2Hz-Electroacupuncture Attenuates Heroin-seeking Behaviors via Adjusts CB1-Rs and CB2-Rs Expression in Relapse-relevant Brain Regions of Heroin Self-administration Rats

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Short Title: 2Hz EA Attenuates Heroin-seeking via Adjusts CB1and CB2 Expression

Summary

Opiate addiction has a high rate of relapse. The accumulating evidence shows that electroacupuncture (EA) may be effective for the treatment of opiate relapse. However, the change of expression of CB1-Rs and CB2-Rs involve in 2Hz EA anti-relapse pathway is still unclear. To explore the changes of expression of CB1-Rs and CB2-Rs, heroin self-administration (SA) model rats were adopted and treated using 2Hz EA. The expressions of CB1-Rs and CB2-Rs were observed using immunohistochemistry method. The results showed that, compared with the control group, active pokes in the heroin-addicted group increased, while the active pokes decreased significantly in 2Hz EA group compared with heroin-addicted group. Correspondingly, the expression of CB1-Rs in prefrontal cortex (PFC), hippocampus (Hip), nucleus accumbens (NAc) and ventral tegmental area (VTA) all increased significantly while the expression of CB2-Rs in those relapse-relevant brain regions decreased obviously in heroin-addicted group when compared with the control group. In addition, the expression of CB1-Rs obviously decreased in the 2Hz EA group while the expression of CB2-Rs in those relapse-relevant brain regions increased significantly when compared with the heroin-addicted group. It indicated that 2Hz EA could attenuate the heroin-evoked seeking behaviors effectively. The anti-relapse effects of 2Hz EA might be related to the decrease of CB1-Rs and increase of CB2-Rs expression in relapse-relevant brain regions of heroin SA rats.

Keywords: Heroin self-administration, 2Hz-Electroacupuncture, heroin-seeking behaviors, CB1-Rs and CB2-Rs

Introduction

A high rate of relapse in drug abuse (such as heroin) during abstinence is defined as a feature of drug addiction (Dong *et al.,* 2017). The ultimate aim in the treatment of heroin abuse is the abstinence and prevention of heroin relapse (Kosten *et al.,* 2003). So far, the prevention of heroin relapse is still an urgent medical and social problem, especially in China (Tang *et al.,* 2007).

Electroacupuncture (EA) is a modification of this technique which small electrical currents were applied to acupoint though needle and appeared to have more effective effects in many specific clinical and research settings (Zhao *et al.,* 2008). EA-induced analgesic effect is used widely to alleviate pain (Chen *et al.,* 2009; Da Silva *et al.,* 2015; Gelbier *et al.,* 2016; Han *et al.,* 2004). EA were applied with great success to attenuate behavior of morphine withdrawal in animals and addicts since 1972 (Cui *et al.,* 2013; Shen *et al.,* 2014; Tseung *et al.,* 1974).

The endocannabinoid system which consisted of the endogenous cannabinoids and cannabinoid receptors also plays an influential role in the mesolimbic dopamine (DA) system and drug addiction (Sagheddu *et al.,* 2015; Wills *et al.,* 2016). Recent studies reported that the discovery and functional presence of CB1-Rs (Mitrirattanakul *et al.,* 2007) and CB2-Rs (Onaivi *et al.,* 2006; Onaivi *et al.,* 2011) in mammalian brain might be crucial in depression and drug abuse. The mesolimbic dopamine system is thought to play a major role in the reinforcing effects of drugs abuse, especially in prefrontal cortex (PFC), hippocampus (Hip), nucleus accumbens (NAc) and ventral tegmental area (VTA) (Koob *et al.,* 1992; Nestler *et al.,* 2004).

Some studies reported that the up-regulation of hippocampal CB1-Rs may contribute to the increasing alcohol consumption (Mitrirattanakul et al., 2007) and the brain cannabinoid CB2-Rs modulated cocaine's addictions in mice probably by a dopamine-dependent pathway (Morales *et al.*, 2012; Xi *et al.*, 2011).

Based on the studies above and our previous finding that 2Hz-EA could significantly increase the anandamide level in inflammatory pain tissues (Chen *et al.*, 2009) and up-regulate the local CB2-Rs expression (Zhang *et al.*, 2010), we hypothesized that the 2Hz-EA could effectively inhibit the heroin addiction behaviors via regulating the expression of CB1-Rs and CB2-Rs in relapse-relevant brain regions of heroin SA rats. To explore the change of expression of CB1-Rs and CB2-Rs, we detected the inhibitory effect of 2Hz EA on heroin priming seeking behavior in SA rats through an extinction/ reinstatement protocol. Meanwhile, the expressions of CB1-Rs and CB2-Rs in the relapse-relevant brain regions were assessed with immunohistochemistry method.

Materials and methods

Animals

Male Sprague-Dawley rats, weighing 280–300 g at the beginning of the experiments, were purchased from Vital River Laboratory Animal Technology Co., Ltd, Beijing, China. The animals were maintained under a 12 h reversed light/dark cycle (with darkness starting from 8:00 am) with controlled room temperature and humidity. Tap water was made available ad libitum and food was restricted to 20 g

per day. All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Surgery and experimental apparatus

Procedures of surgery and experimental apparatus followed our previous study (Yue *et al.,* 2012). Briefly, after 1 week of acclimation, a permanent intravenous catheter was surgically implanted and secured to the right jugular vein under deep anesthesia. Following surgery, each rat was housed and was allowed at least 7 days of recovery during which they received a daily intravenous infusion of gentamicin (0.16 mg/kg) and heparinized (1%) sterile saline solution to keep the catheter clean and unobstructed. Heroin SA was carried out in operant chambers which encased in sound- and light-attenuating cubicles and fans that provided ventilation (Anilab Software & Instruments Co., Ltd., China) (Wang *et al.,* 2010). The front panel was armed with two nose-poke operandi (ENV-114M; Med Associates, USA) located 9 cm above the floor of the chambers. A red stimulus light was placed in each nose-poke and a white house light was fixed on the opposite wall. A single active nose poke resulted in a 5 s heroin infusion meanwhile the pump is on. Concurrently, the red cue light in this nose-poke was on for 5 s while the white house light was turned off. A 20 s timeout period was then allowed, during which the red cue light was turned off and further nose pokes had no additional consequence, but were recorded. After the timeout period, the white house light was once again turned on. Inactive nose pokes were also recorded. Each SA session started when the white house light was turned on.

Heroin self-administration training (acquisition, maintenance and reinforcement)

Heroin self-administration training was developed with fixed ratio program. In short, after recovered, rats were allowed to self-administer heroin (30 μg/kg per inf) under an fixed-ratio 1 (FR1) schedule of acquisition and maintenance and to nose-poke for 3 h each day. The acquisition sessions were carried out until stable heroin intake was reached (typically within 12-14 days). Responding was considered stable when animals displayed accurate discrimination between the active and the inactive nose-poke, with the day-to-day difference in the number of active nose pokes of less than 15% for 3 consecutive days. Rats not satisfying the acquisition criterion were excluded from the experiment. The same experimental procedures were used for the rats of the control group (n=8) except the heroin was substituted with the same volume of saline. Twenty-four rats developed a stable pattern of heroin intake. Thereafter, these rats were randomly divided into three groups (n=8 per group), that is heroin-addicted group, sham EA group and 2Hz EA group. The rats of 2Hz EA group were treated with 2Hz EA stimulus (1 mA and 0.1 ms pulse width) for 30 mins on acupoints zusanli (ST36) and sanyinjiao (SP6) 30 min before the experimental sessions according to the methods reported in the existing literature (Han *et al.,* 1993). The same experimental procedures were used for the rats of sham EA group without electrical stimulus. The same experimental procedures were used for the rats of control and heroin-addicted group without 2Hz EA stimulus. The reinforcement effects (numbers of inactive/active nose poke on an FR 1schedule) were recorded at

the last day of the training. Rats were then allowed to training return to their basal rate of responding to heroin before being switched to the subsequent extinction phase.

Extinction and sham/2Hz EA treatment

Those rats were abstinent from heroin for two weeks, during which they stayed in the operant chambers for 2h per day without cue light, house light, sound of pump and heroin infusion. Extinguished behavior, in most cases, took place at around day 10-12. Previous study found that treatment with acupuncture at ST36 or SP6 during the withdrawal period inhibited alcohol withdrawal syndrome of rats undergoing ethanol injection (Kim *et al.,* 2005), so the rats of sham/ 2Hz EA groups continued to be treated by sham or 2Hz EA stimulus (1 mA and 0.1 ms pulse width) for 30 mins on acupoints zusanli (ST36) and sanyinjiao (SP6) for every day before the extinction procedure according to the methods reported in the existing literature (Han et al., 1993) in our present study. The same experimental procedures were used for the rats of control and heroin-addicted group without EA stimulus.

Reinstatement

After extinction, heroin-induced reinstatement was examined. During testing, the rats were reintroduced into the operant cages for 2h training. Before the beginning of the training, the rats were administrated with heroin priming (subcutaneous injection of heroin at 0.25 mg/kg). A single active nose-poke obtained with a programmed consequences but no heroin injection. The numbers of active nose-poke and inactive nose-poke were both recorded.

Locomotor testing

To further characterize the sedative properties of sham EA and 2Hz EA, twenty-four heroin-free rats were tested for the effects of EA on locomotor activity. Rats were tested for their locomotor responses using an automated photocell system (Anilab Software & Instruments Co., Ltd., China) consisting of eight identical black Plexiglas chambers (43×43×35 cm) in light- and sound-controlled cubicles (Cheng *et al.*, 2011). Each chamber was equipped with a video camera on the top, which was interfaced with a computer to record the movement of the rats in the chambers. The locomotor activity of each rat was analyzed by employing an AniLab ver 4.3 analysis software package (Anilab Software & Instruments Co., Ltd., China) (Zhang *et al.*, 2006) and was expressed as the total distance traveled (in millimeters) during a 2-h period that began 30 min after sham EA or 2Hz EA administration.

Immunohistochemically staining

Immediately after behavioral testing, the expression of CB2-Rs in prefrontal cortex (PFC), hippocampus (Hip), nucleus accumbens (NAc) and ventral tegmental area (VTA) were investigated. The rats were systemically perfused with 4% paraformaldehyde during 2-3% isoflurane-induced anesthesia. The brain were then isolated post-fixed overnight in 4% paraformaldehyde in PBS. After paraffin embedding, brain paraffin tissue blocks were cut into 4 μm-thick slices. After dewaxing and rehydration treatment, microwave antigen retrieving and endogenous peroxidase blocking, slices were then incubated with rabbit anti-CB1-Rs (1: 200;

Cayman, USA) and rabbit anti-CB2-Rs (1: 200; Cayman, USA) diluted in PBS for overnight at 4℃ separately. After 3 rinses in PBS, the sections were then incubated with the secondary antibody (HRP conjugated goat anti-rabbit, 1:1000; Abcam, Cambridge, UK) at 37 ℃ for 40 min. After three washes again in PBS, slices were mounted in glycerol and covered. The sections were examined with microscopy (Olympus BX51, Japan) and taking photographs. Optical densities of CB1-Rs and CB2-Rs were counted in a blinded fashion on 12-16 randomly selected brain brain sections from 8 animals per each condition. The results were expressed as relative optical density from these sections. Negative controls in which PBS was used instead of the primary antibody were processed in the same manner.

Statistical analysis

All results were expressed by mean ± S.E.M. statistical significance of results was analyzed with one-way or two-way ANOVA. All statistical analyses were performed by utilizing SPSS for Windows, version 11.5 (SPSS Inc., Chicago, IL, USA). $P< 0.05$ was considered to be statistically significant.

Results

Effects of 2Hz EA on heroin reinforcement

Heroin reinforcement effect refers to the coercive behavior of pursuing heroin without self-restraint which reflects the degree of heroin craving. After a stable pattern of heroin intake was developed in rats, the heroin SA reinforcement effects of these four groups were tested (sham EA or 2Hz EA treatment performed 30 mins

before the experimental session). The effects on heroin SA reinforcement of rats on an FR 1 schedule were shown in Fig. 1. Significant differences ($F_{3, 51}$=28.36, $p < 0.01$) were observed (presented as the number of active nose pokes) among control group and other three heroin-treated groups which indicated the heroin SA model was successfully built. The number of active nose pokes of 2Hz EA group significantly decreased compared with that in the heroin-addicted group ($F_{3, 51}$=32.87, $p < 0.01$). Comparisons also revealed a significant difference between 2Hz EA groups and the sham EA group ($F_{3, 51}$=32.87, $p < 0.01$). No significant differences were observed in the number of inactive nose pokes ($F_{3, 51}$=200.36, $p > 0.05$). These results indicated that 2Hz EA could effectively inhibit the heroin reinforcement.

Effects of 2Hz EA on heroin-induced reinstatement

Our result showed that the effect of 2Hz EA on heroin-induced reinstatement (Fig.2). The number of active nose-pokes significantly increased in heroin treated groups when compared to the control group ($F_{3, 56}$=5.566, $p < 0.01$). And the number of active nose-pokes significantly decreased in 2Hz EA group as compared with heroin-addicted group ($F_{3, 56}$=8.362, $p < 0.01$). There was a significant difference between sham EA group and the heroin-addicted group in the number of active nose pokes ($F_{3, 56}$=8.362, $p < 0.05$). By contrast, no significant differences were observed in the number of inactive nose-pokes during reinstatement group ($F_{3, 56}$=32.1, $p > 0.05$), suggesting that rats retained a good discrimination between the active nose-poke and inactive nose-poke. It was showed that 2Hz EA stimulation could significantly reduce the heroin-induced reinstatement behavior in

heroin-addicted rat.

Effects of 2Hz EA on locomotor activity

To further examine the sedative properties of 2Hz EA, the effects of heroin, sham EA and 2Hz EA on locomotors activity (total distance traveled/2 h) on normal rats were examined as shown in Fig. 3 (n=5). Heroin treatment could reduce the locomotors activity of rats significantly ($p < 0.05$). The results showed that there was no significant reduce in locomotor activity in these two EA-treated groups compared to the control group ($p > 0.05$). It was indicated that EA-therapy itself had no ataractic influence on heroin self-administration reinforcement or heroin-induced reinstatement.

Effects of 2Hz EA on CB1-Rs *and CB2-Rs Protein expression in PFC, Hip, NAc and VTA of heroin SA rats*

Immunohistochemical technique is beneficial to the localization and semi-quantification of the receptor at the cellular level. We applied this technique to explore the differences of CB1-Rs and CB2-Rs expressions in the brain regions related to relapse. The present results showed that the expression of CB1-Rs in *PFC, Hip, NAc and VTA of* brain areas significantly increased in heroin-addicted group when compared to the control group ($p < 0.05$), but decreased significantly in 2Hz EA group as compared with heroin-addicted group ($p < 0.01$) (Fig. 4). Interestingly, the expression of CB2-Rs in *PFC, Hip, NAc and VTA of* brain areas significantly reduced in heroin-addicted group when compared to the control group ($p < 0.05$),

but enhanced significantly in 2Hz EA group as compared with heroin-addicted group ($p < 0.01$) (Fig. 5). These results indicated that 2Hz EA stimulation could significantly attenuate the expression of CB1-Rs and enhance the expression of CB2-Rs in addiction-relative brain areas in heroin-addicted rats.

Discussion

Previous animal studies found that the underlying neurobiological mechanisms of EA preventing opiate withdrawal syndrome and relapse might be mediated by μ- and δ-opioid receptors and probably via accelerating the synthesis and release of enkephalin in the NAc (Cui *et al.,* 2013; Han *et al.,* 1993; Liang *et al.,* 2010; Shi *et al.,* 2003; Wu *et al.,* 1999). Our present study firstly reported that the 2Hz-EA could effectively inhibit the heroin addiction behaviors which induced by heroin priming via regulating the expression of CB1-Rs and CB2-Rs in relapse-relevant brain regions of heroin SA rats.

Accumulated animal studies adopt the CPP rat model to study the effectiveness of EA. Compared with other models of addiction, the heroin SA model is widly used to study the neurobiological mechanisms involved in drug addiction(Garcia Pardo *et al.,* 2017) because of the great similarity between the results obtained from the animal model and those human addictive behaviors (Mead *et al.,* 2014). Both our and other related studies also showed that 2Hz EA could effectively prevent the reinstatement to heroin seeking elicited by conditional cue in the heroin SA models (Chen L *et al.,* 2014; Liu *et al.,* 2012; Zachariou *et al.,* 2006). Here, we continued to adopt the heroin

SA rat model to observe the influence of 2Hz EA on seeking-behaviors which induced by heroin priming and to explore the potential mechanism. A significantly difference was observed in the number of active nose pokes (represent the heroin self-administration) among control group and heroin-treated group which suggested that the heroin SA model was successfully built in our present study.

The withdrawal syndrome in morphine-dependent rats could be effectively suppressed by 100-Hz EA (Han *et al.,* 1993; Wu *et al.,* 1999). Morphine-induced CPP could be successfully suppressed by 2- or 100-Hz EA (Liang *et al.,* 2010), (Shi *et al.,* 2003). Our present results showed that both heroin SA reinforcement and reinstatement (representes heroin seeking and relapse behaviors, showed in active nose pokes) induced by heroin priming in 2Hz EA group decreased significantly when compared with the heroin-addiction group. Meanwhile, there was no significant reduction of locomotor activity in 2Hz EA-treated group when compared with control group. It indicated that 2Hz EA could effectively inhibit the reinforcement and reinstatement of heroin self-administration rats and the 2Hz EA-therapy itself had no ataractic influence on heroin-seeking and relapse behaviors.

Generally speaking, the inhibitory effects of 2Hz EA on the expression of the morphine CPP might be mediated by μ- and δ-opioid receptors, possibly via accelerating both the release and synthesis of enkephalin in the brain (Liang *et al.,* 2010). Nowadays, the mesolimbic dopamine system is thought to play a major role in the reinforcing effects of drug abuse, especially in PFC, Hip, NAc and VTA (Koob *et al.,* 1992; Nestler *et al.,* 2004). Study found that acupuncture significantly decreased

dopamine release and behavioral hyperactivity which induced by a systemic morphine challenge but the detailed mechanism involved in it has not been fully investigated (Kim *et al.,* 2005).

Recently, it was proved that drugs which enhanced brain reward had common actions on the DA reward system and on animal behaviors closely relates to endocannabinoid system's function (Gardner *et al.,* 2005). Studies reported that the endocannabinoid system also involved in modulating animal drug-seeking behaviors (Chen *et al.,* 2017; Xi *et al.,* 2011). The presence of CB1-Rs throughout brain reward circuits and the rewarding effects produced by CB1-Rs activation allows for the possible influence on the rewarding effects produced by non-cannabinoid substances. In general, drugs activate CB1-Rs appear to facilitate the rewarding effects of non-cannabinoid drugs (Parsons *et al.,* 2015). CB1-Rs agonists increase the motivational and reinforcing effects of alcohol, nicotine and opiates indexed by animal models of drug reward while CB1-Rs antagonism diminished CB1-Rs signaling (either genetic deletion or pharmacological antagonism) attenuates the motivational and rewarding effects of these drugs (Panagis *et al.,* 2014; Serrano *et al.,* 2011). A low density of CB2-Rs expression was found in mesolimbic DA neurons, which might modulates cocaine's addictions in mice probably by a dopamine-dependent mechanism (Morales *et al.,* 2012; Xi *et al.,* 2011). Activation of CB2-Rs ($G_{i/o}$ coupled receptor (Bayewitch *et al.,* 1995)) on DA neurons in the midbrain VTA could directly inhibit dopaminergic neurons and decrease NAc DA release (Bayewitch *et al.,* 1995; Xi *et al.,* 2011). Therefore, the CB1-Rs and CB2-Rs

involved in the mesolimbic dopamine system (such as PFC, Hip, NAc and VTA) might play important roles in heroin addiction. Our results demonstrated that the expression of CB1-Rs significantly increased while the expression of CB2-Rs decreased significantly in PFC, Hip, NAc and VTA in heroin SA rats when compared with those control rats.

Our previous study showed that CB2 receptors contribute to the analgesic effect of 2Hz EA in inflammatory pain. 2Hz EA could significantly upregulate the local CB2-Rs expression (Zhang *et al.,* 2010). In addition, study reported that the endocannabinoid system might be a primary mediator and regulatory factor of acupuncture's beneficial effects in drug addiction (Hu *et al.,* 2017). We hyperthesised that 2Hz EA could down-regulate the expression of CB1-Rs and up-regulate the expression of CB2-Rs in relapse-relevant brain regions and help attenuating the heroin seeking behaviors. Our results demonstrated that the expression of CB1-Rs significantly decreased while the expressions of CB2-Rs significantly increased in PFC, Hip, NAc and VTA of 2Hz EA treated rats compared with heroin SA rats.

Conclusion

Our present study adopted the heroin SA model rats to explore whether 2Hz EA inhibited the heroin-evoked seeking behaviors and regulated the expression of CB1-Rs and CB2-Rs in mesolimbic brain. The results showed that 2Hz-EA could attenuate the heroin evoked seeking and relapse behaviors. The inhibitory effect of 2Hz-EA might be related to down-regulate the expression of CB1-Rs and

up-regulation the expressions of CB2-Rs in relapse-relevant brain regions in heroin-addicted rats. The CB1-Rs and CB2-Rs in mesolimbic brain were potential targets for 2Hz EA treatment of drug addiction and relapse.

Conflict of interest

Authors declare that they have no conflict of interest.

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Figure Legends

Fig.1. 2Hz EA stimulation attenuated heroin self-administration reinforcement in rats. Values are given in mean ± SEM of eight animals per group. ## $p < 0.01$, as compared with control group. && $p < 0.01$, as compared with heroin-addicted group. $$ $p < 0.01$, as compared with sham EA group.

Fig.2. 2Hz EA stimulation attenuated heroin-induced reinstatement in rats. Values are given in mean ± SEM of eight animals per group. ## $p < 0.01$, as compared with control group; && $p < 0.01$, as compared with heroin-addicted group; & $p < 0.05$, as compared withheroin-addicted group.

Fig.3. 2Hz EA stimulation did not affect the locomotor activityon normal rats. Values are given in mean ± SEM of eight animals per group. ## $p < 0.01$, as compared with control group.

Fig.4. The different expressions of CB1-Rs in PFC, Hip, NAc and VTA among control group, heroin-addicted group, Sham EA and 2Hz EA group. Values are given in mean ± SEM of eight animals per group. # $p < 0.05$, ## $p < 0.01$, as compared with control group; & $p < 0.05$, && $p < 0.01$, as compared with heroin-addicted group. Scale bar, 100 μm.

Fig.5. The different expressions of CB2-Rs in PFC, Hip, NAc and VTA among control group, heroin-addicted group, Sham EA and 2Hz EA group. Values are given in mean ± SEM of eight animals per group. # $p < 0.05$, ## $p < 0.01$, as compared with control group; & $p < 0.05$, && $p < 0.01$, as compared with heroin-addicted group. Scale bar, 100 μm.

Fig. 1

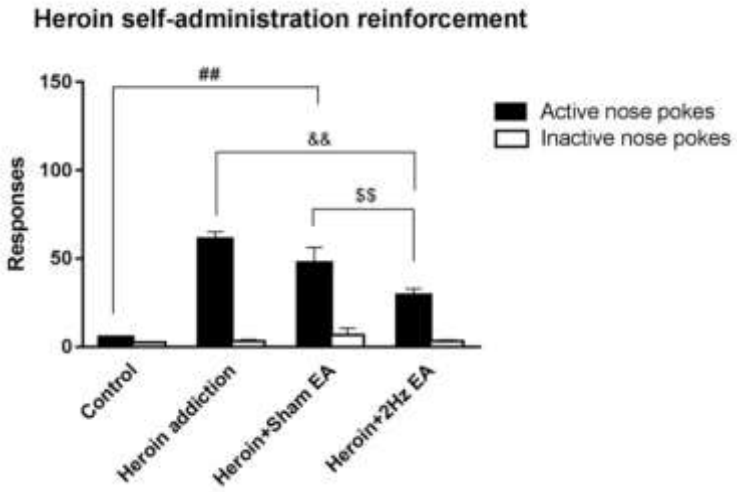


Fig. 2

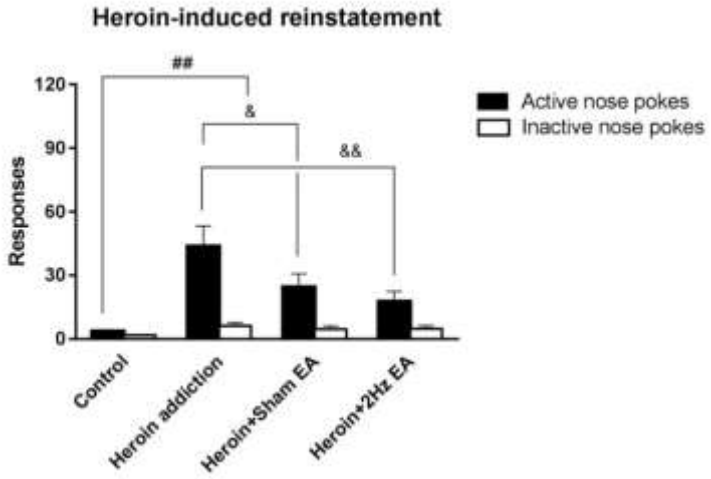


Fig. 3

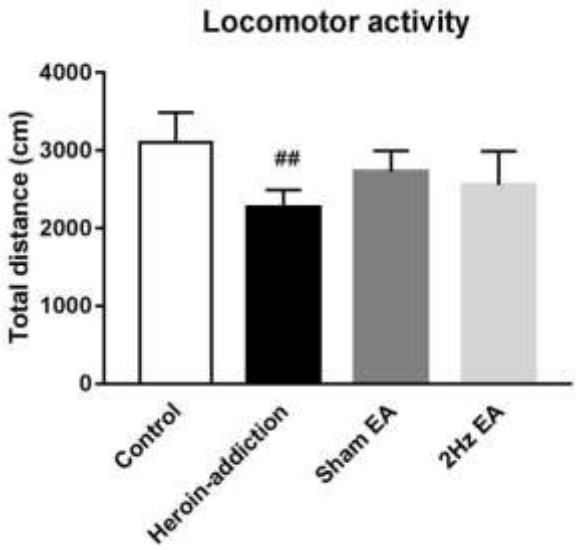

Locomotor activity
Total distance (cm)
4000
3000
2000
1000
0
##
Control
Heroin-addiction
Sham EA
2Hz EA

Fig. 4

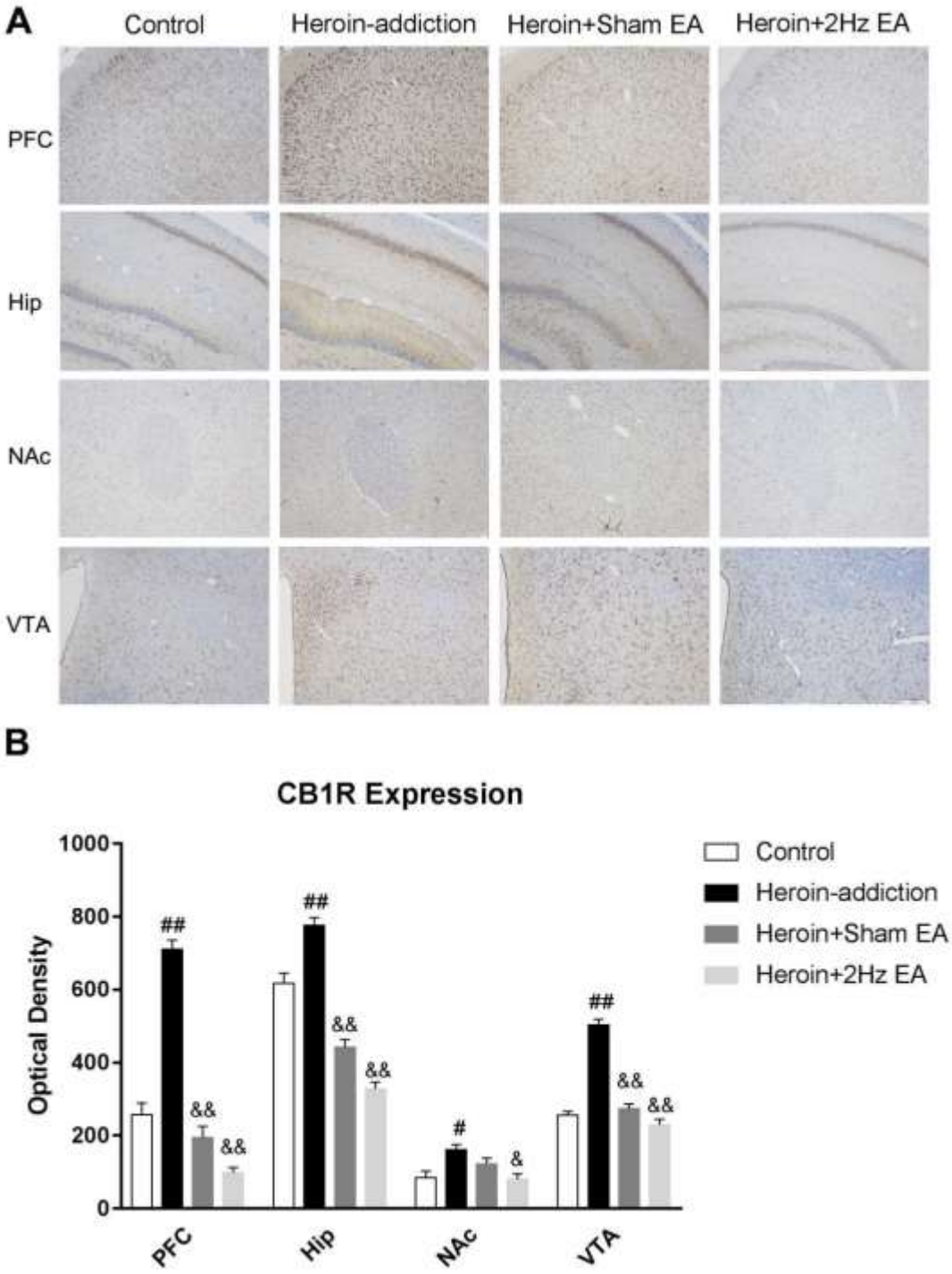
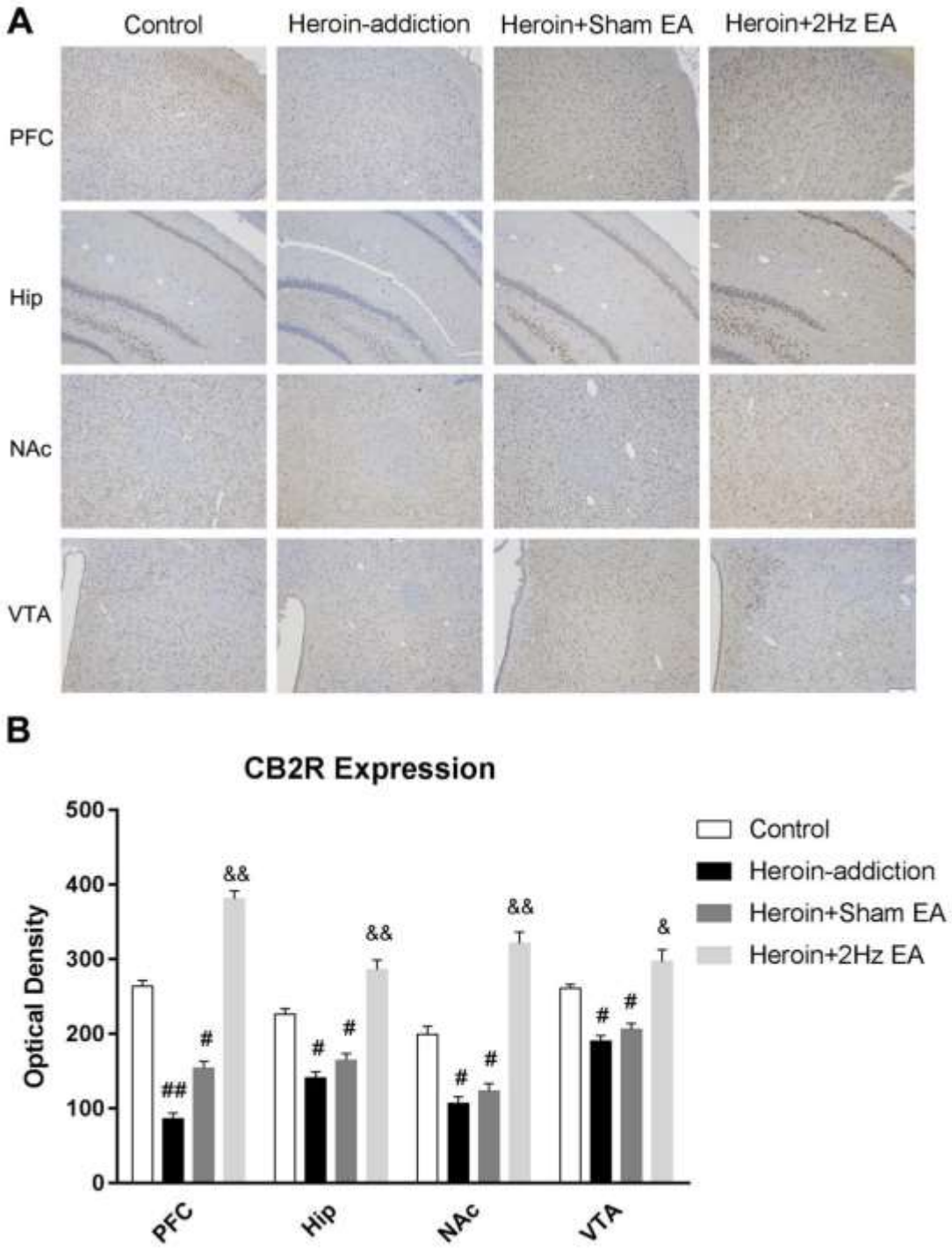

A
Control
Heroin-addiction
Heroin+Sham EA
Heroin+2Hz EA
PFC
Hip
NAc
VTA
B
CB1R Expression
Optical Density
1000
800
600
400
200
0
Control
Heroin-addiction
Heroin+Sham EA
Heroin+2Hz EA
##
&&
&&
##
&&
&&
#
&
##
&&
&&
PFC
Hip
NAc
VTA

Fig. 5


A
Control
Heroin-addiction
Heroin+Sham EA
Heroin+2Hz EA
PFC
Hip
NAc
VTA
B
CB2R Expression
Optical Density
500
400
300
200
100
0
Control
Heroin-addiction
Heroin+Sham EA
Heroin+2Hz EA
PFC
Hip
NAc
VTA