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ORIGINAL INVESTIGATION

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cAMP-dependent protein kinase and reward-related learning: intra-accumbens Rp-cAMPS blocks amphetamine-produced place conditioning in rats

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Abstract *Rationale:* Dopamine may produce reward-related learning by activating D_1-like receptors in the nucleus accumbens (NAc) and stimulating the formation of cyclic adenosine monophosphate (cAMP) and the activation of cAMP-dependent protein kinase (PKA). *Objectives:* This hypothesis was tested using the conditioned place preference (CPP) based on NAc injections of amphetamine (amph) and evaluating the effects of PKA inhibition with Rp-cAMPS. *Methods:* The CPP procedure consisted of three phases: pre-exposure (three 15-min sessions in a chamber consisting of two distinct compartments connected by a tunnel), conditioning (four 30-min placements into one compartment with the tunnel blocked following drug injection into the NAc alternating with four similar placements into the other side following NAc injection of saline), and test (one 15-min session with the tunnel open). A CPP was defined as an increase in time spent on the drug-paired side from mean pre-exposure to test. *Results:* Dose-response experiments showed that 15.0 or 20.0 but not 5.0 or 10.0 μg/0.5 μl per side of amph produced a CPP. The amph (20.0 μg) CPP was blocked by Rp-cAMPS co-injections of 25.0 and 250 but not 2.5 ng/0.5 μl per side. Rp-cAMPS or the PKA activator Sp-cAMPS (50.0, 250, 500, 600 ng/0.5 μl per side) alone had no effect on side preference. Co-injection of 10.0 μg amph+Sp-cAMPS (25.0, 50.0, 250, 500 ng) did not result in a CPP but co-injection of 20.0 μg amph+Sp-cAMPS (250 ng) led to a loss of the CPP normally seen with that dose of amph. Doses of Rp-cAMPS that blocked CPP did not block the locomotor stimulatory effect of amph during conditioning sessions. *Conclusions:* Results supported the hypothesis that PKA activation in NAc is necessary for reward-related learning.

Keywords Accumbens · cAMP-dependent protein kinase · Locomotor activity · Amphetamine · Conditioned place preference · PKA · Place conditioning · Reward · Rp-cAMPS · Sp-cAMPS

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Introduction

Dopamine (DA) has been implicated in reward-related incentive learning. DA antagonists block the rewarding effects of food (Wise et al. 1978), water (Gramling and Fowler 1985), sex (Pfaus and Phillips 1991) and other rewarding stimuli in a number of species (Beninger 1983). DA agonists such as amphetamine (amph; Pickens and Harris 1968) and cocaine (Pickens and Thompson 1968) produce rewarding effects that are blocked by DA antagonists, as are the rewarding effects of opiates, nicotine and alcohol (Di Chiara 1999), implicating DA in the rewarding effects of agents that do not directly stimulate DA neurons. Rewarding stimuli produce DA release (Kiyatkin 1995), increase activity in DA neurons in monkeys (Schultz et al. 1997; Schultz 2000) and increase metabolic activity in the NAc in humans (Elliott et al. 2000; Knutson et al. 2001). It should be noted that DA is also released by aversive stimuli (Horvitz 2000). DA is strongly implicated in reward-related learning.

Second messenger pathways play a critical role in learning (Sheng and Kim 2002) and the cyclic adenosine 3',5'-monophosphate (cAMP) cascade, leading to the activation of cAMP-dependent protein kinase (PKA), is involved. This pathway is implicated in activity-dependent long term sensitization in *Aplysia* (Kandel 2001), odor discrimination learning in *Drosophila* (Waddell and Quinn 2001), classical conditioning to olfactory stimuli in honeybees (Fiala et al. 1999), discrimination learning in chicks (Rose 2000), late phase long term potentiation in the rat hippocampus (Kandel 2001), inhibitory avoidance

learning in rats (Izquierdo et al. 1999), cocaine self-administration in rats (Self et al. 1998), fear (Schafe and LeDoux 2000) or appetitive (Jentsch et al. 2002) conditioning in rat amygdala, working memory in the frontal cortex (Taylor et al. 1999; Aujla and Beninger 2001) and conditioned activity based on intra-NAc injections of amph in rats (Sutton et al. 2000). There is a remarkable preservation of the mechanisms involving the cAMP-PKA pathway in learning across a wide range of species.

DA may produce reward-related learning by activating the cAMP-PKA pathway. Many results show that D_1-like receptors, those that stimulate the cAMP-PKA pathway, play a critical role. For example, D_1-like agonists are rewarding (Self et al. 1996; Abrahams et al. 1998) and D_1-like antagonists block the effects of reward (Beninger and Miller 1998). D_2-like agents similarly affect behaviour but a number of studies show important differences between D_1- and D_2-like agents in their effects on reward. D_1-like agonists impair responding for a number of rewards, whereas D_2-like agonists often enhance rewarded responses; D_1-like agonists may mask (occlude) the signal generated in DA neurons by reward, producing the observed impairments (Beninger and Miller 1998). Both D_1- and D_2-like antagonists block responding for reward, but detailed analyses have shown a dissociation of reward-blocking from anti-locomotor effects for D_1- but not D_2-like antagonists (Fowler and Liou 1994). Thus, the critical role for D_1-like receptors in reward-related learning provides indirect support for the hypothesis that stimulation of the cAMP-PKA pathway may also play an important role.

The present experiments used the conditioned place preference (CPP) paradigm to test the hypothesis that stimulation of the cAMP-PKA pathway plays a critical role in reward-related learning. The NAc has been shown to be an important structure for CPP learning (Carr and White 1986). In the present experiments, we established the dose-response function for CPP with NAc amph. Then we tested the effects of co-injections of amph plus the PKA inhibitor Rp-adenosine 3',5'-cyclic monophosphosphonothioate triethylamanine (Rp-cAMPS) to evaluate the hypothesis that activation of the second messenger pathway was critical for amph reward in the NAc. Control experiments evaluated the effects of Rp-cAMPS alone. Additional experiments assessed the ability of the PKA activator Sp-adenosine 3',5'-cyclic monophosphonothioate triethylamanine (Sp-cAMPS) to produce a CPP when injected either alone or along with a subthreshold dose of amph into the NAc. Studies with Sp-cAMPS failed to produce a CPP, possibly because of intracellular "noise" in second messenger pathways produced by the activator. A final study evaluated this idea by co-injecting Sp-cAMPS with a suprathreshold dose of amph to test the hypothesis that the usual rewarding effects of amph would be impaired by the activator.

Materials and methods

Subjects

Treatment was in accordance with the guidelines of the Animals for Research Act, the Canadian Council on Animal Care and Queen's University. Experimentally naive male Wistar rats (Charles River, St Constant, Quebec, Canada) weighing 225–250 g were group-housed (four per cage) in hanging wire cages, prior to surgery, in a temperature-controlled (21±1°C) colony room on a 12-h light-dark cycle (lights on at 0700 hours). Food (Purina rodent lab chow #5001) and water were freely available in the home cages. All rats were handled daily for at least 5 days prior to surgery. After surgery, rats were housed (two per box) on wood shavings in clear plastic cages.

Surgery

Rats were anesthetized with an oxygen flow containing 4% halothane (Halocarbon Labs, River Edge, N.J., USA); during surgery, the flow was decreased to 0.5–2.0% in response to the rat's breathing. Using standard stereotaxic surgical procedures rats were implanted with chronic bilateral stainless steel guide cannulae (0.64 mm diameter, 13 mm long) at the coordinates:1.2 mm anterior to bregma, 2.0 mm lateral to the midline and 7.0 mm ventral to the skull surface (Paxinos and Watson 1998). Stainless steel pins (0.31 mm diameter) were inserted into the guide cannulae to keep them unoccluded. Each rat received 0.2 ml SC injections of the analgesic buprenorphine (10% in saline) at the onset of surgery and 8–12 h after for pain relief. Bupivacaine (Marcaine 0.3 ml) was injected around the incision for local analgesia. One week of recovery preceded testing.

Drugs

d-Amphetamine sulfate (Bureau of Drug Surveillance, Health Canada) was dissolved in sterile saline each day 30 min prior to testing. Rp- or Sp-cAMPS (RBI, Oakville, Ontario, Canada) were dissolved in distilled water, aliquotted and frozen until needed. Injections were made in a volume of 0.5 μl per side. Doses for amph were 5.0, 10.0, 15.0 or 20.0 μg per side. Those of Rp- or Sp-cAMPS were 2.5, 25.0, 50.0, 250, 500 or 600 ng per side.

Intracranial drug injections

A 10.0 μl microsyringe (Hamilton Co., Reno, Nev., USA) mounted on an infusion pump (Sage Instrument Model 355) infused the drug at a constant rate of 1.0 μl/min. Injection cannulae, cut 1.0 mm longer than the guides, were made from stainless steel tubing (0.31 mm diam). Polyethylene tubing attached injection cannulae to the microsyringe. Drug injections were delivered over a 30-s interval and injection cannulae remained in place for an additional 30 s to allow for diffusion.

Apparatus

Four rectangular wooden boxes, with removable Plexiglas covers, consisted of two compartments (38×27×36 cm) that were connected by a closable tunnel (8×8×8 cm). The compartments were different: walls consisted of urethane-sealed wood or Plexiglas with black and white stripes (1 cm wide); floors were galvanized steel mesh or stainless steel bars (1 cm apart). Tunnel floors were galvanized sheet metal. The arrangement of walls and floor types had a unique configuration for each box. Photo emitters and detectors were located in each box: two in each compartment (height 5 cm) and one at each end of the tunnel (height 3 cm). A micro-controller hosted by a Macintosh computer, recorded the time spent in each compartment and number of beam breaks. Boxes were housed in

Styrofoam-insulated chambers, indirectly illuminated by a 7.5 W light bulb, and ventilated with a small electric fan. For further details, see Brockwell et al. (1996).

Procedure

Pre-exposure (Pre-ex)

Rats received three (one per day) 15-min sessions with the tunnel open. No drugs were administered. Half of the rats started in the left compartment and the other half in the right. The amount of time spent in each compartment and in the tunnel was recorded.

Conditioning

Rats received eight 30-min sessions with the tunnel blocked. Drug injections were paired with one compartment on days 1, 3, 5, and 7, and saline injections were paired with the other on days 2, 4, 6, and 8. For half of the rats the start compartment was paired with drug and for the other half with saline. Activity (number of beam breaks) was recorded.

Testing

Rats received one 15-min session with the tunnel open. The amount of time spent in each compartment and in the tunnel was recorded.

Groups

Groups were defined by the drug treatment they received during conditioning. Injections always were given immediately before placement into the conditioning chamber. In amph dose-response experiments, four groups received amph doses of 5.0, 10.0, 15.0 or 20.0 μg/0.5 μl per side. In Rp+amph experiments, four groups received amph (20.0 μg/0.5 μl per side) plus Rp-cAMPS doses of 0, 2.5, 25.0 or 250 ng/0.5 μl per side. In Rp alone experiments, three groups received Rp-cAMPS doses of 2.5, 25.0 or 250 ng/0.5 μl per side. In Sp alone experiments, four groups received Sp-cAMPS doses of 50.0, 250, 500 or 600 ng/0.5 μl per side. In Sp+amph (10.0 μg) experiments, four groups received amph (10.0 μg/0.5 μl per side) plus Sp-cAMPS doses of 25.0, 50.0, 250 or 500 ng/0.5 μl per side. In the Sp+amph (20.0 μg) experiment, one group received amph (20.0 μg/0.5 μl per side) plus an Sp-cAMPS dose of 250 ng/0.5 μl per side. The amph (20.0 μg/0.5 μl per side) group from the amph dose-response experiment was used for comparison.

The experiments were performed in two parts separated by over a year. The amph+Rp and Rp alone studies were done by P.L.N. The remaining studies were carried out by I.S.

Histological analyses

Coronal brain sections (70 μm) were mounted on glass slides and stained with Nissl cell body stain. Verification of injection sites was performed by an observer who was blind to the behavioral results. Animals were classified as hits if the tips of both cannulae were located in the region of core or shell of NAc. Animals with one or both cannulae placements outside of NAc and those with infections or procedural or health problems were excluded from the main analyses.

Statistical analyses

For place conditioning, the dependent variable was amount of time (s) spent on the drug-paired side during the average of the 3 pre-ex days and during the test. Scores were compared across groups within experiments using analyses of variance (ANOVA) and planned *t*-tests for each group. Planned *t*-tests evaluated the hypothesis that amph, Rp- or Sp-cAMPS alone or together would produce a change in time spent on the drug-paired side. Locomotor activity (counts/5 min) on drug and saline days during conditioning for the groups from each experiment was compared using ANOVA with independent groups and repeated measures on treatment (drug versus saline) followed by post hoc tests where appropriate.

Results

Histology

The numbers of rats operated for each experiment, found to have bilateral cannulae in NAc core or shell, and with placements outside of NAc, are indicated in Table 1. Placements for the rats included in the amph dose-response experiment are shown in Fig. 1; placements for the other experiments were similar. For the amph dose-response experiment, rats with placements outside of NAc were included in additional analyses of time spent on the drug-paired side to provide information on anatomical specificity of the effects. For the Rp+amph experiments, the animals that did not have bilateral NAc placements were further dissociated into subgroups with one cannula

Table 1 Total number of rats used in each experimental group and the summary of histological results indicating the number of rats with bilateral cannulae placements in the nucleus accumbens (*NAc*) and those with placements in adjacent regions

Experiment	*n*	Placements		
		Inside NAc	Outside NAc	
Amphetamine dose-response				
5.0 μg/0.5 μl/side	17	11	6	
10.0 μg/0.5 μl/side	21	14	7	
15.0 μg/0.5 μl/side	14	12	2	
20.0 μg/0.5 μl/side	17	9	8	
			One in NAc	Caudal to NAc
Rp-cAMPS+amphetamine (20 μg)				
0.0 ng/0.5 μl/side Rp-cAMPS	34	15	9	10
2.5 ng/0.5 μl/side Rp-cAMPS	19	14	3	2
25.0 ng.0.5 μl/side Rp-cAMPS	20	12	3	5
250.0 ng/0.5 μl/side Rp-cAMPS	24	13	7	4
Rp-cAMPS alone				
2.5 ng/0.5 μl/side	7	6	1	
25.0 ng.0.5 μl/side	25	17	8	
250.0 ng/0.5 μl/side	16	15	1	
Sp-cAMPS alone				
50.0 ng/0.5 μl/side	21	16	5	
250.0 ng/0.5 μl/side	8	6	2	
500.0 ng/0.5 μl/side	19	13	6	
600.0 ng/0.5 μl/side	15	8	7	
Sp-cAMPS+subthreshold dose of amphetamine (10.0 μg)				
25.0 ng/0.5 μl/side Sp-cAMPS	9	9	0	
50.0 ng.0.5 μl/side Sp-cAMPS	19	13	6	
250.0 ng/0.5 μl/side Sp-cAMPS	13	11	2	
500.0 ng/0.5 μl/side Sp-cAMPS	11	8	3	
Sp-cAMPS+suprathreshold dose of amphetamine (20.0 μg)				
250.0 ng/0.5 μl/side Sp-cAMPS	12	11	1	

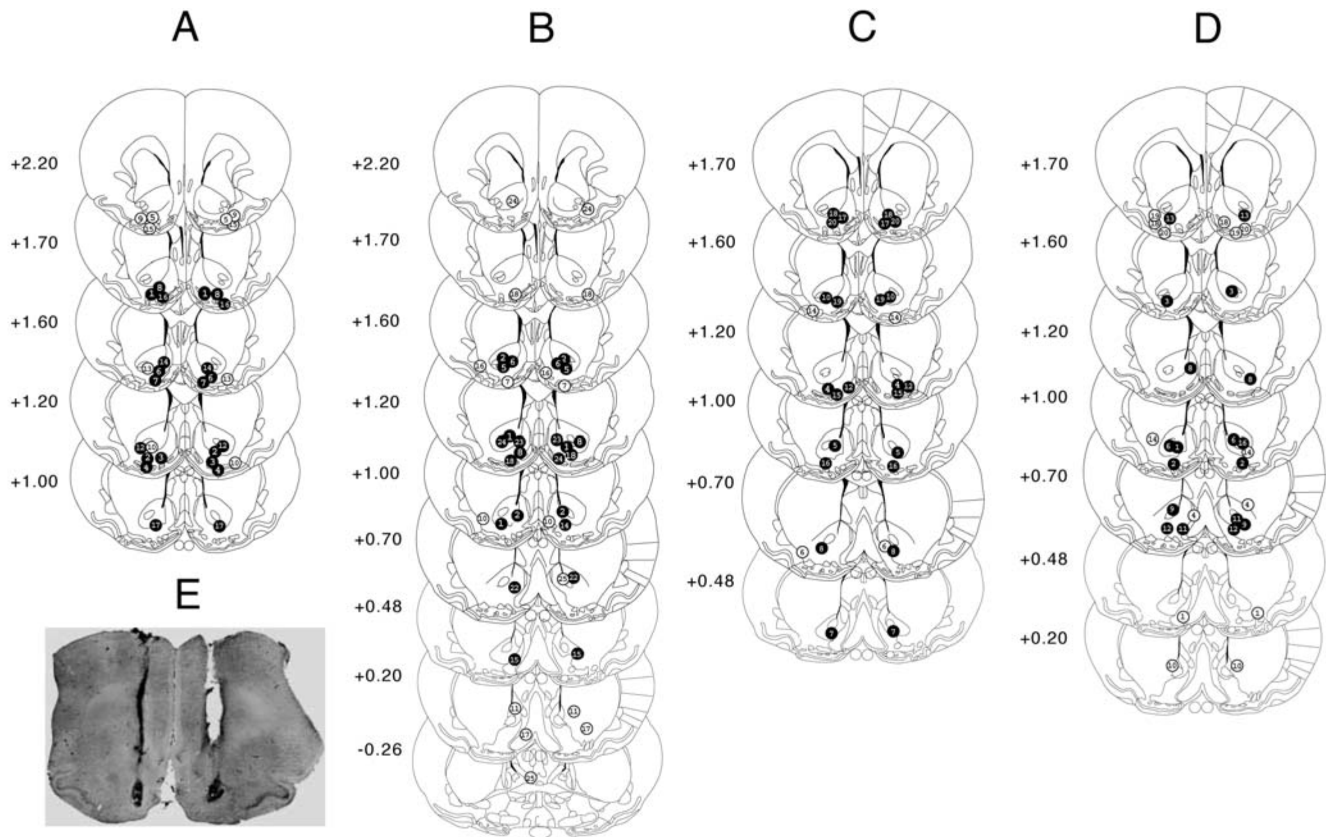

Fig. 1 Location of cannulae tips in and around the nucleus accumbens (*NAc*) for the groups from the amphetamine dose-response experiments (**A**–**D**: 5, 10, 15, 20 μg/0.5 μl per side; **E** representative photomicrograph showing cannulae tracts and tips in NAc). *Filled circles* indicate rats with both cannulae tips within the NAc and *open circles* indicate rats with one or neither cannulae tip within the core or shell region of the NAc. Drawings adapted from Paxinos and Watson (1998)

in NAc and one in an adjacent region and subgroups with both placements caudal to NAc; these groups similarly were analysed as anatomical controls.

Place conditioning

CPP was defined as spending more time in the drug-paired compartment during the test compared to the averaged pre-ex sessions. To evaluate possible side-bias during pre-ex, time spent on each side for each group in each experiment was compared using paired t-tests (Table 2). The difference was significant ($P<0.05$) for the 15.0 μg amph group (more time on the side to be paired with vehicle) and the 500.0 ng Sp-cAMPS+10.0 μg amph group (more time on the side to be paired with drug). Although the 15.0 μg amph group showed a CPP, similar effects were seen in other groups that did not show a significant saline side-bias (see below). The group receiving 500.0 ng Sp-cAMPS+10.0 μg amph did not show a significant CPP but neither did the other groups in that experiment (see below). Thus, side biases during pre-ex appear not to account for the CPPs.

Amph dose-response experiments

These studies provided a basis for selecting doses for subsequent studies. Amph produced a dose-dependent CPP (Fig. 2A). A two-way mixed design ANOVA comparing the four groups at pre-ex and test revealed a significant main effect of phase [$F(1,42)=10.64$, $P<0.01$] and interaction [$F(3,42)=3.05$, $P<0.05$]. Planned t-tests revealed a significant CPP for the two higher doses of amph [$t(8)=2.68$, $P<0.05$ for the 20.0 μg group, and $t(11)=3.34$, $P<0.01$ for the 15.0 μg group] revealing the source of the interaction. Thus, intra-NAc amph produced CPP in a dose-dependent manner.

A number of rats had cannulae placements outside of NAc (Table 2, Fig. 1). Analyses of these rats provided an anatomical control. Even though the placements were somewhat heterogeneous, analyses provided some information concerning the importance of the NAc for the CPP effect. The missed placements from the 5.0 and 10.0 μg amph groups were combined, as neither of these groups showed a CPP, and those from the15.0 or 20.0 μg groups were combined, as both of these doses produced a CPP in the groups with NAc placements. Neither of the groups of missed placements showed a significant CPP (Table 3).

Table 2 Mean (±SEM) time (s) spent on the drug- and saline-paired sides and in the tunnel during the 3 pre-exposure days averaged together for each experiment. *NS* not significant, *Amphet* Amphetamine, *Rp* Rp-cAMPS, *Sp* Sp-cAMPS

Experiment	*n*	Drug-paired	Tunnel	Saline-paired	*P*-value
Amphetamine alone					
5.0 μg/0.5 μl/side	11	435.4 (±38.3)	40.0 (±5.5)	424.5 (±39.6)	NS
10.0 μg/0.5 μl/side	14	427.7 (±26.0)	41.3 (±4.7)	429.5 (±25.3)	NS
15.0 μg/0.5 μl/side	12	394.0 (±28.5)	31.5 (±3.8)	476.5 (±28.7)	<0.03
20.0 μg/0.5 μl/side	9	382.2 (±36.9)	37.9 (±4.9)	479.9 (±34.7)	NS
Amphet (20 μg)+Rp-cAMPS					
0.0 ng/0.5 μl/side Rp	15	396.1 (±21.2)	46.6 (±5.2)	456.0 (±22.1)	NS
2.5 ng/0.5 μl/side Rp	14	420.4 (±22.1)	47.2 (±4.1)	429.4 (±21.1)	NS
25.0 ng/0.5 μl/side Rp	12	441.3 (±30.7)	33.2 (±3.7)	420.6 (±30.1)	NS
250.0 ng/0.5 μl/side Rp	13	472.5 (±36.7)	38.8 (±4.2)	385.7 (±35.6)	NS
Rp-cAMPS alone					
2.5 ng/0.5 μl/side	6	396.6 (±31.1)	52.7 (±7.6)	444.2 (±29.6)	NS
25.0 ng/0.5 μl/side	17	425.9 (±21.0)	47.4 (±4.0)	423.5 (±20.3)	NS
250.0 ng/0.5 μl/side	15	414.0 (±34.2)	45.6 (±4.3)	437.4 (±33.7)	NS
Sp-cAMPS alone					
50.0 ng/0.5 μl/side	16	435.4 (±28.9)	46.6 (±4.6)	414.8 (±28.3)	NS
250.0 ng/0.5 μl/side	6	445.4 (±32.0)	45.7 (±9.1)	406.0 (±31.6)	NS
500.0 ng/0.5 μl/side	13	435.9 (±20.2)	41.4 (±4.4)	419.6 (±19.7)	NS
600.0 ng/0.5 μl/side	8	390.5 (±37.1)	41.8 (±6.4)	464.3 (±38.4)	NS
Amphet (10 μg)+Sp-cAMPS					
25.0 ng/0.5 μl/side Sp	9	421.9 (±37.0)	52.0 (±7.4)	426.2 (±35.6)	NS
50.0 ng/0.5 μl/side Sp	13	385.1 (±25.6)	46.9 (±5.0)	468.0 (±26.3)	<0.06
250.0 ng/0.5 μl/side Sp	11	430.7 (±31.2)	51.0 (±5.5)	418.3 (±31.1)	NS
500.0 ng/0.5 μl/side Sp	8	496.5 (±34.1)	33.2 (±4.0)	370.3 (±33.8)	<0.05
Amphet (20 μg)+Sp-cAMPS					
250.0 ng/0.5 μl/side Sp	11	438.1 (±27.1)	40.0 (±5.3)	421.9 (±25.9)	NS

Rp+amph (20 μg) experiments

These studies evaluated the effect of co-injections of the PKA inhibitor on the CPP produced by intra-NAc amph (Fig. 2B). Rp-cAMPS dose-dependently antagonized the amph effect: 2.5 ng had little effect and 25.0 and 250.0 ng blocked the effect.

A mixed design ANOVA comparing amph alone and the three doses of Rp-cAMPS (2.5, 25.0 and 250.0 ng/0.5 μl per side)+amph revealed a significant effect of phase [$F(1,50)=4.11$, $P<0.05$]. This result occurred when groups were combined and indicated a greater amount of time spent on the drug-paired side in the test. Planned *t*-tests performed separately on each group revealed that the 0 and 2.5 ng Rp-cAMPS+amph groups spent significantly more time on the drug-paired side [$t(14)=2.32$, $P<0.05$ and $t(13)=1.94$, $P<0.05$]. The results for the 25.0 and 250.0 ng Rp-cAMPS+amph groups were not significant. Thus, the ability of intra-NAc amph to produce a CPP was replicated and treatment with a PKA inhibitor dose-dependently blocked this effect.

Rats with cannulae placements outside of the NAc (Table 1) were assigned to one of two categories: those with one cannula in NAc and one adjacent to but not in NAc and those with both cannulae placements caudal to NAc. Because the NAc groups that received doses of 0 or 2.5 ng Rp-cAMPS+amph showed a CPP and those receiving doses of 25.0 or 250.0 ng+amph did not, the missed-placement groups were combined to form four subgroups: 1) one cannula in NAc and 0 or 2.5 ng Rp-cAMPS+amph; 2) one cannula in NAc and 25.0 or 250.0 ng Rp-cAMPS+amph; 3) both cannulae caudal to NAc and 0 or 2.5 ng Rp-cAMPS+amph; 4) both cannulae caudal to NAc and 25.0 or 250.0 ng Rp-cAMPS+amph. None of the groups showed a significant CPP (Table 3).

Rp alone experiments

These experiments evaluated the possibility that injections of Rp-cAMPS alone, at the doses used in the co-injection with amph experiments, would produce place conditioning (Fig. 2C). No significant differences were found.

Sp alone experiments

These experiments determined whether Sp-cAMPS (50.0, 250.0, 500.0, and 600.0 ng/0.5 μl per side) alone would produce a CPP. There was little effect of any of these doses on place preference (Fig. 2D).

Sp+amph (10.0 μg) experiments

The lack of effect of Sp-cAMPS on place conditioning may have resulted from its general activation of the PKA pathway in the absence of a specific signal associated with reward. These studies tested this hypothesis by co-injecting Sp-cAMPS (25.0, 50.0, 250.0 and 500.0 ng/

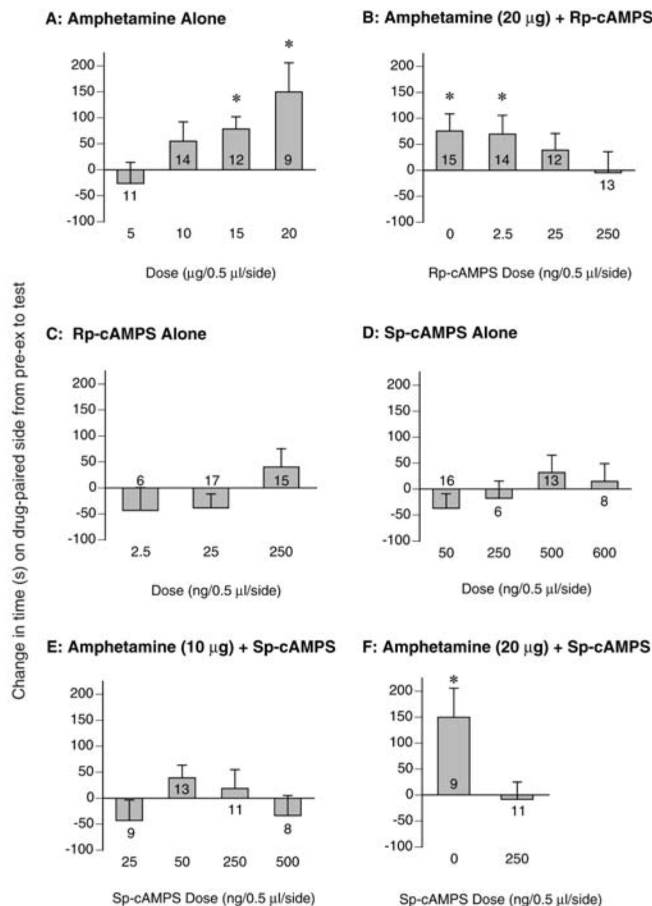

Fig. 2. A–F Mean (±SEM) change in time spent on the drug-paired side from mean pre-exposure (*pre-ex*) to test for each group in each experiment. Note that the group receiving 0 ng Sp-cAMPS+20.0 µg amphetamine in **F** is the 20.0 µg amphetamine group from the dose-response experiment in **A**. *Asterisks* represent a significant difference ($P<0.05$) between the mean of the 3 pre-ex days and the test day. The figures within, above or below the bars indicate the number of animals per group

0.5 µl per side) with a subthreshold dose of amph (10.0 µg/0.5 µl per side) into NAc. It was postulated that if 10.0 µg amph into NAc produced a weak signal in the cAMP-PKA second messenger pathway, the addition of the PKA activator might augment that signal. However, none of the experiments produced a significant effect (Fig. 2E). Thus, treatment with Sp-cAMPS, a PKA activator, either alone (previous experiments) or in the presence of a sub-threshold dose of amph failed to produce a CPP.

Sp+amph (20.0 µg) experiment

The failure of Sp-cAMPS alone or in combination with a subthreshold dose of amph to produce CPP may be related to second messenger "noise" (occlusion) produced by PKA activation. This experiment tested this hypothesis by co-injecting Sp-cAMPS with a suprathreshold dose of amph. It was postulated that the putative second messenger "noise" created by Sp-cAMPS might disrupt the normal signal produced by DA acting at D_1-like receptors as a result of the actions of amph. Results supported this hypothesis (Fig. 2F). Co-injection of Sp-cAMPS (250.0 ng/0.5 µg per side)+amph (20.0 µg/0.5 µl per side) disrupted the amph CPP. For analyses, the Sp-cAMPS+20 µg amph group was compared to the 20.0 µg amph dose group from the dose-response experiment and to the group receiving 20.0 µg amph+0 ng Rp-cAMPS from the Rp+amph experiments. ANOVA revealed a significant main effect of phase [$F(1,18)=5.13$, $P<0.05$] and an interaction [$F(1,18)=6.40$, $P<0.05$] for the first comparison but no significant effects for the second. Because the co-injection experiment was done as part of the set of studies that included the amph dose-response experiment, comparison of the co-injection group to the amph alone group from the dose-response study is the most appropriate comparison. Planned *t*-tests showed the amph effect to be significant for both comparison groups (see above) and no significant CPP for the co-injected group, revealing the source of the interaction. Thus, Sp-cAMPS disrupted the usual rewarding effects of amph.

Unconditioned activity

Figure 3 illustrates the difference scores between the mean number of sensor beam breaks/5 min in the drug-

Table 3 Mean (±SEM) change in time (s) spent on the drug-paired side from pre-exposure to test for groups classified as having cannulae placements outside of the nucleus accumbens. *NS* not significant in *t*-test, *pre-ex* pre-exposure

Group	*n*	Mean (±SEM) change in time (s) on drug-paired side from pre-ex to test	*P*-value
Amphetamine 5.0 or 10.0 µg	13	15.0 (±36.2)	NS
Amphetamine 15.0 or 20.0 µg	10	9.6 (±54.0)	NS
One cannula in NAc and one adjacent to but not in NAc			
Rp-cAMPS (dose/0.5 µl/side)			
0, 2.5	12	14.8 (±21.7)	NS
25.0, 250.0	10	44.5 (±26.3)	NS
Both cannulae placements caudal to NAc			
Rp-cAMPS (dose/0.5 µl/side)			
0, 2.5	12	26.2 (±45.0)	NS
25.0, 250.0	9	−12.1 (±48.4)	NS

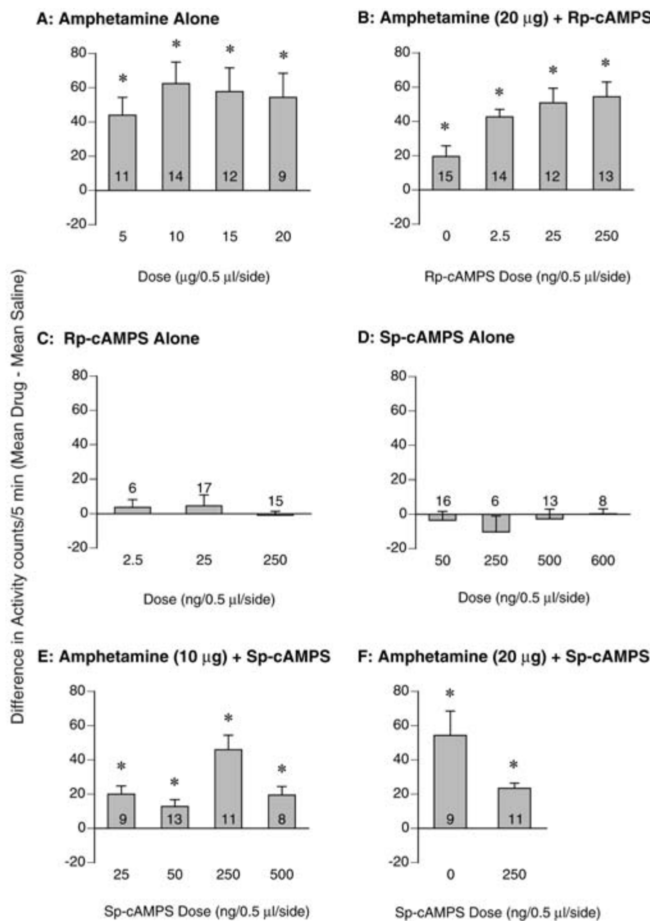

Fig. 3. A–F Mean (±SEM) difference in activity counts/5 min (mean drug days–mean saline days) for each group in each experiment. Note that the group receiving 0 ng Sp-cAMPS+20.0 µg amphetamine in **F** is the 20.0 µg amphetamine group from the dose-response experiment in **A**. *Asterisks* represent a significant difference (P<0.05) between mean activity on the drug-paired side and on the saline-paired side. The figures within, above or below the bars indicate the number of animals per group

and saline-paired compartments averaged over the 4 days of the conditioning phase for each set of studies. Groups treated with amph either alone or in combination with Rp- or Sp-cAMPS showed increased activity on the drug-paired side; groups treated with Rp- or Sp-cAMPS alone showed little difference in the level of activity on the saline- and drug-paired sides.

Amph dose-response experiments

All groups showed increased drug-induced activity (Fig. 3A). Mean (±SEM) saline values (counts/5 min) ranged from 48.4 (±3.3) to 59.5 (±7.4) and amph values ranged from 93.1 (±10.2) to 114.4 (±13.4). A two-way mixed-design ANOVA revealed a main effect of side [F(1,42)=69.23, P<0.0001], with no significant effect of group or interaction. Thus, all doses of amph elevated unconditioned activity.

Rp+amph experiments

Treatment with Rp-cAMPS+amph produced greater activity than saline (Fig. 3B). Mean (±SEM) counts/5 min on the saline side ranged from 49.6 (±3.0) to 59.5 (±4.5); for the three co-injected groups, drug side values ranged from 100.5 (±8.6) to 108.1 (±10.5), similar to the corresponding ranges from the amph dose-response experiments above. The exception was the drug value for the amph (20.0 µg) alone group, 71.6 (±6.8) counts/5 min; this value was lower than the 113.8 (±16.3) counts/5 min observed in the corresponding group from the dose-response experiments and resulted in the reduced difference score shown in Fig. 3B. ANOVA yielded main effects of group [F(3,50)=3.69, P<0.02], side [F(1,50)=143.21, P<0.0001], and their interaction [F(3,50)=5.32, P<0.01]. One-way ANOVA were conducted to determine the source of the interaction. The group effect was significant only for the drug-paired side [F(3,50)=4.98, P<0.01]. Newman-Keuls post hoc tests revealed that the animals in each of the three Rp-cAMPS+amph groups were significantly more active in the drug-induced state than the animals in the drug-induced state that received amph alone, thus revealing the source of the interaction.

The group receiving 20.0 µg/0.5 µl per side amph in the dose-response experiment had drug and vehicle side means (±SEM) of 113.8 (±16.3) and 59.5 (±7.4) counts/5 min and the group receiving 20.0 µg amph+0 ng Rp-cAMPS from this experiment had corresponding values of 71.6 (±6.8) and 52.1 (±3.3). ANOVA yielded a significant interaction [F(1,22)=6.65, P<0.02]. One-way ANOVA for groups on each side revealed a significant effect on the drug side only [F(1,22)=7.65, P<0.02]. Thus, although 20.0 µg amph injected bilaterally into the NAc had a significant stimulant effect in both experimental groups, the effect was larger in the dose-response experiment.

Rp alone experiments

The groups showed similar activity when they were treated with drug or saline (Fig. 3C). Mean saline values ranged from 44.5 (±1.7) to 49.7 (±3.2) and drug values ranged from 43.6 (±2.1) to 54.3 (±6.0) counts/5 min. ANOVA revealed no significant effects.

Sp alone experiments

Like Rp-cAMPS alone, Sp-cAMPS alone did not have a significant effect on locomotor activity counts (Fig. 3D). Mean saline values ranged from 47.9 (±2.5) to 53.5 (±5.8) and Sp-cAMPS counts ranged from 42.6 (±2.7) to 50.8 (±3.5) counts/5 min.

Sp+amph (10.0 μg) experiments

Groups showed increased drug-induced unconditioned activity (Fig. 3E). For statistical analyses, the 10.0 μg amph group from the dose-response experiment was included; for this group the mean (±SEM) saline value was 48.4 (±3.3) and the drug value was 110.8 (±13.7). With the exception of the group receiving 250 ng Sp-cAMPS, the corresponding values for the Sp-cAMPS groups ranged from 42.7 (±2.7) to 51.2 (±3.3) for saline and from 62.7 (±6.3) to 70.6 (±4.8) counts/5 min for drug. Values for the 250 ng Sp-cAMPS group were 61.6 (±3.2) and 107.5 (±10.0), respectively. Thus, with the exception of 250 ng Sp-cAMPS, groups co-treated with Sp-cAMPS+amph were less active than groups treated with amph alone; however, the saline value was higher for the 250 ng Sp-cAMPS group suggesting that overall activity was greater for this group. ANOVA revealed main effects of group [$F(4,50)=6.23$, $P<0.001$], side [$F(1,50)=73.92$, $P<0.0001$], and their interaction [$F(4,.50)=6.08$, $P<0.001$]. One-way ANOVA for the saline side yielded a significant group effect [$F(4,50)=4.40$, $P<0.01$], and Newman-Keuls tests showed that the saline-side activity scores for the 250 ng Sp-cAMPS group were significantly higher than those of the other four groups, which did not themselves differ from each other. One-way ANOVA for the drug side also yielded a significant group effect [$F(4,50)=6.39$, $P<0.001$]. Post hoc tests showed the amph alone and 250 ng Sp-cAMPS+amph groups to differ from the other three but not from each other. Thus, Sp-cAMPS appeared to attenuate the stimulant effect of amph at all doses but 250 ng but this group was more active during saline trials.

Sp+amph (20.0 μg) experiment

Sp-cAMPS also seemed to attenuate the stimulant effect of 20.0 μg amph injected into NAc (Fig. 3F). ANOVA comparing this co-injected group with the 20.0 μg amph group from the dose-response experiment yielded a significant effect of group [$F(1,18)=8.82$, $P<0.01$], side [$F(1,18)=34.60$, $P<0.0001$], and interaction [$F(1,18)=5.47$, $P<0.05$]. One-way ANOVA for groups on each side revealed a significant effect on the drug side [$F(1,18)=8.58$, $P<0.01$], with the effect on the saline side being near significance [$F(1,18)=4.37$, $P=0.051$]. Thus, amph stimulated motor activity but this effect was attenuated by co-treatment with Sp-cAMPS. On the other hand, when the 20.0 μg amph group from the Rp-cAMPS+amph experiments was used to evaluate activity effects in the Sp-cAMPS+amph (20.0 μg) experiment, only a main effect of side was seen [$F(1,24)=31.25$, $P<0.0001$]. Similarly, when the two 20.0 μg amph groups were combined and compared to the Sp-cAMPS+amph group, only a main effect of side was seen [$F(1,33)=25.71$, $P<0.0001$]. It was clear that amph stimulated activity when injected on its own or co-injected with 250.0 ng Sp-cAMPS but whether Sp-cAMPS attenuated the amph effect was less clear and depended on the comparison group.

Discussion

Results can be summarized as follows: 1) the CPP paradigm was unbiased with most groups showing no significant difference in time spent on the two sides during pre-ex; 2) intra-NAc amph produced a dose-dependent CPP; 3) the CPP seemed to be somewhat specific to the NAc as groups injected in anatomical regions outside the NAc failed to show a CPP; 4) intra-NAc Rp-cAMPS, while having no effect on place preference on its own, dose-dependently blocked CPP produced by NAc amph; 5) intra-NAc Sp-cAMPS alone or co-injected with10.0 μg amph (that did not produce CPP on its own) did not lead to a CPP but co-injection of Sp-cAMPS with a 20.0 μg dose of amph (that did produce CPP on its own) eliminated the CPP effect; 6) Rp- and Sp-cAMPS alone did not affect motor activity; 7) intra-NAc amph increased locomotor activity during conditioning and this stimulant effect was not blocked by co-treatment with either Rp-cAMPS or Sp-cAMPS.

The observation that intra-NAc amph produced a CPP agrees with previous findings (Tzschentke 1998). Amph increases synaptic concentrations of DA by blocking uptake and increasing neurogenic release (Scheel-Krüger 1971; Westerink 1979) and this action is thought to be responsible for its ability to produce a CPP. Thus, DA receptor antagonists injected into the NAc block the ability of amph to produce a CPP (Tzschentke 1998). Findings support the hypothesis that DA plays a critical role in reward-related learning (Beninger 1983).

DA D_1-like receptors may play a critical role in reward-related learning (Beninger and Miller 1998; Berke et al. 2000). As D_1-like receptors activate the cAMP-PKA signalling pathway, one test of this hypothesis would be to evaluate the effects of inhibition of PKA in NAc on CPP produced by co-injected amph. The present finding that Rp-cAMPS dose-dependently decreased the amph CPP provides further support for the D_1-like receptor hypothesis. The further observation that Rp-cAMPS injected alone had no significant effect on side preference confirms that its action on CPP cannot simply be attributed to additive effects of the two treatments.

The present finding that PKA plays a role in reward-related learning assessed in CPP is consistent with the observation that conditioned activity based on NAc amph was blocked by Rp-cAMPS (Sutton et al. 2000) and the recent finding from A. Kelley's lab showing that acquisition of lever pressing for food was impaired by PKA inhibition in NAc (Baldwin et al. 2002). Others have identified a role for PKA in learning and memory in a range of species (see Introduction).

The doses of Rp-cAMPS that blocked CPP were 25.0 and 250.0 ng/0.5 μl per side whereas 1.0 μg blocked the establishment of conditioned activity in our previous study (Sutton et al. 2000). The difference may reflect the

higher dose of amph (25.0 μg/0.5 μl per side) used by Sutton et al. (2000) and the fact that conditioned activity based on amph is more robust, being significant after three drug conditioning sessions versus the four used here. Baldwin et al. (2002) found that Rp-cAMPS doses of 2.2–8.9 μg/1.0 μl per side into NAc slowed the acquisition of operant lever pressing for food. Perhaps this high dose reflects the use of a food-reinforced behavioral paradigm in which increased levels of DA in the NAc and the dorsal striatum have been observed (Blackburn et al. 1986; Joseph et al. 1989). This consideration might also provide a basis for understanding the finding of Baldwin et al. (2002) that learning was delayed but not blocked by NAc Rp-cAMPS. Schafe and LeDoux (2000) blocked the establishment of fear conditioning with an Rp-cAMPS dose of 18.0 μg/0/5 μl per side into the lateral and basal amygdala. The fear conditioning was strong, being established in one session and the injections were given after conditioning providing possible variables affecting the least effective dose of the PKA inhibitor. It is important to note that all of the studies implicating NAc or amygdala PKA in learning used paradigms involving externally presented rewarding or aversive stimuli in contrast to the CPP based on intra-NAc amph used here and the conditioned activity procedure based on intra-NAc amph used by Sutton et al. (2000). The substantially lower doses of Rp-cAMPS used in these latter studies may reflect this use of a locally injected rewarding stimulus.

Sp-cAMPS is a PKA activator. Following the logic that intra-NAc PKA inhibition blocks CPP based on intra-NAc amph, it was postulated that PKA activation in NAc might produce a CPP. However, results showed no significant effect. One possibility is that this negative finding reflects the relatively low doses of Sp-cAMPS tested (50–600 ng/0.5 μl per side). However, when a dose of 250 ng was added to amph in the final experiment, it blocked the CPP. Furthermore, co-injection of Sp-cAMPS in this dose range with amph (10.0 μg/0.5 μl per side) generally decreased the stimulant effect of amph (Fig. 2). Thus, the doses of Sp-cAMPS employed in these studies were sufficiently high to produce significant behavioral effects. Normally the cAMP-PKA pathway is activated by a number of different receptors including, for example, adenosine A_{2A} and A_{2B}, adrenergic β_1, β_2 and β_3, DA D_1, γ-aminobutyric acid $GABA_B$, histamine H_2, serotonin 5-HT_4 and some peptides including corticotrophin releasing factor and vasopressin. Perhaps local injection of Sp-cAMPS leads to indiscriminant activation (occlusion) of cAMP-PKA pathways that is not linked to the activity of a particular receptor (e.g. D_1) and CPP learning is not seen. A similar argument might explain the observation of Taylor et al. (1999) that intra-prefrontal cortical injections of Sp-cAMPS impaired working memory performance. However, Jentsch et al. (2002) observed enhanced approach responses to a conditioned stimulus for food with intra-amygdala injections of Sp-cAMPS at one dose (2.23 μg/0.5 μl per side) but impairment at a higher dose (8.93 μg). Whether these differences are related to the structures being studied, the nature of the rewarding stimulus, the dose range of the PKA activator or some other variable will have to await further studies.

One possibility for linking activation of the cAMP-PKA pathway to stimulation of DA receptors might be to co-inject Sp-cAMPS with a sub-threshold CPP dose of a compound known to lead to activation of those receptors. However, in the present study, this strategy also failed. If the hypothesis that Sp-cAMPS fails to produce a CPP because it creates “noise” in the cAMP-PKA signalling pathway is correct, it follows that co-injection of the PKA activator with a dose of amph that normally produces a CPP might lead to an impairment of the CPP effect. This prediction was confirmed by the final experiment. Thus, the PKA activator Sp-cAMPS may have failed to produce a CPP when injected alone or in combination with a sub-threshold CPP dose of amph because it indiscriminately activated cAMP-PKA signalling normally controlled by a number of effectors.

The Rp-cAMPS experiments revealed dissociation between the motor and learning effects of amph. Thus, although Rp-cAMPS dose-dependently decreased the rewarding effects of amph in CPP, it did not block locomotor stimulation produced by amph. This finding is in agreement with our previous results showing that Rp-cAMPS blocked the establishment of conditioned activity based on NAc injections of amph without blocking its motor stimulant effects (Sutton et al. 2000). Furthermore, Rp- and Sp-cAMPS alone also failed to affect locomotor activity. Results suggest that the cAMP-PKA signalling pathway activated by DA acting at D_1-like receptors plays a critical role in learning but not in motor activation.

In conclusion, the ability of intra-NAc injections of amph to produce a CPP appears to depend on activation of the cAMP-PKA signalling pathway. However, activation of this pathway in NAc is not sufficient to produce a CPP possibly because agents that activate this pathway do so indiscriminately and may thereby produce noise in signalling pathways normally activated in a more discrete manner by neurotransmitter signals. Continued investigation of the mechanisms of DA-mediated reward-related learning may reveal that this type of learning depends on the same mechanisms as a range of other types of learning in a variety of species.

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