The effect of electroacupuncture on extinction responding of heroin-seeking behavior and FosB expression in the nucleus accumbens core

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HIGHLIGHTS
- Electroacupuncture facilitated the extinction responding of heroin seeking.
- Electroacupuncture did not alter locomotor activity in drug-naive rats.
- Electroacupuncture attenuated FosB expression in the nucleus accumbens core.
- Acupuncture with extinction therapy may be a novel strategy for drug relapse.

ABSTRACT
Augmentation of extinction with learning enhancing therapy may offer an effective strategy to combat heroin relapse. Our lab previously found that electroacupuncture (EA) not only significantly reduced cue-induced reinstatement of heroin seeking but also exhibited a promoting effect on the ability of learning and memory. In the present study, we further investigated the effects of EA on the extinction of heroin-seeking behavior in rats with a history of intravenous heroin self-administration. We trained Sprague–Dawley rats to nose-poke for i.v. heroin either daily for 4 h or 25 infusions for 14 consecutive days; then the rats underwent 7 daily 3 h extinction sessions in the operant chamber. To assess EA’s effects on the extinction response of heroin-associated cues, 2 Hz EA was administered 1 h before each of the 7 extinction sessions. We also applied immunohistochemistry to detect FosB-positive nuclei in the nucleus accumbens core. We found that EA treatment facilitated the extinction response of heroin seeking but did not alter the locomotor activity in an open field testing environment. EA stimulation attenuated the FosB expression in the core of the nucleus accumbens, a brain region involved in the learning and execution of motor responses. Altogether, these results suggest that EA may provide a novel nonpharmacological approach to enhance extinction learning when combined with extinction therapy for the treatment of heroin addiction.

Keywords: Electroacupuncture Extinction Heroin Nucleus accumbens core

1. Introduction
Relapse to drug-seeking and drug-taking behaviors is a common manifestation of heroin addiction and perhaps the most serious impediment to effective treatment. One factor that contributes to drug-seeking behavior is the presence of cues and contexts that it previously has associated with past drug use. One of the promising strategies is the extinction therapy targeting the conditioned drug cues. Extinction is an active process where an organism learns that a stimulus is no longer predictive of a reward, and therefore, future presentations of the stimulus no longer produce behaviors to seek that reward. Extinction does not cause “forgetting” of the original stimulus–reward relation [1], but promotes new learning that counters the motivating impact of drug-related cues [7]. However, clinical studies using extinction therapy have reported little success, possibly reflecting the context-dependent nature of extinction therapy [4] as well as the drug-induced dysfunction of memory systems critical for effective extinction learning and consolidation [17]. Therefore, the development of therapy to augment extinction therapy for substance addiction is of high priority.

Recently, acupuncture or electroacupuncture (EA) has been applied successfully to attenuate various conditions related to drug addiction. Morphine-induced conditioned place preference (CPP) can be successfully suppressed by 2 Hz EA [3]. Acupuncture or EA...
can prevent the reinstatement to drug seeking elicited by foot shock stress or conditioned drug cue in the self-administration model of reinstatement [23,13]. Interestingly, several lines of evidence have suggested the beneficial effects of EA in treating senile patients with memory deficit and patients in the early stage of Alzheimer’s disease [19]. In animal model, EA also exhibited a promoting, rather than a deteriorating, effect on the ability of spatial memory [3]. These findings have led to the hypothesis that EA can enhance extinction learning of heroin-associated cues to reduce cue-induced reinstatement, bearing significance in clinical practice that the combination of EA with extinction training may provide an innovative method to reduce cue-induced relapse in addicts.

The goals of our present study were to evaluate the effect of EA on extinction response of heroin seeking. Given that EA can suppress cue-evoked heroin seeking and c-fos expression in the nucleus accumbens (NACc) core [13], we also determined the FosB expression in the NACc core after the extinction session and EA administration. In contrast to c-Fos proteins, FosB is induced in response to acute drug administration; however because of its unique stability, after repeated drug administration, FosB gradually accumulates in the striatum and stays elevated for weeks or months after discontinuation of drug exposure [9,2]. Overexpression of FosB protein in the NACc has been shown to increase cocaine-seeking behaviors [24,10]. We hypothesized that the effects of EA on extinction response of heroin seeking were mediated by suppressing FosB in the NACc core.

2. Materials and methods

2.1. Self-administration procedure

Male Sprague–Dawley rats (250–300 g) from the Zhejiang Center of Experimental Animals were used. All animal treatments were performed in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The animals were catheterized in the right jugular veins under sodium pentobarbital (50 mg/kg, i.p.) anesthesia. Seven days after surgery, behavioral training started as previously described [13]. In brief, the rats were trained to self-administer heroin in operant chambers equipped with two nose-pokes under an escalating fixed ratio schedule for 4 h daily. Each trial began with illumination of a green light inside the active nose-poke hole. Responding in the active hole resulted in an infusion of heroin (0.05 mg/kg) delivered by an infusion pump. The green nose-poke light was turned off during heroin infusions. A 30 s inter-trial interval (time out) followed and then another trial began. Responding in the inactive hole had no consequences. The response requirements started with one and increased one after each five-heroin infusion. Each earned heroin infusion was also paired with a 5 s cue-light that served as the CS. Training sessions were conducted daily for 14 consecutive days, and sessions ended after 4 h or 25 heroin infusions, whichever occurred first.

2.2. Extinction procedures

Extinction training was conducted in 3 h daily sessions in the presence of drug-associated cues (i.e., presentation of the light/tone stimulus complex for 2 s following each active nose poke, followed by a 20 s timeout), since it has been observed that such procedures produce drug-seeking behavior that is more resistant to extinction than that observed during extinction in the absence of drug-associated cues [18]. No drug solution was infused during extinction sessions, and pressing the inactive pores during extinction had no programmed consequences. One hour before each extinction training session, animals were administered EA or restraint according to their group assignment and returned to their home cages. All rats underwent 7 daily extinction sessions.

2.3. EA stimulation

Rats were kept in special holders, with their hind legs and tails exposed. Two stainless steel needles of 0.3 mm diameter were inserted into each hind leg in the acupoints ST36 (5 mm lateral to the anterior tubercle of the tibia) and SP6 (3 mm proximal to the superior border of the medial malleolus at the posterior border of the tibia) at a depth of 3 mm. Constant current squarewave electric stimulation produced by an EA apparatus (Model G–6805-2, Shanghai Medical Electronic Apparatus, China) was administered via the two needles. The frequency of stimulation used was 2 Hz. The intensity of the stimulation was increased stepwise from 0.5 to 1.5 mA, with each step lasting for 15 min. This EA paradigm was successfully used to suppress cue-evoked heroin-seeking and morphine-induced CPP [13,3]. According to the theory of traditional Chinese medicine, ST36 belongs to stomach meridian, and SP6 belongs to spleen meridian. It has been shown that EA stimulation at these two points induces analgesia [6], controls gastrointestinal peristalsis [5], and suppresses addictive drug’s withdrawal and craving [13,3,21]. Sham EA was preformed using same parameters via non-acupoints. For sham EA, needle was placed into non-acupoints (1/5 tail length from the proximal region of the tail). The other needle (anode) was inserted into the anterior tibial muscle 3 mm distal to ST36. Anodal and cathodal leads from an electrical stimulator were connected to the two needles.

2.4. Locomotor activity assessment

In a control experiment, drug-naive rats were administered daily EA (n = 6), sham EA (n = 6) or restraint (n = 6) for 7 days. On day 7, the rats were placed into a photocell apparatus for 2 h to measure the locomotor response to a novel open field environment 1 h after EA or restraint. The locomotor activity assessment apparatus consisted of a 30 cm × 30 cm open field testing arena equipped with infrared photobeams that measured horizontal locomotor activity. Each open field testing arena was located in a sound-attenuating cubicle equipped with a house light and fan to mask external noise and odors, and was interfaced to a PC computer. Distance traveled (in cm) was recorded by the computer. The same experimental procedures were used for the control rats (no EA and no Restraint, n = 6).

2.5. FosB immunohistochemistry

Four rats from each group (except sham EA group) were randomly selected for FosB immunohistochemistry. Two hours after the last extinction training, rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and transcardially perfused with ice-cold 0.9% saline followed by ice-cold 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4. The brains were removed and stored overnight in 4% paraformaldehyde and then stored in 30% sucrose at 4 °C for 3–5 days. Coronal sections (40 μm thickness; 1.6 mm from bregma according to the atlas of Paxinos and Watson [16]) were cut on the cryostat at −25 °C. Sections were rinsed in 0.01 M phosphate-buffered saline (PBS) and incubated in PBS containing 5% normal goat serum and 0.3% Triton X-100 for 30 min and then in FosB antibody (sc-48; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted at 1:500 in PBS at 4 °C for 48 h. Sections were rinsed three times in PBS and then incubated for 2 h with the secondary antibody (biotinylated goat anti-rabbit 1:200, Sigma, USA). After three rinses in PBS, all corresponding sections were placed in the avidin-biotin–peroxidase complex solution for 60 min. Finally, DAB was used for visualization of
FosB immunoreactivity. The reaction was stopped by several PBS washes. Sections were then mounted on gelatin-coated slides, air-dried, dehydrated through graded alcohols, cleared in xylene, and coverslipped with Eukitt.

Sections were scanned using an Olympus BX51 microscope with Nomarski DIC optics. Images were captured using a Spot Insight QE digital camera and Image-Pro Plus software under identical, calibrated exposure conditions. Image analysis was carried out with the aid of an image analysis system (40× magnifications). Three consecutive sections were taken from each animal, and the FosB-positive nuclei were counted bilaterally, based on a randomization procedure. A computer-generated rectangle (250 μm × 600 μm) was placed in a fixed area of the NAcc core of each section, and the analysis software counted stained nuclei within the area.

2.6. Statistical analysis

Experimental data were expressed as mean ± SEM. Effects of EA on the number of active responses during extinction sessions were analyzed by a two-way repeated measures analysis of variance (ANOVA) with treatment group and session as factors. The FosB protein expression and the locomotor activity were analyzed using one-way ANOVA. When significance was found using ANOVA procedures, post hoc analyses were conducted using Fisher LSD test. 

p < 0.05 was considered statistically significant.

3. Results

3.1. Effect of EA on extinction responding of heroin seeking

All heroin-trained rats demonstrated reliable heroin self-administration, as indicated by the increase in active responses and reached stable levels of active responses within 14 days of heroin self-administration training. The heroin-trained rats were divided randomly into five groups: (1) extinction (EXT, n = 8); (2) extinction and EA (EXT + EA, n = 8); (3) extinction and sham EA (EXT + Sham EA, n = 8); (4) extinction and restraint (EXT + Restraint, n = 8), the animals were kept in the special holders, without needle insertion and electrical stimulation; and (5) no extinction (No EXT, n = 6). The rats were abstinent from heroin for 7 days in their home cages, without restraint and EA stimulation. As shown in Fig. 1, there were no differences between groups in total active responses calculated as an average of the last 2 days of self-administration. Only minimal responding was observed at the inactive nose-poke during self-administration training and extinction sessions (data not shown). Analysis of the effects of administration of EA 1 h prior to daily extinction on active responses during 7 daily extinction sessions revealed a significant effect of treatment group (F(3, 28) = 15.9, p < 0.001), session (F(6, 168) = 19.7, p < 0.001) as well as a treatment group × session interaction (F(18, 168) = 17.1, p < 0.001). Post hoc analyses revealed that animals treated with EA emitted significantly fewer active responses during 1, 2, 4, 5, 6 and 7 extinction sessions as compared with Sham EA, EXT and EXT + Restraint groups. The first day of extinction was analyzed in hourly intervals, and the primary inhibitory effect of EA on extinction active responses was during the first hour (Fig. 1B). Total active responses in No EXT rats were higher after 7 days of abstinence, but this effect did not achieve statistical significance (p > 0.05).

3.2. Effect of EA on the locomotor activity in an open-field environment

Open-field locomotor activity was assessed in a separate set of drug-naive animals (see Fig. 2). A significant effect of EA was not observed in the locomotor response to an open field environment (F(3, 20) = 2.37, p > 0.05), indicating a lack of motor impairing effects of EA treatment. Although there was a trend for restraint stressor to increase the distance traveled (in cm) in open field environment, this effect did not achieve statistical significance (p > 0.05).

3.3. Effect of EA on the FosB protein expression in the NAcc core

Coronal sections of the brain were obtained and FosB-positive nuclei were imaged in the NAcc core of the different groups (Fig. 3).

Fig. 1. The effect of electroacupuncture (EA) on extinction responding following heroin self-administration. Data were expressed with mean ± S.E.M. (A) EA prior to extinction training sessions reduces extinction responding (active responses that previously resulted in heroin infusions); *p < 0.05 vs. EXT and EXT + Restraint animals on the same day of extinction (extinction day 1, etc.). (B) Data were analyzed in hourly intervals indicating that the effect of EA on day 1 extinction responding occurred primarily in the first hour. SA represents the average number of active responses emitted during the last 2 days of heroin self-administration.
Fig. 2. Distance counts showing that EA did not alter behavioral responding in a novel open field environment compared with control group and Restraint group. (A) Distance traveled in 5 min intervals during 2 h locomotor activity. (B) Total distance traveled during 2 h locomotor activity.

Fig. 3. The effects of EA on the expression of FosB in the NACc core following extinction training. (A) Quantitative analysis of FosB-positive nuclei in the NACc core. Data are expressed as mean ± SEM. *p < 0.05; *p < 0.01 versus EXT group and EXT+Restraint group. (B) Representative coronal sections showing FosB immunoreactivity in the NACc core. Scale bar, 200 μm.

As illustrated in Fig. 3, ANOVA demonstrated significant differences among treatment groups in the number of FosB-positive nuclei in the NACc core (F(3, 44) = 26.7, p < 0.001). Of note, post hoc comparisons indicated that the EXT group and EXT+Restraint group exhibited a decrease in FosB-positive nuclei in the NACc core, as compared with the No EXT group. EA stimulation further attenuated the FosB expression relative to the EXT group and EXT+Restraint group (Fisher’s LSD test; p < 0.01). No differences in the FosB expression were noted between the EXT group and EXT+Restraint group.

4. Discussion

A number of studies have shown that EA or acupuncture can reduce drug self-administration and the reinstatement to drug seeking [23,13,22]. Our results further indicated that 2 Hz EA also facilitates the extinction of drug seeking evoked by drug-associated cues. The findings suggest that EA combined with extinction therapy may constitute a rational strategy for the clinical management of heroin relapse. Extinction is a process of new and active learning. Given that EA can significantly improve the learning and memory
capacity [19,5,3], administration of EA combined with extinction training may promote new learning that countered the impact of the previously conditioned heroin cue rather than the reinforcing effect of heroin per se. This was also supported by our control experiment, wherein we found that EA did not alter the locomotor activity in a novel open-field testing environment, suggesting that reductions in extinction response by EA are not likely because of nonspecific decreases in the locomotor activity. These data indicate that EA may provide a novel nonpharmacological approach to enhancing extinction learning when combined with extinction therapy for the treatment of heroin addiction. Notably, given the fact that the inhibitory effect of EA commences during the first hour of extinction training, pointing to a potential nonspecific effect of EA, further characterization is necessary to discern the behavioral mechanisms of the apparent facilitation of extinction learning.

It is notable in our present study that EA did not affect the locomotor activity in drug-naive rats, indicating that EA by itself did not produce any significant increase or decrease in behavioral activity. However, several lines of evidence demonstrate that acupuncture or EA significantly decreased the locomotor activity in cocaine, morphine and amphetamine dependent rats [11,21,12]. The discrepancy between these studies and the present one might be explained by differences in animal model and experimental protocols. Repeated exposure to psychostimulants can produce behavioral sensitization, as evidenced by an enhanced locomotor response that occurs with repeated exposure to such psychostimulants. Acupuncture or EA might inhibit increased the locomotor responses induced by the repeated psychostimulants challenge, rather than the locomotor activity itself. In fact, numerous examples reveal that the regulatory action of acupuncture is bidirectional. Its therapeutic actions are achieved by normalizing metabolism or pathogenic changes toward homeostasis. The specific direction of the acupuncture effect may depend on physical condition of subjects and appropriate selection of certain acupuncture points and variation in technique.

In the present study, we found that EA stimulation attenuated FosB expression in the NACC core. The FosB primary antibody binds to both ∆FosB and the FosB protein. As there are no antibodies available to distinguish between FosB and the different isoforms of the truncated FosB protein (∆FosB), we cannot definitely say which form of the protein we are measuring. However, previous research has indicated that acute doses of cocaine reportedly no longer elevate FosB levels but instead lead to elevations in ∆FosB after chronic exposure to cocaine [14,8]. Because of the prolonged period of the experiments (14-day self-administration training and 7-day extinction sessions) and the shorter half-life of the FosB protein, it can be assumed that we are primarily measuring ∆FosB and not FosB especially. ∆FosB expression is known to acutely and cumulatively increase within a subset of brain regions, particularly NACC, after exposure to many classes of abused drugs (reviewed by Nestler et al. [15]). The increased expression of ∆FosB in the NACC after drug exposure has been shown to increase cocaine-seeking behaviors [24,10]. The NACC can be further divided into anatomically distinct core and shell subregions. The NACC core has been proposed to be a critical site for acquisition of goal-directed instrumental learning [20]. Infusions of ∆FosB restricted to the NACC core increased cocaine-reinforced instrumental learning [15]. Extinction involves new instrumental learning processes. Our current findings that EA stimulation attenuated the FosB expression in the core of the NACC seem to suggest the effects of EA on extinction learning of heroin-associated cues. However, we did not measure the FosB expression in the NACC shell using the same groups of rats. Further studies must be performed to clarify this issue.

In conclusion, EA treatment facilitated the extinction of drug seeking following heroin self-administration and attenuated the FosB expression in the core of the nucleus accumbens. The present data provide a strong preclinical indication that EA with extinction therapy may provide a novel nonpharmacological strategy for drug relapse. This is supported not only by the inhibition of drug-seeking induced by drug-associated cues but also by the facilitation of extinction training.

Conflict of interest

The authors do not have any conflicts of interest or any circumstances that could be perceived as a potential conflict of interest.

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