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The phosphodiesterase-4 inhibitor rolipram attenuates heroin-seeking behavior induced by cues or heroin priming in rats

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Abstract

Inhibition of phosphodiesterase-4 (PDE4), an enzyme that specifically hydrolyzes cyclic adenosine monophosphate (cAMP) increases intracellular cAMP/cAMP-response element binding protein (CREB) signaling. Activation of this signaling is considered as an important compensatory response that decreases motivational properties of drugs of abuse. However, it is not known whether PDE4 is involved in heroin seeking. Self-administration of heroin (50 μg/kg/infusion) was performed under the fixed ratio 1 (FR1) schedule for 14 d and then drug seeking was extinguished for 10 d. The progressive ratio schedule was used to evaluate the relative motivational value of heroin reinforcement. After training, the conditioned cue or heroin priming (250 μg/kg) was introduced for the reinstatement of heroin-seeking behavior. Pretreatment (i.p.) with rolipram (0.03–0.3 mg/kg), a prototypical, selective PDE4 inhibitor, failed to inhibit heroin self-administration under the FR1 schedule, but decreased the reward values under the progressive ratio schedule in a dose-dependent manner. In addition, rolipram decreased the reinstatement of heroin seeking induced by cues or heroin priming even at the lowest dose (0.03 mg/kg); in contrast, the highest dose (0.3 mg/kg) of rolipram was required to decrease sucrose reinforcement. Finally, the effects of rolipram on heroin-seeking behavior were correlated with the increases in expression of phosphorylated CREB in the nucleus accumbens. The study demonstrated that rolipram inhibited heroin reward and heroin-seeking behavior. The results suggest that PDE4 plays an essential role in mediating heroin seeking and that PDE4 inhibitors may be used as a potential pharmacotherapeutic approach for heroin addiction.

Introduction

Relapse to heroin use after abstinence is a major clinical problem in the treatment of heroin addiction (O'Brien, 1997). Opiate withdrawal produces aversive properties, which are considered to promote heroin-seeking and taking behaviors (Kenny et al., 2006; Zhou et al., 2009). Using a rat model of drug relapse, we have recently found that heroin seeking induced by re-exposure to drug-associated cues or a heroin-priming injection persists for over two months after withdrawal from heroin self-administration (Zhou and Kalivas, 2008). The magnitude of heroin seeking induced by drug-related cues is enhanced by spontaneous withdrawal or naltrexone-precipitated withdrawal (Kuntz et al., 2008; Zhou et al., 2009).

Cyclic adenosine monophosphate AMP (cAMP) signaling plays an important role in drug abuse and dependence. Infusions of cAMP signaling inhibitors such as protein kinase A (PKA) inhibitors directly into the nucleus accumbens (NAc), a brain region important for motivation and reward, reduce responses of animals to rewarding actions of cocaine and opiates (Self and Nestler, 1995; Self and Nestler, 1998). In addition, deficiency of the isoform (CREBα∆) of cAMP-response element-binding protein (CREB), the primary downstream target of cAMP/PKA signaling, decreases the rewarded responses to a low dose of morphine, while it increases the rewarding properties at a high dose of morphine (Walters et al., 2005). The latter is supported by the findings that overexpression of CREB in the NAc

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using viral-mediated gene transfer decreases the rewarding effects of cocaine and opiates, whereas expression of a dominant negative mutant CREB (mCREB) in the NAc increases drug rewarding effects, in addition to producing antidepressant activity (Carlezon et al., 1998; Pliakas et al., 2001; Barrot et al., 2002). Moreover, drug-induced activation of CREB signaling is considered an important compensatory response that decreases the motivational properties of the drug (McClung and Nestler, 2003). This is in agreement with the role of striatal miR-212, which dramatically amplifies the stimulatory effects of cocaine on CREB signaling and decreases responsiveness to the motivational properties of the drug (Hollander et al., 2010). Taken together, the cAMP/PKA/CREB signal pathway is importantly involved in rewarded responses of drugs.

Cyclic AMP is hydrolyzed by phosphodiesterase-4 (PDE4), which is critical in the control of intracellular cAMP concentrations. PDE4 inhibitors increase intracellular cAMP and activate the cAMP/PKA signaling pathway, leading to CREB phosphorylation (Rutten et al., 2008; Rutten et al., 2009). It has been well documented that PDE4 plays an important role via this signaling pathway in the mediation of a variety of CNS functions, including antidepressant and anxiolytic activity (O'Donnell and Zhang, 2004; Li et al., 2009; Zhang, 2009), memory-enhancing effects (Barad et al., 1998; Rutten et al., 2008; Rutten et al., 2009; Li et al., 2011), and reversal of various memory deficits (Imanishi et al., 1997; Zhang and O'Donnell, 2000; Gong et al., 2004; Cheng et al., 2010; Wang et al., 2012). Comparably, much less is known about the role of PDE4 in drug dependence. Limited studies have shown that PDE4 is involved in the physical dependence and rewarding properties of morphine (Itoh et al., 1998; Thompson et al., 2004). Co-administration of rolipram, a prototypical, selective PDE4 inhibitor, and morphine decreases the signs of morphine withdrawal in mice and rats (Itoh et al., 1998; Gonzalez-Cuello et al., 2007; Nunez et al., 2009). This appears to be consistent with increased PDE4 activity induced by the naloxone challenge in morphine-treated rats (Kimura et al., 2006). These results suggest that rolipram may block the development of morphine dependence. In addition, PDE4 inhibitors may also block rewarded responses to drugs of abuse, as evidenced by rolipram-induced reduction of conditioning in morphine conditioned place preference (Thompson et al., 2004) and blockade of cocaine operant self-administration (Knapp et al., 1999). However, the role of PDE4 in heroin dependence has not been investigated. Most recently, we found that decreases in expression of pCREB in the NAc by stimulation of the vagus nerve contribute to heroin-seeking behavior (Liu et al., 2011). Thus, we hypothesize that rolipram benefits the treatment of heroin seeking via activation of cAMP/CREB signaling. In the present study, we characterized the role of PDE4 in heroin seeking after withdrawal by examining the effects of rolipram on heroin

self-administration and motivation of reward, and the reinstatement of heroin seeking induced by cues or heroin priming. The results provided novel evidence for the role of PDE4 in heroin reward and heroin-seeking behavior.

Materials and methods

Subjects and drugs

Adult male Sprague–Dawley rats (Zhejiang Experimental Animal Center, China), weighing 250–280 g at the beginning of the experiment, were housed individually in a temperature-controlled, ventilated colony room with a 12-h light/dark cycle (lights on 07:00.–19:00 hours). All experiments were conducted during the dark period according to specifications of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23, revised 1996). Food and water were available *ad libitum*.

Heroin (diacetylmorphine HCl) was obtained from the National Institute of Forensic Science (Beijing, China). The heroin dose (50 µg/kg/injection) used for the self-administration experiment was chosen on the basis of our previous studies (Zhang et al., 2004; Zhou et al., 2004). Heroin was dissolved in sterile saline at a concentration of 0.2 mg/ml, which was prepared daily. Rolipram (A.G. Scientific, San Diego, CA) was dissolved in dimethyl sulfoxide (DMSO) as the stock solution (1–3 mg/ml), which was diluted before use with saline to the concentrations needed in experiments. The final solutions of rolipram contained less than 2% DMSO, which was used as vehicle. Sodium pentobarbital (30 mg/ml; Sigma), heparin sodium (Qianhong Bioscience, China), benzylpenicillin sodium (Shiyao Bioscience, China) were dissolved in sterile saline.

Surgery procedure

Rats were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.) and implanted with chronically indwelling intravenous catheters as described previously (Zhou et al., 2005; Zhou et al., 2007). The catheters were flushed daily with 0.3 ml saline containing penicillin B (60 000 units) and heparin (5 units) to prevent potential bacterial infections and maintain catheter patency. All the animals were allowed to recover for at least 7 d before the beginning of tests.

Heroin self-administration training

During the daily 4-h training session of self-administration starting with the blue light on inside the active nose-poke hole, the rat received a single heroin infusion (50 µg/kg per infusion) following completion of the fixed ratio 1 (FR1) schedule. Each infusion was paired with 5-s illumination of the house light and in combination with the noise of the infusion pump, therefore serving as discrete conditioned stimulus (CS) paired
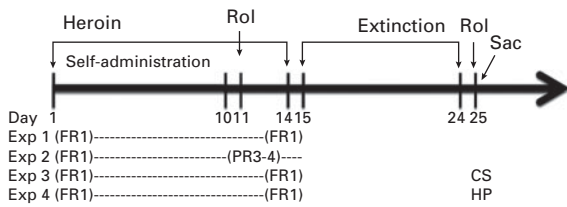
Fig. 1. Schedules of rolipram (Rol) treatments and tests. Rats were trained under the fixed ratio 1 (FR1) for self-administration of heroin (50 μg/infusion), 4 h per session each day for 14 d, followed by the 2-h extinction session for 10 d. During the extinction training, rats were returned to the chambers, but no heroin or cues delivered. Experiment (Exp) 1: On day 11, rolipram (0.03–0.3 mg/kg) was given (i.p.) 60 min before the training session. Exp 2: Also on day 11, the FR1 schedule was switched to progressive ratio (PR) for the 4-h session of heroin self-administration 60 min after rolipram treatment. Exp 3: Conditioned stimulus (CS)-induced reinstatement on day 25 after the 14-d self-administration followed by 10-d extinction. Rats were exposed to the CS; each subsequent nose-poke, which was previously paired with heroin, elicited a CS but no heroin injection. Rolipram was given 60 min before the beginning of the session, which lasted for 2 h after initial cue exposure. Exp 4: Heroin priming (HP)-induced reinstatement on day 25. Fifty minutes after rolipram administration, rats were injected with heroin (250 μg/kg, s.c.) and 10 min later, the 2-h session was started. Each active nose-poke response also resulted in another presentation of the CS. Rats were sacrificed (Sac) once the reinstatement session was completed.

with the drug infusion. A timeout period was imposed for 20 s, during which the responding produced no programmed consequences but was still recorded. Illumination of the blue light in the active nose-poke signaled the end of the 20-s timeout period. Responding in the inactive nose-poke produced no programmed consequences. The session ended after 4 h. Each rat received one heroin self-administration session per day for a total of 14 d (day 1–14; Fig. 1).

Break point of heroin self-administration

The progressive ratio (PR) reinforcement schedule (i.e. the third response requirement is four lever presses) required animals to increase nose poking progressively for each successive reward in the following series, within a self-administration session. There was a timeout of 20 s following an infusion in the PR schedule. The progression of response requirements was calculated by the equation: Response ratio = $(5 \times e^{(0.2 \times \text{infusion number})}) - 5$, which was rounded to the nearest integer (Roberts and Bennett, 1993). For example, over 28 reinforcers, the nose poking requirements were as follows: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 603, 737, 901, 1102, and 1347. The last ratio successfully completed was registered as the break point for that session (Duvauchelle et al., 1998; Wang et al., 2009).

Extinction and cues or heroin-induced reinstatement of heroin seeking

After two weeks of heroin self-administration training, the rats underwent the extinction procedure with one 2-h session daily for 10 d (day 15–24; Fig. 1). The extinction criterion was that the last active responses are less than 10% of the average responding on the active nose-poke during maintenance (Lai et al., 2013). During the extinction session, the rats were brought to the self-administration chambers in the absence of heroin infusions and the previously relevant cues. On day 25 after the completion of extinction (Fig. 1), all rats were tested for cues or heroin-induced reinstatement of heroin seeking. In the reinstatement induced by cues, the discrete CS were presented for 5 s, after which each active nose-poke response resulted in another presentation of the CS. Nose-pokes during this phase of CS reinstatement were accumulated for over 120 min. In the reinstatement induced by heroin priming, the rats were injected with heroin (250 μg/kg, s.c.) 10 min before the beginning of the test; each active nose-poke response also resulted in another presentation of the CS. Nose-pokes during this phase of heroin-primed reinstatement were also accumulated for over 120 min.

Oral sucrose self-administration

Twenty-four rats were trained to nose-poke for sucrose pellets. The paradigm was similar to heroin self-administration described above, except that rats received a 45 mg sucrose pellet (Research Diets, Inc., USA) delivered via a sucrose cup. The operant chambers were equipped with two holes. The active nose-poke stimulus light was illuminated when a sucrose pellet was available. Rats were trained to nose-poke on a fixed-ratio 5 (FR5) schedule for a sucrose pellet for 30 min each day. They received a sucrose pellet following completion of the ratio requirement in the active hole, which was followed by a 20-s timeout signaled by illumination of the house lights. After they acquired sucrose self-administration for 10 d, the rats were randomly divided into four groups (n = 6 in each group), which were treated (i.p.) with vehicle or rolipram at 0.03, 0.1, or 0.3 mg/kg 60 min prior to the training session.

Specific experiment procedures

Experiment 1: Effect of rolipram on heroin self-administration

A total of 64 rats were used to start the heroin self-administration, but three of them were excluded because they did not meet the training standard for heroin self-administration or extinction.

Thirty-two rats were tested for the effect of rolipram on the reinforcement of heroin self-administration. After heroin self-administration training for 10 d under the FR1 schedule, the rats were randomly divided into four

groups ($n=8$ per group), which were treated (i.p.) with vehicle or rolipram at 0.03, 0.1, or 0.3 mg/kg 60 min prior to the FR1 training session on day 11 (Fig. 1). The results were accumulated for 4 h. After rolipram testing, heroin self-administration continued without drug treatment during day 12–14.

Experiment 2: Effect of rolipram on heroin-induced reward motivation

A separate set of 30 rats was tested for the effect of rolipram on the reward motivation of heroin self-administration. After heroin self-administration training for 10 d under the FR1 schedule, the rats were randomly divided into four groups and injected (i.p.) with vehicle ($n=8$) or rolipram at 0.03 mg/kg ($n=8$), 0.1 mg/kg ($n=7$), or 0.3 mg/kg ($n=7$) at 60 min prior to the training session on day 11, when the training procedure was switched to the PR schedule for 4 h. After rolipram testing, heroin self-administration continued without drug treatment during day 12–14.

Experiment 3: Effect of rolipram on cue-induced reinstatement of heroin seeking

After 14-d heroin self-administration under the FR1 schedule followed by 10-d extinction (i.e. on day 25; Fig. 1), the rats that had been tested in Experiment 1 were tested for evaluating the effects of rolipram on cue-induced reinstatement of heroin seeking. The rats were injected (i.p.) with rolipram (0.03, 0.1, or 0.3 mg/kg) or vehicle ($n=8$ per group) 60 min before they were re-introduced into the training chambers. When the test started, the rats were presented with the CS, and each subsequent nose-poke in the hole previously paired with heroin elicited a CS without heroin injection. CS-induced reinstatement was observed for 2 h after initial cues presentation.

Experiment 4: Effect of rolipram on heroin-priming-induced reinstatement of heroin seeking

Also on day 25 after the 10-d extinction period, reinstatement tests were performed in rats that had been tested in experiment 2 to evaluate the effects of rolipram on heroin seeking induced by heroin priming. Rats were injected (i.p.) with rolipram at 0.03 mg/kg ($n=8$), 0.1 mg/kg ($n=7$), or 0.3 mg/kg ($n=7$), or vehicle ($n=7$) 50 min prior to heroin (250 μg/kg), which was given (s.c.) 10 min before the beginning of the 2-h testing session as described above.

Western blot analysis

Under deep anesthesia with pentobarbital (80 mg/kg), additional rats of extinction training on day 24 and rats of experiment 4 on day 25 were decapitated immediately after reinstatement testing (Fig. 1) and NAc tissues were dissected from the brain for Western blotting analysis.

Briefly, the NAc tissues were directly lyzed in the sodium dodecyl sulfate sample buffer and incubated at 95 °C for 10 min before loading on a 10% sodium dodecyl sulfate-polyacrylamide gel. Proteins (50 μg) were separated by electrophoresis (SDS-PAGE) before transferred to a nitro-cellulose membrane (BioRad, USA). The membrane was blocked in 5% milk-TBST, probed with anti-pCREB and anti-CREB antibodies (both 1:1000; sc-7978-R for pCREB; Santa Cruz Biotechnology Inc., USA), both in 3% milk-TBST, and then reacted with the horseradish peroxidase-conjugated secondary antibody (1:2000) also in 3% milk-TBST. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal protein control (1:2000). Immunoreactive protein bands were detected by using the Odyssey CLx infrared imaging system for analyzing the changes in pCREB/CREB expression in the NAc (for Fig. 4) during the extinction and heroin priming. The immunoblotting bands in Experiment 4 (for Fig. 5) were analyzed by integrating densitometry using GeneSnap and GeneTools (Chemigenius Gel Documentation System, Syngene, UK).

Data statistics

The data from the self-administration and reinstatement tests were analyzed separately. The mean number of infusions or responses for active and inactive holes during self-administration was analyzed using a one-way analysis of vaviance (ANOVA). Newman–Keuls multiple comparisons were used for *post-hoc* analysis between groups. Statistical significance was considered when the p value was less than 0.05.

Results

Effect of rolipram on the heroin reinforcement and motivation value

As shown in Fig. 2, rolipram tended to decrease the active responses, but the one-way ANOVA revealed neither significant changes in the active responses ($F_{(3,28)}=1.39$, $p=0.27$) nor inactive nose-pokes ($F_{(3,28)}=2.26$, $p=1.1$) compared to the vehicle control (Fig. 2a). The total heroin injections received were not altered among the groups ($F_{(3,28)}=1.69$, $p=0.19$; Fig. 2b).

To determine whether rolipram attenuated the reward motivation of heroin self-administration, rats were examined under the PR schedule following rolipram treatment. The one-way ANOVA revealed a significant effect of rolipram on the accumulated responses of active nose-pokes ($F_{(3,26)}=10.36$, $p<0.01$), but not that of inactive nose-pokes ($F_{(3,26)}=0.92$, $p=0.44$; Fig. 2c). *Post-hoc* Newman–Keuls tests indicate that rolipram at doses of 0.03–0.3 mg/kg significantly decreased the break point of the active responses ($p<0.01$) in a dose-dependent manner, while it did not affect the break point of the inactive responses relative to the corresponding vehicle control. Rolipram at the same doses also decreased the total
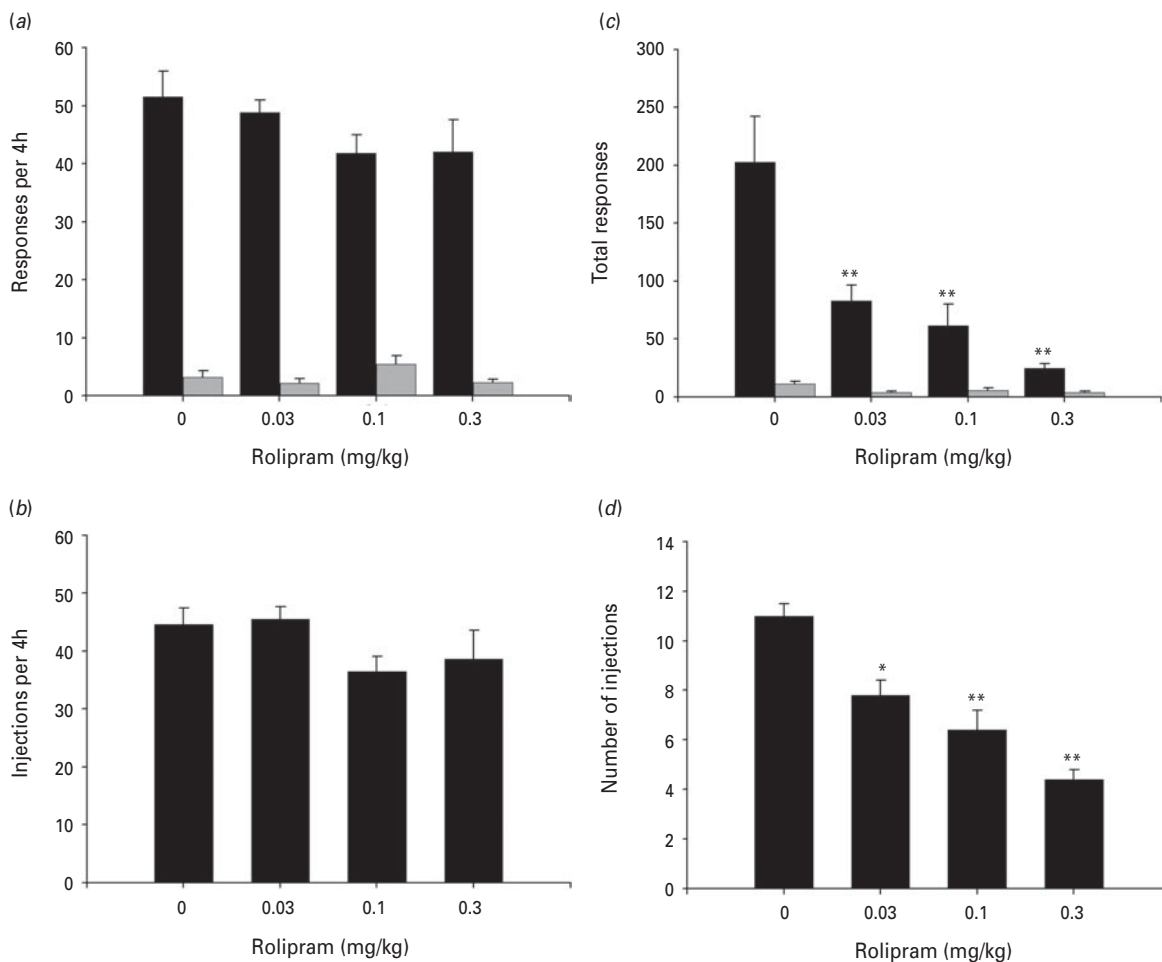
Fig. 2. Effects of pretreatment with rolipram on heroin reinforcement and heroin reward values in rats. While rolipram did not significantly change the active or inactive nose-pokes (*a*) or heroin infusions (*b*) during the training of heroin self-administration using the FR1 schedule, it decreased the mean total responses of active nose-pokes (*c*) and infusion breakpoints (*d*) for intravenous heroin on the progressive ratio schedule (PR) of reinforcement. Rats were administered (i.p.) vehicle, 0.03, 0.1, or 0.3 mg/kg rolipram 60 min prior to the 4-h training session on day 11. Data shown are means±S.E.M; *n*=7–8 per group. *, **, Significant difference from vehicle ($p < 0.05$, $p < 0.01$, respectively).

heroin injections received in a dose-dependent manner ($F_{(3,26)} = 18.22$, $p < 0.01$; Fig. 2*d*); at the lowest dose (0.03 mg/kg), rolipram significantly decreased the total injections under the PR schedule ($p < 0.05$).

Effect of rolipram on heroin seeking induced by cues or heroin priming

To determine whether rolipram affected cue-induced seeking behavior, we designed Experiment 3 to evaluate the effect of rolipram on cue-induced reinstatement of heroin seeking after extinction for 10 d. As shown in Fig. 3*a*, one-way ANOVA revealed that the cue-induced active nose-pokes were reduced by rolipram at doses of 0.03–0.3 mg/kg ($F_{(3,28)} = 5.95$, $p = 0.003$), while the inactive nose-pokes were not significantly different from the vehicle control ($F_{(3,28)} = 0.93$, $p = 0.44$). *Post-hoc* Newman–Keuls comparisons indicate that rolipram at any of the doses (0.03–0.3 mg/kg) used significantly decreased cue-induced active responses ($p < 0.01$; Fig. 3*a*).

To determine whether rolipram affected heroin priming-induced seeking behavior, we designed Experiment 4 to evaluate the effect of rolipram on heroin-induced reinstatement of seeking following heroin-pretreatment after 10-d extinction. One-way ANOVA revealed that heroin-induced active nose-pokes were reduced by rolipram ($F_{(3,25)} = 12.65$, $p < 0.01$), whereas the inactive nose-pokes were not significantly different from the vehicle control ($F_{(3,25)} = 2.71$, $p = 0.07$; Fig. 3*b*). *Post-hoc* Newman–Keuls comparisons indicate that rolipram at any of the doses (0.03–0.3 mg/kg) used significantly decreased heroin-induced active responses ($p < 0.01$).

Effect of rolipram on expression of pCREB in the NAc

To compare pCREB levels in the extinction and heroin priming and determine whether CREB signaling was involved in the effects of rolipram on heroin seeking, expression of pCREB and total CREB (T-CREB) was examined using immunoblotting analysis in the NAc of
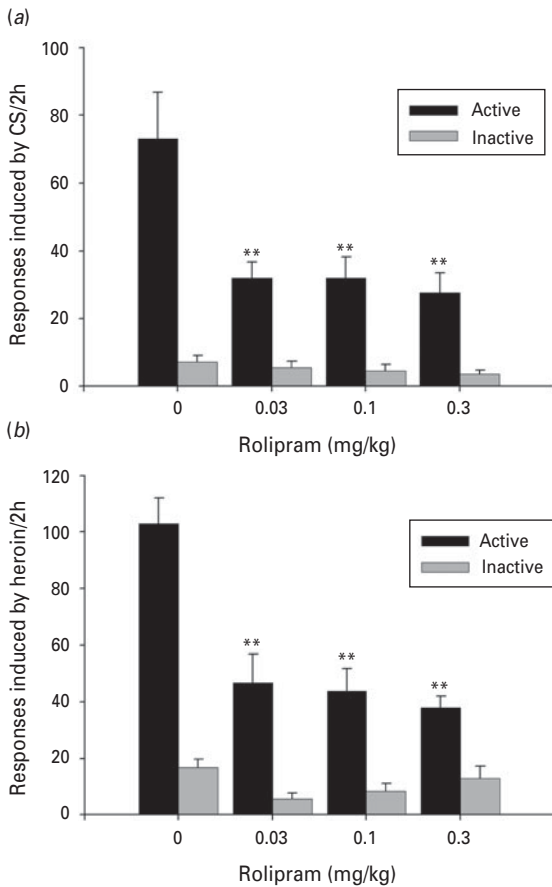
Fig. 3. Effect of rolipram on heroin seeking induced by cues or heroin priming in rats. Rolipram decreased active, but not inactive responses during reinstatement induced by cues (*a*) or heroin priming (*b*). Rats were administered (i.p.) vehicle, 0.03, 0.1, or 0.3 mg/kg rolipram 60 min before returned to the operant chambers and exposed to the cues previously paired with heroin infusions (for *a*) or 60 min before the injection of heroin (0.25 mg/kg, s.c.) and returning to the operant chamber (for *b*). The test was performed for both *a* and *b* on day 25 after the 10-day extinction following the 14-d self-administration training (Fig. 1). Data shown are means±S.E.M; *n*=8 for A or 7–8 for B; **, Significant difference from vehicle (*p*<0.01).

Fig. 4. Expression of pCREB in the nucleus accumbens (NAc) of rats during the extinction (Ext) and heroin priming. (*a*) The representative immunoblotting bands show the expression of pCREB, total CREB (T-CREB), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (the internal control) in the same brain tissue samples. (*b*) Quantification analyses using Odyssey CLx infrared imaging system show the relative levels of pCREB and CREB proteins over GAPDH. (*c*) The responses during the extinction or heroin priming. Bars shown represent means±S.E.M; *n*=4; **, Significant difference from Extinction (*p*<0.01).

rats immediately after the last extinction training and completion of the reinstatement test induced by heroin priming, during which rolipram or its vehicle was given. One-way ANOVA revealed that heroin priming produced significantly higher active responses ($F_{(1,14)}=235.5$, $p<0.001$) and lower levels of pCREB expression ($F_{(1,6)}=478.8$, $p<0.001$), without altering T-CREB levels ($F_{(1,6)}=0.029$, $p=0.87$) in the NAc (Fig. 4). Post-hoc Newman–Keuls comparisons indicate a significant decrease in pCREB levels in the NAc ($p<0.01$; Fig. 4a, b) and an increase in active responses ($p<0.01$; Fig. 4c), relative to the last extinction training. Rolipram (0.03–0.3 mg/kg) increased expression of pCREB, without altering T-CREB levels in the NAc of rats following the heroin-induced reinstatement test, relative to the vehicle control (Fig. 5a); one-way ANOVA revealed significance for pCREB
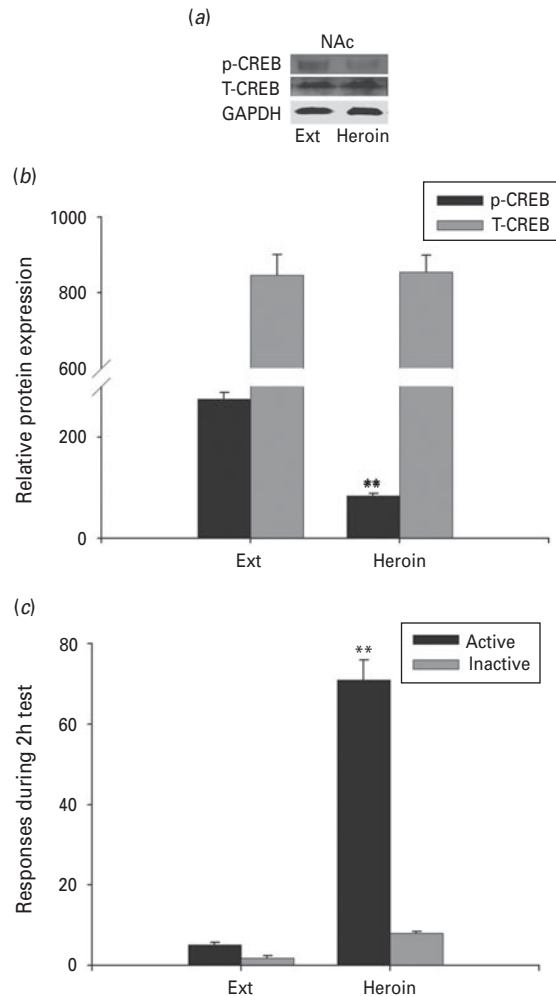
($F_{(3,8)}=122.3$, $p<0.001$), but not T-CREB ($F_{(3,8)}=1.45$, $p=0.30$; Fig. 5b]. Post-hoc Newman–Keuls comparisons indicate significant increases in pCREB levels in the NAc following treatment with rolipram at doses of 0.03, 0.1, or 0.3 mg/kg ($p<0.01$).

Effect of rolipram on the sucrose reinforcement

To determine whether rolipram specifically affected heroin seeking, the effect of rolipram on sucrose self-administration was examined in a separate set of rats using similar procedures. One-way ANOVA revealed overall decreases in the active nose-pokes ($F_{(3,20)}=17.15$,
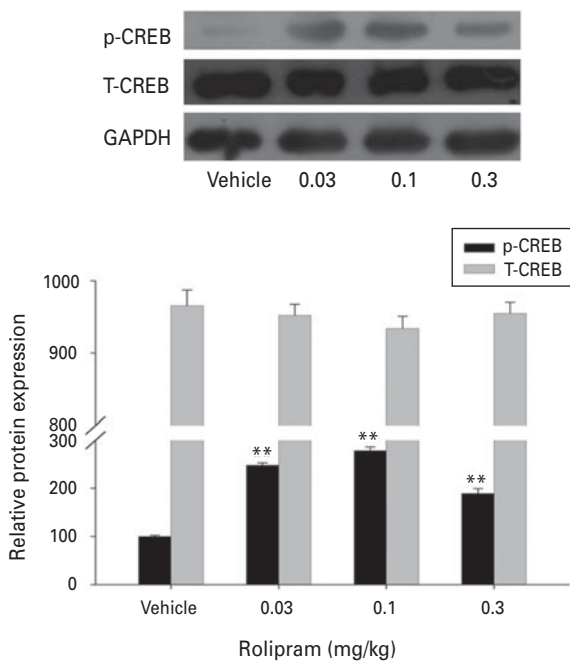
Fig. 5. Effects of rolipram on expression of pCREB in the nucleus accumbens (NAc) of rats following the heroin-induced reinstatement test. The representative immunoblotting bands (upper panel) show the expression of pCREB, total CREB (T-CREB), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (the internal control) in the same brain tissue samples. Quantification analyses (lower panel) show the relative levels of pCREB and CREB proteins over GAPDH. Bars shown represent means±S.E.M; $n=4$; **, Significant difference from vehicle ($p < 0.01$).

Fig. 6. Effect of pretreatment with rolipram on sucrose reinforcement in rats. Rolipram at 0.3 mg/kg decreased active, but not inactive nose-pokes (*a*) and sucrose pellets (*b*) during sucrose self-administration training. Rats were administered (i.p.) vehicle, 0.03, 0.1, or 0.3 mg/kg rolipram 60 min prior to the 30-min FR5 training session. Data shown are means±S.E.M; $n=6$ per group. **, Significant difference from vehicle ($p < 0.01$).

$p < 0.01$) and total pellets received ($F_{(3,20)}=16.93$, $p < 0.01$), but unaltered inactive nose-pokes ($F_{(3,20)}=0.48$, $p=0.69$), following treatment with rolipram (0.03–0.3 mg/kg; Fig. 6a, b). *Post-hoc* Newman–Keuls comparisons indicate that rolipram significantly reduced the active responses and the total number of sucrose pellets only at the highest dose (0.3 mg/kg; $p < 0.01$); at the lower doses of 0.03 and 0.1 mg/kg rolipram was not effective (Fig. 6).

Discussion

While limited studies have shown that PDE4 appears to be involved in physical dependence and tolerance to morphine (Itoh et al., 1998; Mamiya et al., 2001), there is no evidence for the potential contribution of PDE4 to heroin dependence. In the present study, we provided promising demonstration that pretreatment with rolipram decreased the motivational value of heroin under the schedule of progressive ratio; it also inhibited heroin-seeking behavior induced by cues or heroin priming. In contrast, rolipram did not affect heroin self-administration at doses reducing reinstatement of heroin-seeking behavior. The increased expression of pCREB in the NAc by rolipram may contribute to the inhibitory action of rolipram on
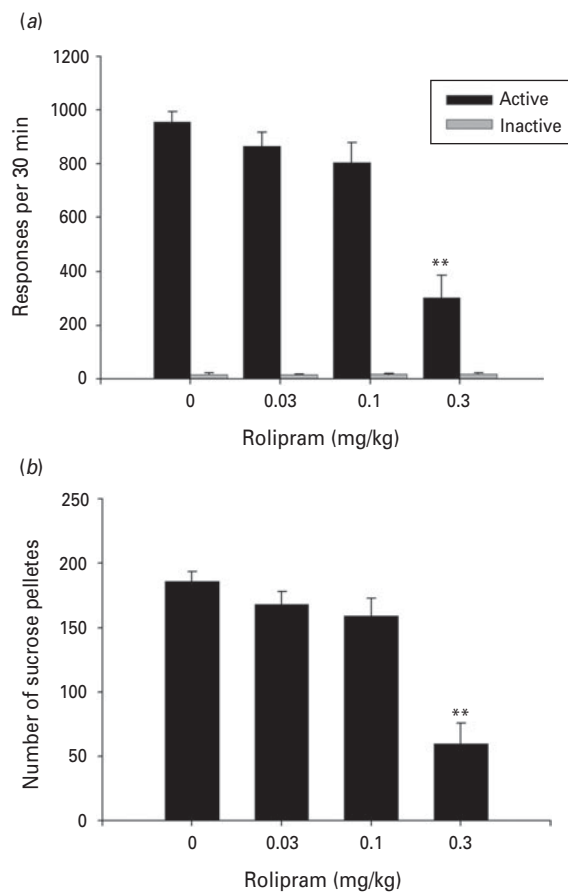
heroin seeking. Our study provides novel evidence for the role of PDE4 in heroin-seeking behavior.

The attenuation by rolipram of heroin-seeking behavior appears to be independent of the sedative action of rolipram. While rolipram produces sedation in rodents, in particular at doses as high as 0.5–1 mg/kg (Silvestre et al., 1999a; Zhang and O'Donnell, 2000; Hu et al., 2011), the hypoactivity induced by rolipram at the dose of 0.3 mg/kg only lasts for approximately 20 min (Wen et al., 2012). In addition, rolipram treatment did not significantly alter inactive responding at the doses used. Further, rolipram at the dose (0.03 mg/kg) that does not alter the operant performance measuring depressive-like behavior in rats (Zhang et al., 2005a) also attenuated the reinstatement of heroin seeking induced by cues or heroin priming. These results suggest that blockade of heroin-seeking behavior by rolipram may not be attributed to the sedative or antidepressant profile of rolipram.

The mechanisms whereby rolipram attenuated heroin-seeking behavior remain unclear. It is noted that rolipram

does not cause euphoria or dependence (Mamiya et al., 2001), indicating that rolipram may not mimic heroin to alter heroin-seeking behavior. However, the effects of rolipram on rewarded responses to opioid, anxiety and opioid withdrawal syndrome should be taken into consideration. The potential action of rolipram on rewarded responses to opioids may also be involved. Rolipram decreases morphine or cocaine place preference conditioning (Thompson et al., 2004). It also inhibits the initiation of operant responding for cocaine, while responding after drug-induced delays tends to be at control levels (Knapp et al., 1999). These results appear to be consistent with inhibition of ethanol self-administration by rolipram in rats (Wen et al., 2012). However, unaltered heroin self-administration following treatment with rolipram at the doses that attenuated heroin seeking indicate that rolipram may not alter the rewarded processes activated by heroin under the conditions in the present study. Interestingly, rolipram at the dose of 0.3 mg/kg inhibited the active responses and sucrose pellet consumption; this is consistent with the previous findings that rolipram reduces response rate for food and water (Knapp et al., 1999; O'Donnell and Frith, 1999).

Cholinergic neurons are involved heroin reward. Enhancing either systemic or NAc acetylcholine (ACh) function inhibits heroin reward and heroin-seeking behavior (Zhou et al., 2007). ACh input into the ventral tegmental area (VTA) from the lateral dorsal tegmental nucleus (LDT) drives reward processes and heroin-seeking behavior, but cholinergic modulation of VTA neurons could be inhibited by ACh via activation of the autoreceptors on LDT cholinergic neurons, which can modify the reinforcing value of natural and drug rewards (Shabani et al., 2010; Liu et al., 2012). Given that ACh is involved in the behavioral effects of rolipram (Silvestre et al., 1999b), rolipram may inhibit the natural reward at least partially through the enhancement of cholinergic transmission in the brain (Hoebel et al., 2007). Meanwhile, other pharmacological effects of rolipram such as sedative effects observed at 0.3 mg/kg also may contribute to its inhibitory effects during the 30 min testing session (Knapp et al., 1999; Wen et al., 2012).

Anxiety disorders are one of the common symptoms in opioid withdrawal and heroin withdrawal produces anxiety-like behavior in rats (Le Roy et al., 2013). It has been noted that rolipram decreases withdrawal signs and severity of withdrawal syndrome precipitated by naloxone in morphine-dependent animals (Hamdy et al., 2001; Mamiya et al., 2001) and produces anxiolytic activity in rodents (Silvestre et al., 1999a; Li et al., 2009). Moreover, rolipram alone or in combination with the classic anxiolytic diazepam attenuates morphine withdrawal syndromes in rodents (Gonzalez-Cuello et al., 2007; Nunez et al., 2009). We did not observe significant indications of heroin dependence in the rats under the present conditions. Nevertheless, studies to date have shown controversial results in terms of the effect of rolipram on

anxiety-like behavior; repeated treatment with rolipram appears to be required to produce anxiolytic activity (Silvestre et al., 1999a; Li et al., 2009). Additionally, a relatively high dose (e.g. 1 mg/kg) of rolipram appears to be required to block the withdrawal syndromes in morphine dependent rodents (Hamdy et al., 2001; Mamiya et al., 2001; Gonzalez-Cuello et al., 2007; Nunez et al., 2009). In the present study, rolipram at a dose as low as 0.03 mg/kg attenuated heroin seeking. While there is no evidence for the capability of rolipram at this dose to block withdrawal behavioral manifestations in opiate dependent animals, it has been demonstrated that a lower dose (0.025 mg/kg) of rolipram does effectively decrease ethanol consumption and preference in rats (Wen et al., 2012). Thus, further studies are needed to clarify the contribution of potential anxiolytic and anti-withdrawal actions of rolipram to attenuated heroin seeking.

It has been well documented that treatment with rolipram increases cAMP/PKA/CREB signaling in brain regions including the striatum and NAc (Schneider, 1984; Barad et al., 1998; Zhang et al., 2002; Monti et al., 2006; Rutten et al., 2008; Li et al., 2009; Rutten et al., 2009; Li et al., 2011). This may be the neurochemical mechanism by which rolipram attenuated heroin-seeking behavior. More specifically, rolipram treatment increases cAMP levels and expression of pCREB in the brain, leading to attenuation of heroin-seeking behavior, as analyzed above. In addition, rolipram-induced increases in pCREB in the NAc may decrease motivational responses of heroin, given that CREB$^{\alpha\Delta}$ mutant mice display increases in opioid rewarding (Walters et al., 2005). In the particular experiment measuring the heroin motivation in rats under the PR reinforcement schedule, animals were required to complete an increasing number of nose-pokes to obtain heroin. Treatment with rolipram increased pCREB in the NAc and consequently decreased this motivation, although it failed to affect heroin self-administration under the FR1 schedule. Consistent with these results, our previous study revealed that increased expression of pCREB in the NAc induced by vagus stimulation also correlates with inhibition of heroin-seeking behavior (Liu et al., 2011).

PDE4 has four subtypes (PDE4A-D) encoded by their distinct genes (O'Donnell and Zhang, 2004; Zhang, 2009). Since rolipram inhibits all the four subtypes at equivalent potency (MacKenzie and Houslay, 2000; Zhang et al., 2005b), the contribution of specific PDE4 subtypes to heroin seeking could not be addressed in the present study. However, as the predominant PDE4 subtype in the striatum and NAc, PDE4B may be the major subtype of PDE4 in regulating heroin-seeking behavior. Further studies using PDE4-subtype knockout mice (Zhang et al., 2002; Zhang et al., 2008) are needed to test this prediction.

In conclusion, our study demonstrated that increased cAMP/CREB signaling by inhibition of PDE4 decreased heroin motivation and heroin-seeking behavior. The

results provide promising evidence for that PDE4 can be a target for mediating heroin seeking. PDE4 inhibitors may be used as the novel approach for treatment of heroin addiction, although the mechanisms of actions and the contribution of specific PDE4 subtypes remain to be elucidated.

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Statement of Interest

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