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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.

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 posterial to the tibia). Square waves generated from a Han's Acupoint Nerve Stimulator (HANS, manufactured at the Beijing University of Aviation and Astronomics) 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 ± 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 μl) by PE20 tubing filled with Rp-cAMPS (Sigma) or saline solution. The dose infused bilaterally was 40 nmol/0.5 μl 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 μg RNA in a 25 μl reaction including 200 U M-MLV reverse transcriptase (Invitrogen), 0.5 mM dNTPs, 30 U RNase inhibitor, and 0.5 μg oligodT_{12-18} primers. Duplicate aliquots of cDNA (2 μl) were used to amplify the fragments by PCR with 1 U Taq DNA polymerase (Invitrogen), 0.2 mM each of dNTPs and 1 μM of each primer in a 20 μl 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 ethidiu 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 × *g* for 5 min. The precipitation was resuspended in 500 μl membrane extraction reagent (NER) and centrifuged at 4 °C, 4000 × *g* for 5 min. The supernatant was discarded and the precipitation was washed in 500 μl 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 μg of protein was loaded in 10% polyacry-