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Conditioned locomotion in rats following amphetamine infusion into the nucleus accumbens: blockade by coincident inhibition of protein kinase A

M.A. Sutton[a,c], K. McGibney[a] and R.J. Beninger[a,b]

Departments of [a]Psychology and [b]Psychiatry, Queen's University, Kingston, Canada, K7L 3N6. [c]Present address: Interdepartmental Neuroscience Program, Sterling Hall of Medicine, L200, Yale University, New Haven, CT 06520-8074, USA

Correspondence to Dr Richard J. Beninger, Department of Psychology, Queen's University, Kingston, Ontario, Canada, K7L 3N6. E-mail: Beninger@Psyc.QueensU.CA

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Recent studies demonstrate a role for cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) in the nucleus accumbens (NAc) in reward-related learning. To clarify this role, we assessed the effect of PKA inhibition on the unconditioned and conditioned locomotor activating properties of intra-NAc amphetamine. Rats underwent three 60 min conditioning sessions, pairing a test environment with bilateral co-infusions of amphetamine (25 μg/side) and the PKA inhibitor Rp-adenosine 3′,5′-cyclic monophosphothioate triethylamine (Rp-cAMPS) (0, 2.5, 250, 500 ng, 1, 10 or 20 μg/side). Two additional groups – receiving amphetamine explicitly unpaired with the environment or saline/environment pairings – served as controls. In a subsequent drug-free 60 min session, animals that received amphetamine/environment pairings demonstrated conditioned locomotion relative to controls. Rp-cAMPS co-treatment during pairing sessions differentially affected conditioned and unconditioned locomotor activation. Amphetamine-induced unconditioned activity was significantly enhanced by 500 ng and 1 μg Rp-cAMPS, locomotor sensitization was enhanced by 250 ng–1 μg Rp-cAMPS, and conditioned activity was attenuated by 1 μg Rp-cAMPS and blocked by 10 and 20 μg Rp-cAMPS. Thus, unconditioned activity and locomotor sensitization were enhanced at doses (250 ng–1 μg) that did not affect or attenuated conditioned activity, while conditioned activity was reduced or blocked at doses (1–20 μg) that enhanced or did not affect overall unconditioned activity. These results demonstrate that the activation of PKA plays a critical role in the process by which properties of drugs become associated with environmental stimuli. © **2000 Lippincott Williams & Wilkins.**

Keywords: cAMP-dependent protein kinase, dopamine, conditioned activity, locomotor sensitization, amphetamine, nucleus accumbens, Rp-cAMPS, rat

INTRODUCTION

Research from several species indicates that learning in a variety of contexts depends critically on the cyclic adenosine monophosphate (cAMP) second messenger system. For example, genetic approaches in *Drosophila* (e.g. Drain *et al*., 1991; Davis, 1996) and mice (Abel *et al*., 1997), as well as pharmacological studies in the honeybee (Menzel and Müller, 1996) have implicated the cAMP pathway, and in particular cAMP-dependent protein kinase (PKA), in associative learning. This critical role for the cAMP system is recapitulated in studies of synaptic plasticity, where the activation of PKA is required for enduring changes in synaptic efficacy in the mammalian hippocampus (Frey *et al*., 1993; Huang *et al*., 1994; Weisskopf *et al*., 1994; Impey *et al*., 1996; Abel *et al*., 1997) and sensorimotor connections in *Aplysia* (Bacskai *et al*., 1993; Kaang *et al*., 1993).

Consistent with these findings, a number of studies have recently suggested an important role for the cAMP pathway in reward-related learning. Dopaminergic neurotransmission, particularly in mesolimbic regions, is necessary for the acquisition and expression of various forms of learning that are established in some manner by reward. Five distinct

dopamine (DA) receptors have been identified, which have been categorized as either D1-like (D1, D5) or D2-like (D2, D3, D4) according to positive or negative coupling, respectively, to the enzyme adenylate cyclase (Niznick and Van Tol, 1992; Civelli *et al*., 1993; Sibley *et al*., 1993). D1-like receptors, acting through a stimulatory G protein (G_s), stimulate the production of cAMP and activate PKA, whereas D2-like receptors inhibit this pathway through the G proteins G_i and G_o. A large body of work using dopaminergic agents with specific actions at different receptor types has demonstrated important, but unique roles for D1- and D2-like receptors (hereafter referred to simply as D1 and D2 receptors) in reward-related learning (for reviews, see Self and Nestler, 1995; Beninger and Miller, 1998; Sutton and Beninger, 1999).

Recently, the investigation of DA-dependent processes in reward-related learning has started to address events downstream from the receptor that may be responsible for the establishment of this form of learning. For example, Kelley and Holahan (1997) examined the effects of upregulating the cAMP pathway on responding for conditioned reward (a previously neutral stimulus that has acquired rewarding properties based on its association with a primary reward). In this study, persistent activation of G_s with cholera toxin in the nucleus accumbens (NAc) enhanced established responding, as well as the acquisition of responding, for conditioned reward. Conversely, Westly *et al*. (1998) have shown that concurrent administration of Rp-adenosine 3′,5′-cyclic monophosphothioate triethylamine (Rp-cAMPS), a drug that inhibits the activation of PKA by cAMP, blocks the enhancement of responding for conditioned reward produced by intra-NAc amphetamine, but not the stimulant effect of this treatment.

Other work has demonstrated that co-treatment with Rp-cAMPS abolishes the conditioned place preference produced by local infusion of amphetamine into the NAc (Beninger *et al*., 1996). Despite this block of reward-related learning, these same animals showed an enhanced unconditioned locomotor response to amphetamine during pairing sessions in the presence of Rp-cAMPS, a behavioural phenotype that is often predictive of reward potentiation. Indeed, another study has shown that administration of intra-NAc Rp-cAMPS reduces intravenous cocaine self-administration and can elicit relapse of cocaine-seeking behaviour (Self *et al*., 1998), behavioural effects consistent with an acute reward-enhancing effect of this treatment. These findings suggest that Rp-cAMPS may disrupt reward-related learning independent of changes in the stimulus properties of reward, perhaps by blocking processes by which properties of the drug become associated with stimuli paired with its presentation.

We have examined this idea by using a learning task where the unconditioned and conditioned effects of particular treatments can be assessed in the same animals using an identical behavioural measure. Systemic administration of stimulant drugs such as amphetamine paired repeatedly with a test environment can produce enhanced locomotor activity in a drug-free test (Barr *et al*., 1983; Beninger and Hahn, 1983); this effect is referred to as conditioned activity. A related behavioural measure is locomotor sensitization, which refers to a progressive increase in the locomotor-activating effects of a drug with repeated treatments. This phenomenon can be distinguished from conditioned activity in that it does not require associative learning (Stewart, 1992). Since the PKA inhibitor Rp-cAMPS does not block the unconditioned locomotor-activating effects of amphetamine, we assessed the role of PKA in the ability of intra-NAc amphetamine to support both locomotor sensitization and conditioned activity under conditions in which the unconditioned stimulant properties of the drug are preserved. Some of these data have appeared previously in abstract form (Sutton *et al*., 1997).

METHODS

Subjects and surgery

Treatment of animals was in accordance with the Animals for Research Act, the Guidelines of the Canadian Council on Animal Care and relevant University Policy, and was approved by the Queen's University Animal Care Committee.

Male Wistar rats ($n = 107$; Charles River Canada) were housed in a temperature-controlled environment (21°C) on a 12 h light-dark cycle (lights on at 06.00 h), initially in groups of three to four. Following at least a 5 day acclimation period to the housing environment, rats were anaesthetized with halothane (3–4%) and implanted bilaterally with guide cannulas (0.6 mm o.d.) aimed at the NAc (A 1.2, L 2.0, V 7.0 according to Paxinos and Watson, 1986). The cannulas were fixed in place with dental acrylic and four skull screws. Immediately following the surgical procedure, all animals received a 0.2 ml intraperitoneal (i.p.) injection of banamine (5 mg/ml) as an analgesic, and four separate intradermal injections (approximately 0.1 ml) of 1.0% xylocaine as a local anaesthetic.

During the first postoperative day, animals recovered in plastic recovery cages (two rats/cage) lined

with soft bedding, after which they were housed individually in wire-mesh cages for the remainder of the postoperative recovery period (at least four additional days). From their arrival to the completion of the experiment, rats were provided freely with food and water.

Apparatus

Behavioural testing was carried out in six Plexiglass chambers (41 × 50 × 37 cm), each containing two sets of seven infrared emitters and detectors (three on the side, four along the front and back walls) spaced 10 cm apart. Thus, the arrangement of the photocells was such that 20 squares of equivalent dimensions (approximately 10 × 10 cm) were enclosed by the infrared beams. The two sets of photocells were located 5 and 15 cm off a wire-rod floor and were used to measure horizontal and vertical locomotor activity, respectively. Photocell beam breaks in both vertical and horizontal dimensions were recorded on an experimenter-controlled circuit board connected to a Macintosh microcomputer, but only horizontal activity was analysed.

Each chamber was equipped with a 2.5 W bulb mounted on the ceiling in the centre of the apparatus, and a fan behind the back wall, which provided ventilation and constant background noise. The activity monitors were individually enclosed in Styrofoam-insulated outer boxes, and located in a small experimental room that was kept dark with the exception of the 2.5 W bulb in each chamber. For further details of the apparatus, see Beninger *et al.* (1985).

Central drug injections

Daily preparations of D-amphetamine sulphate (Health Canada, Therapeutic Products Directorate, Ottawa) were dissolved in saline (0.9% NaCl) to reach a concentration of 50 μg/0.5 μl. Rp-cAMPS (Research Biochemicals Inc., Natick, Massachusetts, USA) was dissolved in distilled water, and stored frozen in aliquots until use. Just prior to infusion, the solution of Rp-cAMPS was added to the amphetamine solution to reach final doses of 2.5 ng (5.6 pmol) to 20 μg (44.8 nmol) Rp-cAMPS and 25 μg amphetamine co-infused in a volume of 0.5 μl.

Drug injections were made through injection cannulas (0.3 mm o.d.) attached via polyethylene tubing to a 10 μl Hamilton micro-syringe mounted in an infusion pump. The injection cannulas were inserted such that they extended 1 mm below the guide cannulas to V 8.0. Infusions were administered over 30 s and the cannulas were left in place for an additional 30 s to allow for further drug diffusion. In cases where sham injections were used, the procedure was identical except that the Hamilton microsyringe was mounted in the pump such that no pressure was applied to it during the 30 s 'infusion' period.

Behavioural procedure

Following surgical recovery, nine groups of rats (n = 8–13) underwent a 12 day experimental protocol (see Figure 1) consisting of three phases: habituation, conditioning, and test. Of these groups, seven were treated with 25 μg amphetamine and 0, 2.5, 250, 500 ng, 1, 10 or 20 μg Rp-cAMPS immediately prior to conditioning sessions. Two additional groups – one receiving vehicle infusions prior to each conditioning session and another receiving 25 μg amphetamine immediately after each conditioning session – served as controls for the effect of the infusion procedure and the non-associative effects of repeated amphetamine treatments.

The habituation phase consisted of five 1 h sessions in the locomotor activity monitors. This extensive habituation procedure was designed to reduce differential sensitivity to novelty, handling and/or the apparatus, as well as exclude differences in habituation as an explanation for differences in conditioned activity.

During three 60 min conditioning sessions, the testing environment was paired with the locomotor stimulant properties of amphetamine or amphetamine/Rp-cAMPS co-infusions into the NAc. In each session, the acute effects of treatments on

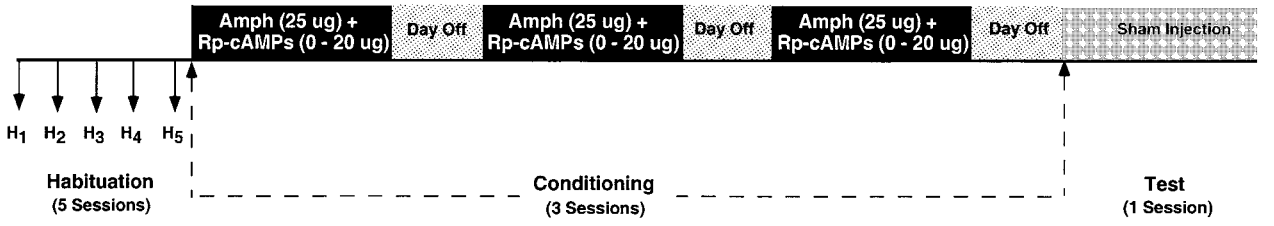

FIGURE 1. The experimental protocol. Amph, amphetamine.

unconditioned locomotor activity were recorded and the degree to which activity was increased by subsequent treatments reflected locomotor sensitization. Each conditioning day was separated by 48 h to ensure that the acute effects of treatments were not carrying over into subsequent sessions.

Forty-eight hours after the last conditioning session, animals were placed back into the activity monitors to test for conditioned activity in a drug-free state. Immediately prior to this test session, all animals received a sham injection (see above) and the number of photocell beam breaks during the 60 min session were recorded.

Histological procedures

On completion of the 12 day protocol, rats were sacrificed through CO_2 inhalation. Immediately after, brains were extracted and stored in a 10% formalin solution. Frozen coronal sections (60 μm) were obtained using a freezing-stage microtome (Reichert-Jung), then stained with thionine for histological evaluation of the injection sites (see Figure 2). All such evaluations were made by an experimenter who was blind to the treatment history of the rat.

Data analysis

To assess whether intra-accumbens amphetamine can support conditioned activity, t-tests were used to compare locomotor responses during the drug-free test session in animals that received 25 μg amphetamine *prior* to the conditioning session (paired group) with animals receiving the same dose of amphetamine *following* each session (unpaired group) and animals receiving saline prior to the session (saline group).

Subsequent analyses assessed the effect of Rp-cAMPS/amphetamine co-treatment on unconditioned and conditioned activity. First, locomotor activity in each conditioning session and in the conditioned activity test session in the two control groups were compared using t-tests, and collapsed into a single mean if there was no significant ($P < 0.05$) difference between them. Locomotor activity in each pairing session and conditioned activity in the drug-free test session for all treatment groups were then analysed with separate single-factor between-groups analyses of variance (ANOVA) to examine the overall treatment effects. Post-hoc Dunnett t-tests were used to compare the seven experimental groups against the controls, while Newman-Keuls tests were used to compare the Rp-cAMPS/amphetamine co-treatments against amphetamine alone. In cases where Rp-cAMPS co-treatment altered the unconditioned stimulant effect of amphetamine in any of the three pairing sessions, t-tests were used to compare the overall unconditioned activity (average locomotor responses over the three conditioning sessions) in these groups and the amphetamine-only group.

Following the above analyses, unconditioned activity was further analysed by a two factor (treatment $\times$ day) mixed ANOVA for evidence of locomotor sensitization. A significant interaction was subjected to an analysis of simple effects to examine under which treatments locomotor activity exhibited sensitization. Finally, conditioned locomotion in a subset of certain groups was compared with amphetamine alone using unpaired t-tests.

RESULTS

Figure 2 illustrates the cannula placements for all animals in three of the nine groups tested; the distribution of placements in these groups is representative of the remaining experimental groups. Only animals with both cannula tips entirely within the NAc were used for the behavioural analyses (102 out of 107 rats that underwent surgery).

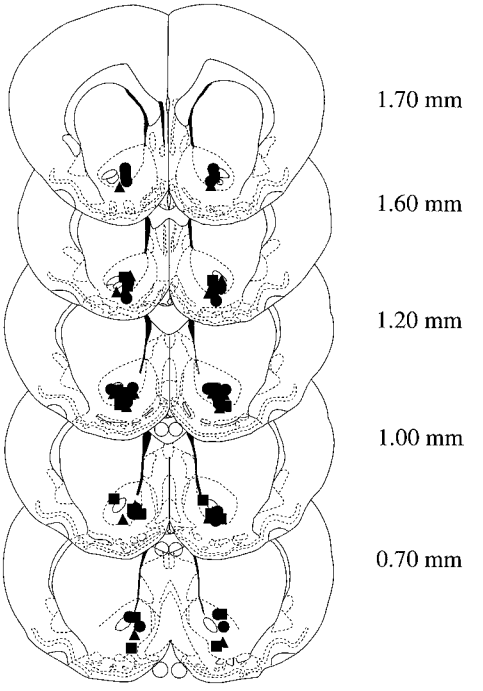

FIGURE 2. Coronal sections modified from Paxinos and Watson (1986) indicating cannula placements for groups receiving amphetamine plus 2.5 ng Rp-cAMPS (●), 250 ng Rp-cAMPS (■) and 1 μg Rp-cAMPS (▲). Cannula placements in these groups are representative of all the animals used for behavioural analyses; numbers to the right of the sections indicate the distance anterior to the bregma.

Unconditioned and conditioned locomotion after amphetamine

Amphetamine infusions directly into the NAc stimulated unconditioned locomotor activity over the entire 60 min in all three conditioning sessions (Figure 3A). Animals that received intra-NAc amphetamine paired with the test environment during conditioning also exhibited enhanced locomotor activity in the drug-free test, relative to both saline-infused animals and animals that received the same amphetamine treatment explicitly unpaired from the environment (Figures 3B and C). Although conditioned locomotor activity collapsed across the entire 60 min session (Figure 3C) it was significantly elevated relative to both control groups ($t_{20} = 2.34$, $P < 0.05$; $t_{22} = 4.06$, $P < 0.05$ for saline and unpaired groups, respectively), Figure 3B demonstrates that this difference was stronger in the first 50 min of the session. For this reason, all subsequent analyses of conditioned activity utilized the data from the first 50 min of the drug-free session as the best index of the conditioned effect. The fact that animals receiving the same dose of amphetamine after conditioning sessions did not show any enhancement during the drug-free test indicates that the conditioned activity effect is a product of associative learning, and not simply a result of repeated amphetamine treatments. Thus, as with reports demonstrating this effect with systemic amphetamine, direct infusions of this drug into the NAc also support conditioned activity.

Unconditioned locomotion: modulation by Rp-cAMPS

Unconditioned locomotor activity after amphetamine or amphetamine/Rp-cAMPS co-treatments on the three conditioning days is shown in Figure 4. Unconditioned activity in all three pairing sessions was similar in both saline-treated animals and those receiving amphetamine explicitly unpaired with the test environment (see Figure 4; $t_{20} = 0.81$, 1.73 and 0.97 for days 1, 2 and 3, respectively; all $P > 0.05$), so the results were collapsed into a single control for the statistical analyses. A single-factor

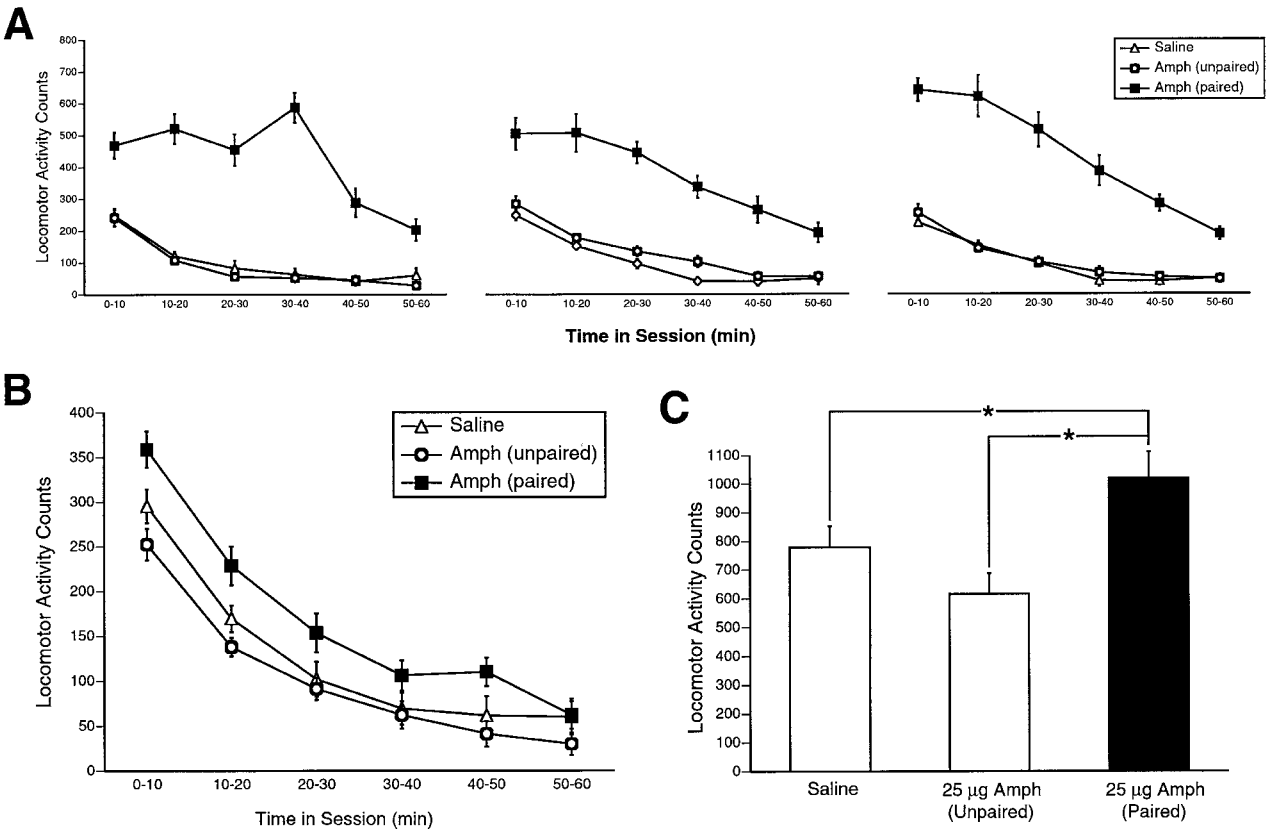

FIGURE 3. Mean (± SEM) number of photocell crossings per 10 min bin for the three pairing sessions (A) and on the drug-free test (B, C) for animals receiving 25 μg amphetamine paired with the test environment [Amph (paired)], animals receiving the same dose of amphetamine explicitly unpaired with the test environment [Amph (unpaired)], and animals receiving saline infusions paired with the test environment (Saline). Unconditioned activity (A) from sessions 1–3 is presented from left to right, while conditioned activity is presented both for each 10 min bin (B) and as a total for the entire 60 min session (C); *, significant ($P < 0.05$) difference by t-test.

ANOVA run on the eight groups for locomotor activity during the first session was significant [$F(7,94) = 16.97$, $P < 0.05$], reflecting locomotor stimulation with amphetamine or amphetamine/Rp-cAMPS treatment relative to the controls. All groups receiving amphetamine and amphetamine/Rp-cAMPS before conditioning demonstrated this locomotor stimulation (all $P < 0.05$, Dunnett's t-test). All Rp-cAMPS/amphetamine groups showed levels of unconditioned activity comparable to that of amphetamine alone (all $P > 0.05$, Newman-Keuls), except for the group receiving 10 μg Rp-cAMPS/amphetamine, which showed significantly reduced locomotor activation ($P < 0.05$). The implication of this specific result is discussed further below.

On the second day of conditioning, all groups receiving amphetamine and amphetamine/Rp-cAMPS treatments again showed locomotor activation relative to controls [$F(7,94) = 11.94$, $P < 0.05$]; all groups were significantly ($P < 0.05$) different from controls by Dunnett's t-test. Importantly, locomotor stimulation in animals receiving the highest doses of Rp-cAMPS (10 and 20 μg) was highly similar to that produced by amphetamine alone (see Figure 4; $P > 0.05$, Newman-Keuls). Moreover, the groups receiving 500 ng and 1 μg co-infused with amphetamine showed an even greater locomotor response than amphetamine alone ($P < 0.05$, Newman-Keuls). Thus, on the second day of conditioning, co-treatment with 500 ng or 1 μg Rp-cAMPS significantly enhanced the unconditioned locomotor activating effects of amphetamine.

Rp-cAMPS co-treatment produced similar effects on unconditioned locomotor activity in the third pairing session, and an inverted U-shaped dose-related potentiation of unconditioned locomotor activity with Rp-cAMPS is particularly evident. Again, relative to controls, locomotor responses were enhanced in all groups receiving amphetamine and amphetamine/Rp-cAMPS treatments [$F(7,94) = 18.04$, $P < 0.05$]; all groups were significantly ($P < 0.05$) different from controls by Dunnett's t-test. Similar to the second conditioning session, locomotor activation produced by intra-NAc amphetamine was significantly enhanced by co-treatment with 500 ng or 1 μg Rp-cAMPS ($P < 0.05$, Newman-Keuls), but was not significantly different with other doses of Rp-cAMPS (including 10 and 20 μg).

To assess the contribution of the modulatory effects of Rp-cAMPS in particular conditioning sessions to overall unconditioned activity during training, locomotor activity across the three pairing days was collapsed into an overall average for groups receiving amphetamine alone or co-treatment with those doses of Rp-cAMPS where effects were observed in particular sessions. The enhancement of locomotor activity in the second and third sessions with 500 ng and 1 μg Rp-cAMPS produced a sig-

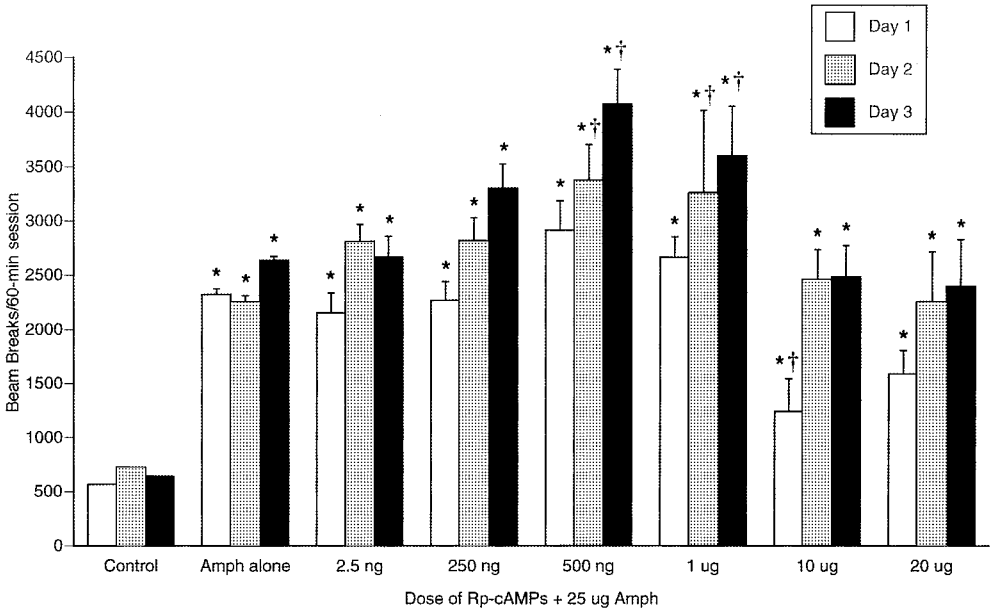

FIGURE 4. Mean (± SEM) number of photocell crossings for each of the three 60 min pairing sessions for controls, amphetamine alone, and all doses of Rp-cAMPS co-infused with amphetamine. *, significant ($P < 0.05$) difference from control; †, significant ($P < 0.05$) difference from amphetamine alone.

nificant increase in overall unconditioned locomotion relative to amphetamine alone ($t_{18} = 2.89$, $P < 0.05$; $t_{22} = 2.65$, $P < 0.05$ for 500 ng and 1 μg Rp-cAMPS, respectively). In contrast, the reduced locomotor activation in the 10 μg Rp-cAMPS co-treated animals (and the non-significant reduction in the 20 μg group) during the first conditioning session did not significantly affect overall unconditioned locomotion relative to amphetamine alone ($t_{21} = 1.12$, $P > 0.05$; $t_{23} = 0.99$, $P > 0.05$ for 10 and 20 μg Rp-cAMPS, respectively).

Locomotor sensitization with amphetamine and Rp-cAMPS

In addition to the general stimulant property of middle doses of Rp-cAMPS on amphetamine-induced unconditioned activity, repeated co-treatments appeared to enhance acute locomotor activating effects progressively (Figure 4). A two-way (treatment day $\times$ group) ANOVA revealed a significant interaction [$F(14,188) = 2.47$, $P < 0.05$], demonstrating that unconditioned locomotor activity changed differentially in the groups across 3 days of drug treatment. The locomotor response to amphetamine (and in the controls) showed little change over the 3 days of drug exposure ($P > 0.05$). In contrast, animals co-treated with Rp-cAMPS at doses between 250 ng and 1 μg showed greater increases in locomotor activity with repeated treatments ($P < 0.05$, simple effect of day). Animals treated with lower (2.5 ng) or higher doses (10 and 20 μg) of Rp-cAMPS also showed increases in motor activity with later treatments ($P < 0.05$), but this effect was largely due to a difference between the first and second sessions (see below). These data demonstrate that the development of locomotor sensitization to intra-NAc amphetamine is enhanced by Rp-cAMPS in a dose-dependent fashion.

Conditioned locomotion: effects of Rp-cAMPS during conditioning

For conditioned activity, the two control groups again did not differ significantly (see Figure 3; $t_{20} = 1.56$, $P > 0.05$), so were collapsed into a single mean. A single-factor ANOVA for the eight treatment groups was significant [$F(7,94) = 7.09$, $P < 0.05$]. Post-hoc Dunnett's t-tests revealed that conditioning with intra-NAc amphetamine produced a significant enhancement of locomotor activity in the drug-free test ($P < 0.05$), demonstrating conditioned activity (Figure 5). This conditioned activity effect was also significant for amphetamine co-infused with low doses of Rp-cAMPS up to 500 ng ($P < 0.05$, Dunnett's t-tests), but not for the higher doses of 1–20 μg ($P > 0.05$). Moreover, groups receiving amphetamine/Rp-cAMPS co-treatments up to 1 μg did not show any enhancement of conditioned activity relative to amphetamine alone ($P > 0.05$, Newman-Keuls), despite significant enhancements of overall unconditioned activity for groups receiving 500 ng and 1 μg Rp-cAMPS co-treatments. In fact, at higher doses of Rp-cAMPS (10 and 20 μg), conditioned locomotor activity was significantly blocked in the drug-free test relative to amphetamine alone (both $P < 0.05$, Newman-Keuls). Thus, the effects of Rp-cAMPS co-treatment on conditioned and unconditioned activity can be dissociated in the same animals. Doses of Rp-cAMPS (500 ng and 1 μg) that significantly enhance unconditioned locomotor activation produced by intra-NAc amphetamine do not enhance conditioned activity (in fact, conditioned locomotor activity in the 1 μg group was not significantly different from controls). Conversely, doses of Rp-cAMPS (10 and 20 μg) that do not affect overall unconditioned activity, completely block the establishment of conditioned activity. These results suggest that the establishment of conditioned activity with intra-NAc amphetamine requires the activation of PKA in this region during conditioning.

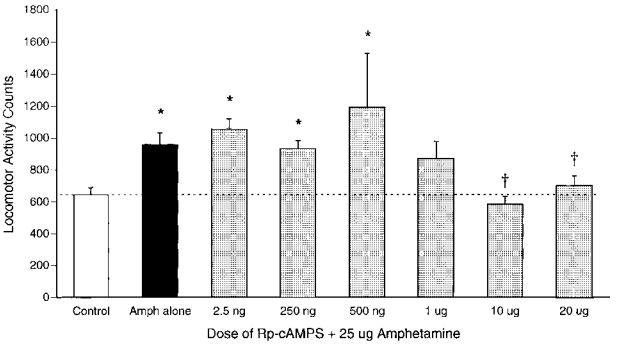

FIGURE 5. Mean ($\pm$ SEM) number of photocell crossings during the first 50 min of the drug-free test session for controls, amphetamine alone, and all doses of Rp-cAMPS co-infused with amphetamine. The dashed line represents the locomotor response in the controls. *, significant ($P < 0.05$) difference from control; †, significant ($P < 0.05$) difference from amphetamine alone.

Since the unconditioned locomotor response to amphetamine on day 1 was reduced in the 10 μg Rp-cAMPS group (and also non-significantly in the 20 μg group), it is possible that this effect could account for the lack of conditioned activity observed in the drug-free test. As noted above for the first conditioning day, locomotor activity in the amphetamine alone group was significantly greater than in the group receiving 10 μg Rp-cAMPS plus amphetamine, but was not significantly different to the 20 μg Rp-cAMPS plus amphetamine group. In the

two following conditioning sessions, unconditioned activity in the 10 and 20 μg groups was similar to that produced by amphetamine alone (see Figure 4), and overall locomotor activity during conditioning (averaged across the three sessions) did not significantly differ between amphetamine alone and either the 10 or the 20 μg groups. Moreover, both the 10 and 20 μg Rp-cAMPS groups showed significant stimulant effects on locomotor activity for all three conditioning sessions when compared with the controls. Despite these characteristics, the lack of a full unconditioned stimulant effect during pairing (relative to amphetamine alone) in these groups could contribute to the absence of conditioned activity. We have examined this possibility by re-analysing conditioned activity in the 10 and 20 μg groups after excluding animals with the lowest overall unconditioned activity, so as to match unconditioned activity in these groups with that of amphetamine. Specifically, animals with the lowest average locomotor activity across the three conditioning sessions were sequentially removed from the 10 and 20 μg groups until the group average on this measure for each group exceeded the group average for amphetamine alone. The resulting data for unconditioned and conditioned activity for these modified groups are shown in Table 1. Despite excluding six and five animals from the 10 and 20 μg groups, respectively, conditioned activity in both these groups was still significantly blocked relative to amphetamine alone ($t_{15} = 3.11$, $P < 0.05$; $t_{18} = 2.20$, $P < 0.05$, for 10 and 20 μg groups, respectively). In light of these results, it is unlikely that the reduced stimulation of unconditioned locomotor activity with these high doses of Rp-cAMPS on the first conditioning day solely accounts for the block of conditioned activity.

DISCUSSION

The results of this study indicate that, like systemic administration, local infusion of amphetamine into the NAc can produce conditioned locomotor activity. This effect depends on a positive contingency between the environment and amphetamine, demonstrating that associative learning, rather than a history of amphetamine infusions *per se*, underlies this conditioned activity effect. To our knowledge, this is the first demonstration of conditioned activity resulting from intra-NAc amphetamine, and suggests that learning-related plasticity within the NAc underlies this effect.

Unconditioned and conditioned locomotion

The locomotor activation induced by intra-NAc amphetamine was enhanced in a dose-dependent manner by co-treatment with middle doses of Rp-cAMPS. This effect followed an inverted U shaped dose-response profile (appearing maximal at the 500 ng dose) across all three pairing sessions, although it reached statistical significance only during the second and third sessions. Nevertheless, overall unconditioned locomotor activity during pairing sessions was significantly enhanced in both the 500 ng and 1 μg Rp-cAMPS groups. At higher doses of Rp-cAMPS (10 and 20 μg), the acute locomotor activation induced by amphetamine appeared to be reduced in the first session; this effect was statistically significant only in the 10 μg group. While the full locomotor stimulant effect of amphetamine in the first session appeared reduced at these doses of Rp-cAMPS, both groups demonstrated significant locomotor activation relative to controls. In the second and third sessions, the acute locomotor response in these groups was similar to that of amphetamine alone. Moreover, neither the 10 nor the 20 μg groups showed significant reductions in overall unconditioned activity compared with amphetamine alone.

Our results suggest that the activation of PKA plays an essential role in initiating the NAc plasticity required for conditioned activity induced by intra-NAc amphetamine. Despite the enhancement of amphetamine-induced unconditioned activity with two doses of Rp-cAMPS (500 ng and 1 μg), locomotor responses during the drug-free test were not similarly enhanced; this result suggests that the increase in unconditioned locomotor activity during PKA inhibition was ineffective in altering the

TABLE 1. Mean $\pm$ SEM locomotor counts over the three pairing sessions and during the drug-free test session for the amphetamine alone group and the 10 and 20 μg Rp-cAMPS/amphetamine groups, excluding animals with the lowest overall unconditioned activity

Treatment	Unconditioned activity				Conditioned activity
	Session 1	Session 2	Session 3	Mean	
25 μg amphetamine alone ($n = 12$)	2323 $\pm$ 187	2253 $\pm$ 158	2636 $\pm$ 198	2404 $\pm$ 144	958 $\pm$ 74.3
25 μg amphetamine + 10 μg Rp-cAMPS ($n = 5$)	1732 $\pm$ 246	3018 $\pm$ 834	3379 $\pm$ 666	2710 $\pm$ 405	568 $\pm$ 72.0*
25 μg amphetamine + 20 μg Rp-cAMPS ($n = 8$)	2023 $\pm$ 216	2918 $\pm$ 485	2978 $\pm$ 444	2639 $\pm$ 340	697 $\pm$ 92.9*

*, significantly ($P < 0.05$) reduced compared with amphetamine alone by t-test.

strength of the conditioned response. More importantly, higher doses of Rp-cAMPS (10 and 20 μg) did not significantly alter overall unconditioned locomotor stimulation of intra-NAc amphetamine, but blocked the conditioned locomotion assessed in the drug-free test. This suggests that the processes by which environmental stimuli paired with intra-NAc amphetamine acquire stimulant-like properties requires the activation of PKA.

Previous studies have shown that the ability of amphetamine or cocaine to produce conditioned activity requires intact dopaminergic neurotransmission (Beninger and Hahn, 1983; Beninger and Herz, 1986). Since D1 receptors stimulate cAMP production and lead to the activation of PKA, our finding that inhibiting such activation blocks the establishment of conditioned locomotion is consistent with previous work showing a critical role for D1 receptors in this form of learning (Drew and Glick, 1990; Mazurski and Beninger, 1991; Vezina and Stewart, 1989). In this context, the previous findings from our laboratory (Beninger *et al*., 1996; Westly *et al*., 1998) and others (Kelley and Holahan, 1997), demonstrating a role for the activation of cAMP in reward-related learning, may specifically reflect modulation of processes involved in associative conditioning rather than effects on hedonic state. One important prediction based on this idea is that the effect of PKA inhibition during associative learning should block both appetitive- and aversively-motivated behaviour (i.e. be independent of hedonic state). In support of this notion, disruption of PKA signalling disrupts associative learning based on aversive stimuli in *Drosophila* (Davis, 1996) and rodents (Abel *et al*., 1997; Vianna *et al*., 1999), and on appetitive stimuli in honeybees (Menzel and Müller, 1996) and rats (Beninger *et al*., 1996 and present study).

Conditioned locomotion and locomotor sensitization

Another interesting dissociation found in the present study is that between locomotor sensitization and conditioned activity. Local infusion of amphetamine into the NAc stimulated unconditioned locomotor activity, but the degree of locomotor activation remained fairly constant over the 3 days of drug treatment. In contrast, unconditioned locomotion after amphetamine/Rp-cAMPS co-treatment was progressively enhanced by repeated treatments over a range of Rp-cAMPS doses (250 ng to 1 μg). Despite this sensitization of the unconditioned response, the conditioned locomotor response was not significantly different from that with amphetamine alone. In fact, the level of conditioned locomotor activity at the 1 μg dose of Rp-cAMPS was not significantly different from controls, suggesting that conditioned activity was attenuated in this group of animals.

As our results suggest that Rp-cAMPS may enhance the development of locomotor sensitization, it is noteworthy that the cAMP system in the NAc has previously been implicated in this effect. Intra-NAc administration of 8-bromo-cAMP (a cAMP analogue that activates PKA) coincident with systemic cocaine administration produced progressively larger stimulation of locomotor activity across three treatment days, while neither treatment alone produced such an effect (Miserendino and Nestler, 1995). Moreover, persistent upregulation of G_s in the NAc with local cholera toxin treatment produced sensitization to the locomotor-activating effects of amphetamine and cocaine (Cunningham and Kelley, 1993). These results suggest that stimulation, rather than inhibition, of PKA in the NAc enhances locomotor sensitization, as well as the acute locomotor response. In fact, Miserendino and Nestler (1995) found that intra-NAc infusion of another PKA inhibitor, 8-(4-chlorophenylthio)-adenosine-3′,5′-cyclic monophosphorothioate, Rp isomer (Rp-CPT-cAMPS), did not enhance the activation of locomotor activity by systemic cocaine, although only one dose of the drug was examined. In the present study, Rp-cAMPS was co-infused with amphetamine into the NAc, whereas the effect of Rp-CPT-cAMPS in the NAc was assessed following systemic cocaine. Whether the differences in the results of these studies are related to the psychomotor stimulant used, their route of administration, the dose or type of PKA inhibitor, or some other variable, will have to await future studies.

The mechanism by which co-treatments of Rp-cAMPS and amphetamine given into the NAc leads to enhanced locomotion and sensitization compared with NAc amphetamine alone is not known. However, by inhibiting PKA, Rp-cAMPS mimics one of the postsynaptic effects of D2 agonists. Amphetamine plus a D2 agonist would be expected to produce a greater locomotor response than amphetamine alone (Dreher and Jackson, 1989). The present results have shown that the roles of PKA in unconditioned activity and sensitization on the one hand, and the establishment of conditioned activity on the other, are dissociable. Perhaps the enhancement of unconditioned activity produced by Rp-cAMPS is related to its D2-like agonist effects, whereas its ability to block conditioning is related to its anti-D1-like effects.

Downstream target(s) of PKA critical for learning

The importance of PKA for conditioned activity, as revealed in the present study, contributes to an ever-increasing literature implicating the cAMP pathway in learning. While several different modes of coincidence detection in the nervous system are likely to contribute to different forms of associative learning (Bourne and Nicoll, 1993), the PKA pathway may subserve a particular mechanism that appears conserved from invertebrates to mammals. To what extent downstream targets of PKA play a role in learning and are similarly conserved is unclear.

DA and glutamatergic inputs terminate on the same spine of striatal medium spiny neurons, and DA release in the NAc and other striatal regions may modify the properties of particular glutamatergic synapses. One idea is that DA may reinforce specific environmental signals carried by glutamatergic inputs in an activity-dependent manner (e.g. Wickens and Kötter, 1995; Beninger and Miller, 1998). In this way, neutral stimuli (activating a subset of glutamatergic afferents) paired repeatedly with an unconditioned stimulus (that stimulates DA neurons; Schultz *et al*., 1997) may come to control behaviour. In support of the idea that the actions of DA depend on concurrent glutamatergic activity, the striatal expression of several immediate early genes (IEGs) induced by D1 agonists is blocked by the *N*-methyl-D-aspartate (NMDA) receptor antagonist 2-amino-5-phosphonopentanoic acid (APV) (Konradi *et al*., 1996). The amount of cAMP stimulated by D1 agonists was not affected by APV treatment, while the D1 agonist-induced phosphorylation of the transcription factor cAMP response element binding protein (CREB) appeared to be inhibited. This suggests that the interaction between D1 receptor activation and NMDA-dependent neurotransmission is downstream of the receptor, its associated G protein and cAMP. Moreover, increased expression of one IEG, FOS, in the NAc has been associated with conditioned locomotion following repeated cocaine treatments, and both the conditioned activity and FOS expression were blocked by the NMDA antagonist MK-801 (Franklin *et al*., 1996); similar behavioural results were found by others (Stewart and Durham, 1993; Cervo and Samanin, 1996). Thus, D1-dependent biochemical and behavioural effects appear to require coincident glutamatergic transmission.

Since D1-mediated transcriptional events depend on functional transmission at NMDA receptors, local postsynaptic activity may have an enabling role for D1-linked second-messengers to initiate nuclear events involved in synaptic strengthening. In this context, PKA could play a critical role in synaptic plasticity underlying associative conditioning, since CREB, a substrate of PKA phosphorylation, initiates such events and appears to be recruited or sustained in an activity-dependent manner (Bito *et al*., 1996; Konradi *et al*., 1996; Liu and Graybiel, 1996). CREB is a transcription factor that has a pivotal role in long-term synaptic plasticity and learning (for reviews see Carew, 1996; Yin and Tully, 1996; Abel and Kandel, 1998). In *Drosophila*, transient induction of an inhibitory CREB transgene prior to training selectively disrupted long-term memory of olfactory learning (Yin *et al*., 1994). Conversely, induction of an activating isoform of CREB prior to training in this task enhanced memory (Yin *et al*., 1995). The enhancement was not observed in transgenic flies expressing the CREB activator with a mutation in a PKA phosphorylation site. A similar importance for CREB in learning has been demonstrated in mammals; mice deficient in two CREB isoforms are profoundly impaired in contextual fear conditioning (Bourtchuladze *et al*., 1994).

In the context of learning and memory, there are likely to be several important downstream targets of PKA, of which CREB is but one. The recognized importance of CREB in learning and memory is due in large part to the experimental attention it has received for this role. In addition to phosphorylating CREB, PKA also seems to recruit additional proteins that are necessary for CREB-initiated transcription (Brindle *et al*., 1995). Moreover, DARPP-32 (a DA- and cAMP-regulated phosphoprotein) may also be an important learning-related substrate, as it exhibits joint regulation by PKA and calcium (for review see Greengard *et al*., 1998). The role of additional downstream targets of PKA, and the manner in which they interact to function in information storage, awaits further research.

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