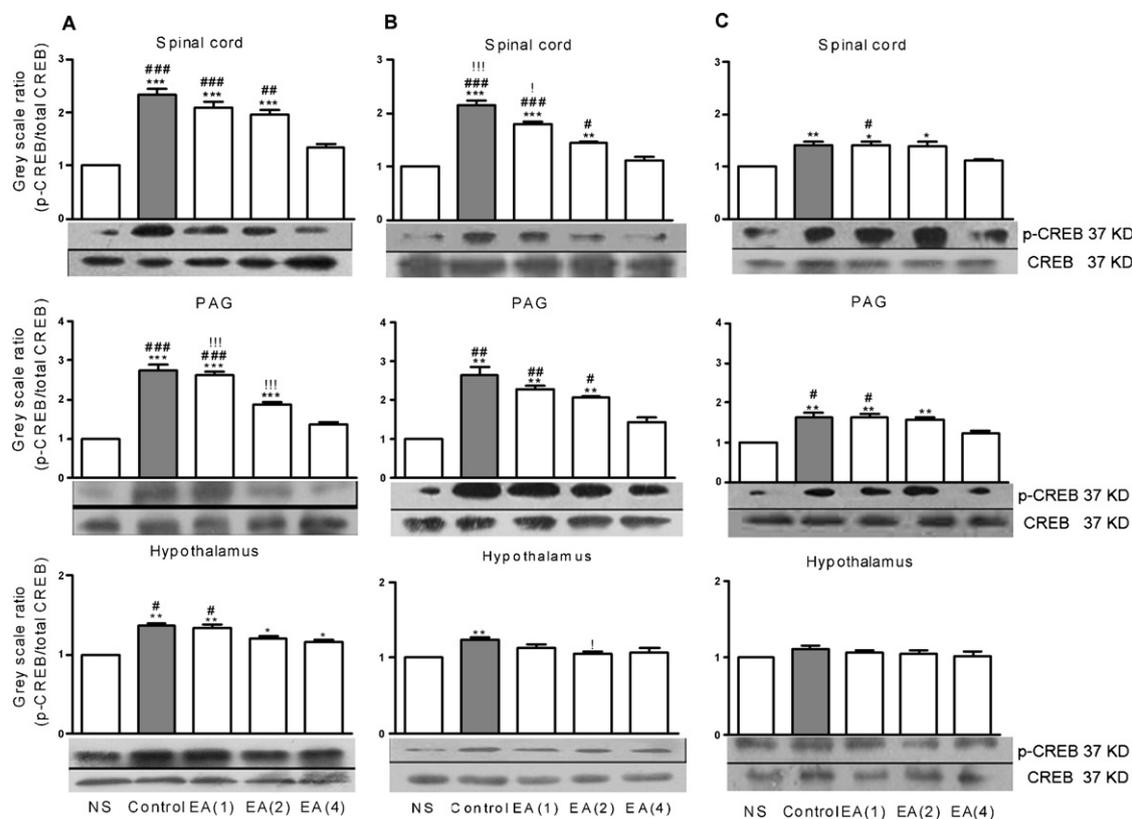


Fig. 5. Effects of multiple 100 Hz EA on the expression of p-CREB in the spinal cord, PAG and hypothalamus 24 h (A), 60 h (B) and 7 d (C) after the last injection. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, versus NS group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, versus EA (4) group. † $p < 0.05$, versus EA(2) group. One way ANOVA followed by Bonferroni post-test.

mu receptor, but also strongly activate the delta and kappa opioid receptor. Hypothetically it may down-regulate the biosynthesis of all endogenous opioid peptides, including endorphin, enkephalin and dynorphin. However, our results showed that a down regulation was observed only in the expression of PPD mRNA (Fig. 4A and B) but not PPE mRNA (Fig. 4C and D). This effect was still evident 2–3 days after the drug abstinence and recovered automatically in 7 days. The decreased expression of dynorphin may be implicated in the development of withdrawal syndrome after morphine abstinence.

Previous studies have shown that 100 Hz EA can accelerate the release of dynorphin in the spinal cord, and increase the expression of PPD mRNA in the brain [23,47]. In the present study, we found that 100 Hz EA can resume the content of the PPD mRNA back to the normal level. The reactivity of the 3 central regions toward EA stimulation was different. For the spinal cord, only one session of EA was enough to bring the suppressed PPD mRNA to the normal level. The hypothalamus and the PAG needed 2 and 4 sessions, respectively (Fig. 4A and B). The potential increase of biosynthesis of dynorphin in the brain and spinal cord may play a key role in the suppression of withdrawal syndrome in morphine abstinence. Several forms of dynorphin such as dynorphin A (1–13), D-ala2-dyn (1–11), dynorphin A (2–17) have been shown to suppress morphine withdrawal of mouse, rat and monkey [1,21,37,45] or heroin withdrawal of human [46].

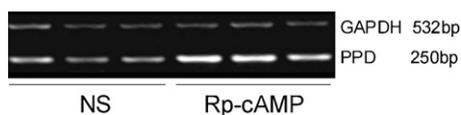


Fig. 6. PPD mRNA level in PAG increased 6 h after intra-PAG Rp-cAMP administration.

It should be mentioned that the efficacy of 100 Hz EA in accelerating PPD mRNA expression seems to depend on the content of the end product. When there is a deficiency of dynorphin supply, 100 Hz EA is very efficient in accelerating the biosynthesis of dynorphin as reflected by the prompt up-regulation of PPD mRNA (Fig. 4A). When the dynorphin supply is in a normal equilibrium status, administration of 100 Hz EA, even 4 times in 12 h, would no longer accelerate the biosynthesis of dynorphin (Fig. 4B).

Previous studies have worked out the time course whereby EA modulated the expression of PPE mRNA in spinal cord and medulla. The PPE mRNA began to increase 4 h after EA, peaked at 48 h, and started to decrease at 72 h [13]. Guo et al. [22] also found that, brain PPD- and PPE-mRNA expression increased 24 h after EA. Based on these findings we dissected the rat brain 48 h after the first EA, namely, 60 h after the last injection of morphine.

It has been reported that during morphine withdrawal, there is an increase of PPE mRNA in the caudal PAG, but not in the rostral PAG [18]. Our results did not show significant difference of PPE mRNA in PAG of rats 60 h after morphine withdrawal, as compared to the saline control rats. This might be related to the time point of tissue sampling and the method of mRNA measurement. In fact, our previous studies have repeatedly shown that the effect of EA in facilitating the biosynthesis of opioid peptide in CNS is frequency specific [23]. 100 Hz EA mainly accelerated the production and release of dynorphin, and only mildly accelerated enkephalin mRNA expression in specific nucleus [22].

Administration of dynorphin (1–17) into PAG has been shown to produce significant antinociception [10]. Kappa-opioid receptor and NMDA receptor might be the target molecules of dynorphin released by multiple 100 Hz EA to analgesia and suppressing withdrawal [28,31,37,41]. In the hypothalamus, dynorphin acted in a retrograde manner to inhibit excitatory synaptic transmission [27]. Whether increased expression of dynorphin would act through this

mechanism to suppress withdrawal syndrome needs to be clarified.

4.3. An impact of CREB phosphorylation

Repeated opiate exposure elicits an increase in activity of cAMP-PKA pathway and CREB phosphorylation, leading to the activation of gene transcription in some specific nucleus and spinal cord. This pathway remained at a high level during drug abstinence, and exhibited very high site specificity. For example, locus coeruleus, PAG and spinal cord were related to the withdrawal syndrome of physical dependence, and striatum was implicated in reward and craving of psychiatric dependence [7]. Morphine treatment and its withdrawal increased the level of p-CREB in the hypothalamus [15,33]. The significant increase of p-CREB in spinal cord, PAG and hypothalamus after morphine abstinence and its decrease by multiple 100 Hz EA observed in the present study (Fig. 5) suggested that p-CREB might be an important transcription modulator implicated in the detoxification effect of EA. The results shown in Fig. 6 indicate that intra-PAG injection of Rp-cAMPS, which has been proved to decrease p-CREB [39,40,49], increased the expression of PPD. This may be one of the pathways for EA modulation of PPD level.

The effect of p-CREB, as a transcription factor, is not limited to the modulation of dynorphin and enkephalin. Many molecules such as AC1, AC8 and BDNF transcription are all regulated by CREB [17,29]. AC1 and AC8 are two isoforms of AC. Recent work demonstrated that deletion of AC1 or AC8 gene dramatically decreased the withdrawal syndrome such as wet-dog shakes, paw tremor, diarrhea and ptosis [48]. BDNF-deficient mice showed a 3 fold reduction in morphine withdrawal symptoms [2]. It is likely that multiple 100 Hz EA decreased CREB phosphorylation and inhibited AC1, AC8 and BDNF subsequently to suppress withdrawal syndrome. Additional work is needed to study the role of these and other genes as downstream targets of p-CREB that mediate the effects of 100 Hz EA.

In summary, our present study indicated that rats received multiple EA during 12–24 h abstinent from morphine exhibited less withdrawal syndrome, and the effect still can be observed even 7 days after abstinence. Dynorphin synthesis increase and CREB phosphorylation decrease in spinal cord, PAG and hypothalamus may account for the accumulated effects of multiple 100 Hz EA in early phase of morphine withdrawal, and the latter may also be implicated the long-term effects of multiple EA. Despite the fact that a line of pharmacological interventions are available to alleviate opiate withdrawal symptoms including methadone [3], buprenorphine [19], clonidine [20] and Chinese traditional herbal medicine [35], etc., acupuncture and related techniques (including EA) have been used as effective complementary approaches to remedy drug addiction [12]. It may help to provide an effective procedure of using EA for clinical detoxification.

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