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Regional expression of extracellular signal-regulated kinase 1 and 2 mRNA in a morphine-induced conditioned place preference model

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Abstract

Chronic morphine administration has been shown to change the expression of extracellular signal-regulated kinase (ERK), which is a molecule known to play an important role in homeostatic adaptations caused by addictive drugs. In the present study, we investigated the expression of ERK messenger ribonucleic acid (mRNA) of the prefrontal cortex (PFC), nucleus accumbens (NAc), hippocampus, and caudate putamen (CPu) in morphine-induced conditioned place preference (CPP) by real-time reverse transcriptase polymerase chain reaction (real-time PCR). CPP was established by alternate morphine (10 mg/kg) injections, extinguished after a 10-day extinction training, and reinstated by a priming injection of morphine (10 mg/kg). During three phases of morphine-induced CPP, the expression levels of ERK1 and ERK2 mRNA were altered in various brain regions. In the PFC, the expression levels of ERK1 and ERK2 mRNA were increased after chronic morphine injection ($p=0.003$, $p=0.000$), and did not return to the basal level after extinction training ($p=0.025$, $p=0.000$), but decreased after a priming injection ($p=0.000$, $p=0.000$). In the CPu, ERK1 mRNA had an abrupt increase following a priming injection ($p=0.000$). Different from other brain regions, the expression levels of ERK1 and ERK2 mRNA were decreased in three phases of morphine-induced CPP in the hippocampus (ERK1: $p=0.000$, $p=0.040$, $p=0.000$; ERK2: $p=0.000$, $p=0.000$, $p=0.000$, respectively). These results suggest region-specific changes of ERK1 and ERK2 mRNA expression during morphine-induced CPP.

Keywords

Morphine; Conditioned place preference; Extracellular signal-regulated kinase

1. Introduction

Opioid addiction manifests as a neuroadaptive disorder characterized by compulsive drug-taking behavior and high rates of drug relapse (Christie, 2008; Koob and Volkow, 2009; Williams et al., 2001). With long-term drug use, homeostatic adaptations can occur within cells and circuits, which can lead to tolerance, dependence, sensitization, craving, and relapse (Cami and Farre, 2003; Robinson and Berridge, 2003). Accumulating evidence has suggested that brain reward circuits, especially the mesolimbic and mesocortical circuits, are involved in this process (Zhai et al., 2008). Dopamine is one of the major neurotransmitters involved in these circuits. Moreover, morphine can elevate the level of dopamine and its metabolites in the nucleus accumbens (NAc) (Ma et al., 2009). In addition to the NAc, the prefrontal cortex (PFC), caudate putamen (CPu), and hippocampus also play a role in both positive and negative effects of drug abuse, including those related to extinction (Christie, 2008; Valjent et al., 2004; Williams et al., 2001).

More recently, the mitogen-activated protein kinase (MAPK) pathway has been shown to be involved in the cellular response to opioids (Christie, 2008; Williams et al., 2001). Extracellular signal-regulated kinases (ERKs) are members of the MAPK pathway that play a key role in intracellular signaling pathways mediating synaptic plasticity, memory formation, and long-term gene expression (Di Benedetto et al., 2007; Ortiz et al., 1995). Eight isoforms of ERKs have been identified and described in the adult rodent brain (Di Benedetto et al., 2007), and of these, ERK1 and ERK2 are the most extensively studied and best-known ERK family members. ERK1 and ERK2 mRNA and protein are ubiquitously expressed in many regions of the adult mouse brain, including the mesolimbic dopamine system (Di Benedetto et al., 2007; Georges et al., 1999), and these proteins can be activated through phosphorylation of the tyrosine and threonine residues by signaling from growth factors and mitogens (Rubinfeld and Seger, 2004). A previous study found that after systemic administration of an ERK inhibitor, mice that had previously established cocaine-induced conditioned place preference (CPP) lost their CPP response when re-exposed to the drug-paired compartment (Valjent et al., 2006). Phosphorylated ERK2 (*p*-ERK2) protein expression levels are reduced in the PFC after chronic morphine injection, but no changes have been observed for total ERK1, total ERK2, or phosphorylated ERK1 (*p*-ERK1) protein levels in the PFC (Li et al., 2008b). Following chronic morphine exposure, *p*-ERK1 and *p*-ERK2 protein levels, but not total ERK1 and total ERK2 levels, were decreased in the NAc, and the protein expression levels of total or phosphorylated ERK1 and ERK2 in the CPu were not altered (Muller and Unterwald, 2004). In the VTA, no change of total ERK1 and ERK2 protein expression levels was observed in the progress of morphine-induced CPP (Lin et al., 2010). Although total ERK1 and total ERK2 protein levels do not significantly change during addiction, it is not clear whether the transcript levels of these proteins are altered under the same conditions. Therefore, we investigated the expression levels of ERK1 and ERK2 mRNA in the NAc, PFC, hippocampus, and CPu during three phases of morphine-induced CPP. The CPP paradigm involves Pavlovian conditioning where the affective state an animal experiences after morphine injection is paired with the particular environment where the affective state occurs (Carboni and Vacca, 2003). This affective state is eliminated after a few days of morphine absence, but when the animal is re-exposed to the paired

environment with morphine, it will recover the response that it previously learned (Aguilar et al., 2009). This model of morphine-induced CPP can mimic the progress of drug-taking, extinction, and relapse in humans. Therefore, we used this model to investigate the neural mechanisms involved with ERK1 and ERK2 mRNA expression levels linked to morphine addiction.

2. Results

2.1. Morphine-induced CPP

Fig. 2 shows the results of morphine-induced CPP. Fig. 2a shows the results of the CPP tests of group 1 and group 2. The repeated measures ANOVA did show a significant effect of group x phase interaction ($F_{(1,14)}=39.783, p=0.000$). Post-hoc comparisons revealed that the CPP score of group 2 in the post-test was significantly increased ($p=0.000$; Fig. 2a) compared with the pretest, but no significant change ($p=0.245$; Fig. 2a) was found in group 1. Fig. 2b shows the results of the CPP tests of group 3 and group 4. The repeated measures ANOVA revealed a significant effect of group x phase interaction ($F_{(1,14)}=19.922, p=0.000$). Post-hoc comparisons revealed that the group 4 established morphine-induced CPP after conditioning training ($p=0.000$; Fig. 2b) and their preference for morphine disappeared after 10-day extinction training ($p=0.593$; Fig. 2b and c) shows the results of the CPP tests of group 5 and group 6. The repeated measures ANOVA revealed a significant effect of group x phase interaction ($F_{(1,14)}=11.755, p=0.001$). Post-hoc comparisons revealed that the mice of group 6 regained their preference for the morphine-paired compartment ($p=0.000$; Fig. 2c) after establishment of morphine-induced CPP ($p=0.000$; Fig. 2c) and 10-day extinction training ($p=0.428$; Fig. 2c).

2.2. ERK1 and ERK2 mRNA expression in NAc, PFC, hippocampus, and CPu of morphine-induced CPP in mice

The changes in ERK1 and ERK2 gene transcription are shown in Fig. 3. In the establishment phase, ERK1 showed significant up-regulation in the PFC and CPu ($t_{14} = -3.590, p=0.003$; $t_{14} = -2.743, p=0.016$; Fig. 3a), and was suppressed in the hippocampus ($t_{14} = 8.996, p=0.000$; Fig. 3a). ERK2 levels were significantly decreased in the NAc, hippocampus, and CPu ($t_{14} = 3.826, p=0.002$; $t_{14} = 5.002, p=0.000$; $t_{14} = 13.403, p=0.000$; Fig. 4a), and only increased in the PFC ($t_{14} = -19.390, p=0.000$; Fig. 4a). In the extinction phase, ERK1 showed a significant up-regulation in the NAc, PFC, and CPu ($t_{14} = -3.689, p=0.002$; $t_{14} = -3.509, p=0.025$; $t_{14} = -7.454, p=0.000$; Fig. 3b), but significant down-regulation in the hippocampus ($t_{14} = 2.261, p=0.040$; Fig. 3b). Similar to ERK1 mRNA expression, ERK2 expression was also increased in the NAc and PFC ($t_{14} = -11.474, p=0.000$; $t_{14} = -35.562, p=0.000$; Fig. 4b) and decreased in the hippocampus ($t_{14} = 15.071, p=0.000$; Fig. 4b). In the reinstatement phase, ERK1 showed significant up-regulation in the NAc and CPu ($t_{14} = -5.027, p=0.000$; $t_{14} = -20.688, p=0.000$; Fig. 3c), but significant down-regulation in the PFC and hippocampus ($t_{14} = 5.387, p=0.000$; $t_{14} = 25.170, p=0.000$; Fig. 3c). ERK2 showed significant down-regulation in the NAc, PFC, and hippocampus ($t_{14} = 4.012, p=0.001$; $t_{14} = 14.725, p=0.000$; $t_{14} = 7.988, p=0.000$; Fig. 4c), but was not significantly altered in the CPu ($t_{14} = -2.095, p=0.055$; Fig. 4c).

3. Discussion

The goal of current research of drug addiction is to focus on the cellular and molecular mechanisms underlying the transition from occasional drug use to compulsive drug-seeking and drug-taking behaviors as well as relapse after long-time extinction from addictive drugs (Lin et al., 2010). Animal models are used to mimic several aspects of addiction, and CPP is currently one of the most popular models for this purpose (Bardo and Bevins, 2000; Valjent et al., 2004). In our experiments, we observed that alternate morphine (10 mg/kg) injections established a significant CPP, which was eliminated after 10 d of extinction training without morphine. Following the establishment and subsequent extinction, CPP was reinstated by a single injection of morphine (10 mg/kg). These results are similar to those reported by Manzanedo et al. (2001). After repeated exposure to the conditioned stimuli (CS) environment without drug, the conditioned effect of morphine disappeared, and a subsequent injection of the same dose of morphine caused the animals to look for the conditioned stimuli associated with the drug. These results are also in agreement with studies showing that the incentive salience and attractiveness of these stimuli are powerful triggers of reinstatement (Aguilar et al., 2009; Mueller and Stewart, 2000). In general, the CPP model is an effective tool for assessing the rewarding effects of addictive drugs and for investigating the mechanisms of drug-induced reinstatement.

ERK1 and ERK2 are widely distributed throughout the entire brain (Boulton et al., 1991; Di Benedetto et al., 2007) and are activated by mitogen-activated protein kinase (MEK) where they subsequently regulate the transduction of extracellular signals (Di Benedetto et al., 2007). Increasing experimental evidence has shown that ERK1 and ERK2 play an important role in mediating the drug rewarding effect and are involved in persistent changes of synaptic plasticity and memory function that are produced by exposure to morphine or other drugs (Liu et al., 2007). Repeated morphine injection has been shown to increase ERK activity in the NAc in mice (Liu et al., 2007). Moreover, microinjection of specific MEK inhibitors PD98059 or U0126 into the lateral cerebral ventricle was found to impair the establishment of morphine-induced CPP (Ozaki et al., 2004). Li et al. found that long-term morphine treatment increased ERK activation in the mouse frontal cortex, hippocampus, and striatum (Li et al., 2008a). Drug relapse and extinction also alter ERK signaling in addition to the pharmacological effect of drugs of abuse. After 30 d of extinction, there was increased ERK phosphorylation in the central amygdala after re-exposure to cocaine cues (Lu et al., 2005). In addition, inhibiting ERK phosphorylation in the central amygdala decreased cocaine seeking behavior after 30 d of cocaine extinction (Lu et al., 2005). ERK2 was also shown to be activated in the NAc when rats were re-exposed to the cocaine-paired compartment (Miller and Marshall, 2005).

In the NAc, our results showed that morphine decreased the level of ERK1 mRNA during the establishment phase and increased during the extinction phase, but significantly decreased after a single injection. Moreover, ERK2 expression levels had similar alterations during morphine-induced CPP. These results appear to be inconsistent with the protein expression levels of ERK1 and ERK2 shown in previous studies. The precise reason for this discrepancy is not clear, but it is possible that the function of NAc is contributing to this observation. NAc is a site of convergence of many limbic areas and receives dopamine

projections from the ventral tegmental area (VTA) and excitatory glutamate projections from the limbic system (hippocampus, amygdala, septal nuclei, and prefrontal cortex). This region also inhibits GABA projections from its intrinsic nucleus and other parts of the basal ganglia (Williams et al., 2001). The nucleus accumbens is a complex area that integrates the information from the PFC, hippocampus, and CPu. Therefore, due to the complexity of the excitatory afferent input and intrinsic inhibitory innervation, it is impossible to reach a consensus on the overall effect of morphine in the NAc during CPP.

In the PFC, an up-regulation of ERK1 and ERK2 mRNA expression was observed after chronic morphine injection, which did not return to the basal level after extinction training, but did decrease after a priming injection. The neurobiological substrates of drug abuse is the brain reward circuitries, and there is a circuitry-based transition from the dopamine-dependent acquisition of drug-seeking behavior to the glutamate-dependent execution of drug-seeking behavior (Kalivas and O'Brien, 2007; Koob and Volkow, 2009). Therefore, during the establishment phase, ERK1 and ERK2 mRNA in the PFC may be activated by dopamine from the VTA. The prefrontal circuit plays an important role during the extinction phase, and drug-trained animals and human addicts have a dramatic elevation in glutamate transmission during extinguished drug-seeking behavior, suggesting that glutamate may stimulate increased expression of ERK1 and ERK2 mRNA. The exact mechanism by which ERK1 and ERK2 mRNA expression decreases during the reinstatement phase is not clear, but we hypothesize that the decrease of ERK1 and ERK2 mRNA expression represents a compensatory mechanism following saturation of ERK1 and ERK2 protein levels.

The response of the hippocampus to morphine-induced CPP was distinct from that of other brain regions examined. Unlike other brain regions, ERK1 and ERK2 mRNA levels were down-regulated in the hippocampus during the three phases of CPP. The hippocampus is typically accorded a major role in declarative memory, which is thought to be involved in learning and the link between affective conditions or circumstances with drug-taking experiences (Koob and Volkow, 2009). Through the extracellular adenosine accumulation in the hippocampus, chronic morphine treatment diminished LTP in hippocampal CA1 and partially impaired spatial memory (Lu et al., 2010). Acute morphine injection induced a decrease in the level of extracellular glutamate in the hippocampus, and an irreversible decrease of extracellular glutamate concentration was observed in the hippocampus after 6 days of morphine treatment (Guo et al., 2005). These results indicate that chronic morphine treatment may suppress the expression of ERK1 and ERK2 mRNA in the hippocampus, which appears to be irreversible. Alternatively, the down-regulation observed in ERK1 and ERK2 mRNA levels may be due to morphine-induced adaption, which subsequently leads to saturation of the ERK1 and ERK2 receptors.

Our data showed that ERK1 mRNA was abruptly increased in the CPu during the reinstatement phase, and ERK2 was also slightly increased. These results indicate that the CPu may be involved in CPP reinstatement. It is currently thought that drug users undergo a transition from voluntary drug use to compulsive drug use, and Everitt et al. (2008) hypothesized that this transition is accompanied by a progression from the ventral striatum to dorsal striatum. Koob and Volkow (2009) also postulated that the transition of engagement of the ventral striatum to the dorsal striatum contributes to certain habits and

compulsive drug use. Our results provide a greater understanding to the function of the CPu during drug relapse. However, the exact mechanism of ERK1 and ERK2 in this region during these processes remains to be elucidated.

Our findings of ERK1 and ERK2 mRNA expression levels during morphine-induced CPP are inconsistent with previous studies exploring changes in protein levels. We postulate that there are several possible reasons for the poor correlations between ERK1 and ERK2 protein and mRNA expression levels in this system. First, transcription is the initial regulatory step in gene expression, and there are many complicated post-transcriptional regulatory mechanisms involved in turning mRNA into protein (Carpentier et al., 2008; Greenbaum et al., 2003). Greenbaum et al. (2003) found that the expression of open reading frames of genes had little or no correlation with the final protein level over the course of the yeast cell cycle. This indicates the cell can control these open reading frames at the translational and/or post-translational level in order to achieve the resulting and desired protein levels. Second, the poor correlation between protein and mRNA suggests that proteins may be synthesized in specific brain regions but expressed in another brain region into which the specific brain region projects (Spangler et al., 2003). For example, Sari et al. (1999) found highest level of 5-hydroxytryptamine_{1B} (5-HT_{1B}) protein in the globus pallidus and substantia nigra where no 5-HT_{1B} mRNA was detectable, but a high 5-HT_{1B} mRNA level was detected in the CPu that project to the globus pallidus and substantia nigra (Boschert et al., 1994). These suggest that the 5-HT_{1B} receptor protein may be synthesized or transported to the globus pallidus and the substantia nigra, into which striatal neurons expressing high levels of 5-HT_{1B} mRNA project. Alternatively, these mismatches may reflect a lower sensitivity of experimental methods in detecting protein and mRNA. Third, the different time points of detection may lead to poor correlations between protein and mRNA expression levels. Due to the different rates of degradation and synthesis, proteins have different half-lives (Greenbaum et al., 2003). Additionally, Cho et al. (1998) demonstrated that the transcript levels of 416 genes of yeast were found to have consistent periodic changes throughout the cell cycle. Thus, the time points of sample preparation may have also contributed to the poor correlation observed.

4. Conclusion

In conclusion, the results of the present study suggest that morphine alters ERK1 and ERK2 mRNA expression in brain regions during different CPP phases, leading to brain region-specific changes. In particular, morphine induces an irreversible suppression of ERK1 and ERK2 mRNA expression in the hippocampus during morphine-induced CPP. These findings contribute to a greater understanding of the potential mechanisms of ERK1 and ERK2 in cellular and molecular processes underlying opioid dependence as well as drug-seeking behaviors. However, further studies are still required to clarify whether the current findings relate to changes at the protein level.

5. Experimental procedures

5.1. Animals

Sixty C57BL/6J male mice (7-weeks-old) (Vital River Laboratory Animal Technology Co. Ltd., Beijing, China) weighing 22–28 g were used in these experiments. Mice were housed in groups of four per cage on a 12 h light/dark cycle (light on between 7:00 A.M. and 7:00 P.M.) with free access to food and water. The animals were randomly assigned to different experimental groups and each animal was used only once. Mice were allowed 1 week to acclimate prior to the start of the experiments. All experiments were executed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 80-23, revised 1996) and were approved by Xi'an Jiaotong University Research and Ethics Committee.

5.2. Conditioned place preference (CPP) protocol

The CPP apparatus (JL Behv-CPPG, Shanghai Jiliang Software Technology Co. Ltd., China) was composed of two compartments of equal size ($15 \times 15 \times 37 \text{ cm}^3$) with a $5 \times 7 \text{ cm}^2$ door in the center. The two compartments had different colors (black or white) and floor textures (mesh or grid). The time spent on each side and the number of crossings between the compartments were recorded by video and analyzed by computerized monitoring software. The CPP protocol consisted of the following three phases (Fig. 1).

5.2.1. Establishment of CPP (EB) phase—On day 0, mice were placed in the compartments with the center door open and allowed to freely explore between both compartments for 15 min. The time spent in each compartment and the number of crossings were recorded as an initial baseline preference. Mice spending less than 300 s in each compartment and less than 20 crossings were removed from the study (Li et al., 2008b). After the pretest, mice were randomly distributed into six groups: three saline groups (group 1, group 3, and group 5) and three morphine groups (group 2, group 4, and group 6) (Fig. 1). Each group consisted of 8 mice. Conditioning training was performed once daily at 8:00 PM for 6 successive days. The three saline groups received saline [10 ml/kg, intraperitoneal (i.p.)] injection in both compartments of the apparatus. The other three groups of mice received saline (10 ml/kg; i.p.) injection in the black compartment and morphine (10 mg/kg; i.p.; Qinghai Pharmaceutical Factory, China) injection in the white compartment. After injection, mice were placed into the apparatus for 30 min with the center door closed. On day 7, all of the animals received a saline injection (10 ml/kg, i.p.) and were then allowed free access to both compartments for 15 min to test the conditioned preference. The mice in group 1 and group 2 were euthanized immediately after the posttest.

5.2.2. Extinction of CPP (ET) phase—Mice in group 3, group 4, group 5, and group 6 underwent extinction training once daily for 10 d. The extinction training was the same as the conditioning training, but not reinforced with morphine. All mice received a saline (10 ml/kg; i.p.) injection in both compartments of the apparatus, training for 30 min with the center door closed after injection. On day 18, all of the animals were tested for the conditioned preference for 15 min after a single saline (10 ml/kg; i.p.) injection. Mice in group 3 and group 4 were euthanized immediately after the extinction test.

5.2.3. Reinstatement of CPP (RI) phase—After the extinction training, mice in group 5 and group 6 received an injection of saline and an injection of morphine (10 mg/kg; i.p.), respectively, 5 min before being placed in the apparatus. The mice were then tested for their conditioned preference for 15 min. After the reinstatement test, mice in group 5 and group 6 were euthanized immediately.

5.3. Sample preparation

After the behavioral tests, mice were euthanized by cervical dislocation at the same time and samples of PFC, NAc, hippocampus, and CPu were dissected on ice from the brains of 8 mice from each group. The samples were stored at -80°C .

5.4. Real-time PCR

Total RNA were extracted from each brain region using the RNA_{fast200} purification kit (Fastagen Biotechnology Co. Ltd., Shanghai, China) according to the manufacturer's protocol. A 1.0 μg sample of total RNA was reverse-transcribed into cDNA using the reagents and the protocol of the Revert AidTM First Strand cDNA synthesis kit (Fermentas, Canada). The RT reaction and PCR amplification were performed with the iCycler iQTM5 System (Bio-Rad, California, USA) using SYBR Green/ROX qPCR Master Mix (2 $\times$) (Fermentas, Canada) based on the manufacturer's instructions. The cycling conditions were set as follows: one cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The reaction mixtures were immediately stored at 4°C once the reaction was complete. All primers and PCR conditions are shown in Table 1. Primers were designed based on the published sequence of the GenBank as follows: (1) NM_011952.2 (ERK1); (2) NM_011949.3 (ERK2); and (3) NM_007393.3 (β -actin). All primers were synthesized by Beijing AuGCT DNA-SYN Biotechnology Co., Ltd. The comparison between samples were based on the threshold cycle (CT), which was considered to be the threshold cycle of PCR at which the fluorescent signal of amplified product was first detected. The relative expression levels of the target genes (*ERK1* or *ERK2*) and reference gene (*β -actin*) were calculated using the formula $2^{-\Delta\text{CT}}$, where $\Delta\text{CT} = \text{CT}_{\text{target}} - \text{CT}_{\text{reference}}$.

5.5. Statistical analysis

For CPP experiments, the amount of time spent in the white compartment minus the time spent in the black compartment was calculated as the CPP score. These results were analyzed by the repeated measures analysis of variance (ANOVA) (group $\times$ phase) and by the Least-Significant Difference test for individual post-hoc comparison. For the RT-PCR experiment, the difference in the target gene expression of the experiment groups compared with the saline groups was calculated using the $2^{-\Delta\text{CT}}$ equation. These values were analyzed by a two-tailed Student's *t*-test. All data are expressed as mean $\pm$ standard error of mean (SEM). Data were analyzed using SPSS17.0 software (IBM, USA). A $p < 0.05$ was considered to be statistically significant.

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Pretest	Conditioning training	Test	Extinction training	Test	Reinstatement
	M S M S M S		S S S S S S S S S S		M
Day 0	1-6	7	8-17	18	19

Fig. 1.

Three phases of the CPP protocol. Establishment phase: Pretest was tested as an initial baseline preference. For the morphine groups (group 2, group 4, and group 6), the mice received morphine (M) (10 mg/kg, i.p.) injections in the white compartment or saline (S) injections in the black compartment on days 1–6 during a 30 min period once daily. The saline groups (group 1, group 3, group 5) received saline injections in both compartments on days 1–6 during a 30 min period once daily. On day 7, the test was administered to examine preference. Extinction of phase: Group 3, group 4, group 5 and group 6 received saline injections in both compartments on days 8–17 and then received 30 min training once daily. The test was administered on day 18 to probe the preference. Reinstatement phase: Group 5 and group 6 received a saline injection and morphine (10 mg/kg, i.p.) injection, respectively, 5 min before the test.

Fig. 2.

The results of morphine (10 mg/kg)-induced CPP. a: The CPP score of group 1 and group 2 from the pretest and posttest. b: The CPP score of group 3 and group 4 in the pretest, posttest, and extinction test. c: The CPP score of group 5 and group 6 in the pretest, posttest, extinction test, and reinstatement test. The CPP score was calculated as the time spent on the drug-paired side minus the time spent on the saline-paired side. Data are shown as mean $\pm$ SEM. * $p < 0.05$ represents statistical significance of change compared to the pretest using the repeated measures ANOVA.

Fig. 3.

The results of ERK1 mRNA expression in the NAc, PFC, hippocampus, and CPU of morphine-induced CPP model. *a*: ERK1 mRNA expression of group 1 and group 2 in the establishment phase. *b*: ERK1 mRNA expression of group 3 and group 4 in the extinction phase. *c*: ERK1 mRNA expression of group 5 and group 6 in the reinstatement phase. Expression levels are calculated relative to β -actin. Data are shown as mean $\pm$ SEM. * $p < 0.05$ denotes a statistically significant difference from the saline group and experiment group according to the Student's *t*-test.

Fig. 4.

The results of ERK2 mRNA expression in the NAc, PFC, hippocampus, and CPu of a morphine-induced CPP model. a: ERK2 mRNA expression of group 1 and group 2 in the establishment phase. b: ERK2 mRNA expression of group 3 and group 4 in the extinction phase. c: ERK2 mRNA expression of group 5 and group 6 in the reinstatement phase. Expression levels are calculated relative to β -actin. Data are shown as mean $\pm$ SEM. * $p < 0.05$ denotes a statistically significant difference from the saline group and experiment group according to the Student's t -test.

Table 1

PCR primer sequences and PCR conditions for ERK1, ERK2, and β -actin PCR.

Gene	Primer sequences	PCR conditions		
		Initial denaturation	Denaturation	Annealing Extension
ERK1	Forward 5'-tggctttctgacggagtatg-3' Reverse 5'-ggctccaggtagtgcctgc-3'	95 °C 10 min 1	95 °C 15 s 40	60 °C 30 s 40 72 °C 30 s 40
ERK2	Forward 5'-cctcaagccttccaacctc-3' Reverse 5'-gccccacagaccataatacaag-3'			
β -actin	Forward 5'-cgacaacggctccggcatgt-3' Reverse 5'-gccctgggtgagcacagg-3'			