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Place Preference Induced by Nucleus Accumbens Amphetamine Is Impaired by Antagonists of ERK or p38 MAP Kinases in Rats

Todor V. Gerdjikov, Gregory M. Ross, and Richard J. Beninger
Queen's University

The nucleus accumbens (NAc) plays a role in conditioned place preference (CPP). The authors tested the hypothesis that inhibition of mitogen-activated protein kinases (MAPKs) would inhibit NAc-amphetamine-produced CPP. Results confirmed that NAc amphetamine increased levels of the MAPK extracellular signal-regulated kinase (ERK). In CPP studies, NAc injections (0.5 μl per side) of the ERK inhibitor PD98059 (1.0–2.5 μg) or the p38 kinase inhibitor SB203580 (15–500 ng) dose dependently impaired CPP. The c-Jun-N-terminal kinase (JNK) inhibitor SP600125 (1.0–2.5 μg) failed to block the CPP effect. The drugs did not block amphetamine-induced motor activity. Results suggest that ERK and p38, but not JNK, MAPKs may be necessary for the establishment of NAc amphetamine-produced CPP and may also mediate other forms of reward-related learning dependent on NAc.

Dopamine (DA) in the nucleus accumbens (NAc) is implicated in reward-related learning (Baldwin, Sadeghian, Holahan, & Kelley, 2002; Beninger, 1983; Carr & White, 1986; Everitt et al., 1999; Setlow, Holland, & Gallagher, 2002; Sutton & Beninger, 1999). Convergent activation of DA and glutamate inputs to NAc medium spiny neurons may strengthen glutamate synapses activated by behaviorally relevant stimuli (Kelley & Berridge, 2002). DA modulates glutamate transmission in the neostriatum (Flores-Hernandez, Galarraga, & Bargas, 1997) and *N*-methyl-D-aspartate-dependent corticostriatal long-term potentiation (LTP) depends on D_1-like DA receptors (Kerr & Wickens, 2001). Hippocampal afferents to NAc produce phasic increases in DA (Blaha, Yang, Floresco, Barr, & Phillips, 1997), and they may partially depolarize medium spiny neurons, allowing prefrontal glutamate inputs to produce action potentials in NAc (Goto & O'Donnell, 2001). Behavioral work has shown that both intact DA transmission and *N*-methyl-D-aspartate receptors in NAc are required for appetitive conditioning (Smith-Roe & Kelley, 2002).

The cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) second messenger cascade may mediate plasticity resulting from convergent DA and glutamate input to NAc. DA activates PKA, which has been implicated in working memory (Aujla & Beninger, 2001; Taylor, Birnbaum, Ubriani, & Arnsten, 1999), fear conditioning (Schafe & LeDoux, 2000), NAc-dependent memory for a conditioned reward (Baldwin et al., 2002), and conditioned place preference (CPP) based on NAc amphetamine (Beninger, Nakonechny, & Savina, 2003). However, other second messenger cascades also may be involved (Berke, Paletzki, Aronson, Hyman, & Gerfen, 1998).

The extracellular signal-regulated kinase (ERK) is a mitogen-activated protein kinase (MAPK; Derkinderen, Enslen, & Girault, 1999) implicated in learning and memory (Adams & Sweatt, 2002). Systemic amphetamine upregulates ERK in the dorsal striatum (Choe, Chung, Mao, & Wang, 2002). Hippocampal ERK is necessary for step-down avoidance learning (Cammarota et al., 2000) and learning in the water maze (Blum, Moore, Adams, & Dash, 1999), and amygdalar ERK mediates freezing to conditioned stimuli paired with footshock (Schafe et al., 2000). Glutamate application to striatal neurons induces ERK activation that leads to phosphorylation of the cAMP-response element-binding protein (CREB) that has been implicated in memory (Choe & Wang, 2002; Silva, Kogan, Frankland, & Kida, 1998). ERK mediates protein kinase C (PKC)-induced CREB phosphorylation. Thus, ERK may participate in learning mediated by DA-glutamate interactions, as crosstalk exists among ERK, PKC, and PKA (Adams & Sweatt, 2002; Cammarota et al., 2000; Choe & Wang, 2002; Grewal, York, & Stork, 1999).

p38 and c-Jun-N-terminal kinases (JNK) are stress-activated MAPKs implicated in apoptosis (Curtis & Finkbeiner, 1999). Limited in vitro evidence suggests that both participate in D_1-like receptor signaling (Zhen, Uryu, Wang, & Friedman, 1998). Striatal DA activates the p38 MAPK in a PKA-dependent manner (Vincent, Sebben, Dumuis, & Bockaert, 1998), and p38 activates CREB (Choe & McGinty, 2000; Curtis & Finkbeiner, 1999). Cerebellar p38, but not JNK, has been shown to play a role in classical conditioning (Zhen, Du, Romano, Friedman, & Harvye, 2001). Hippocampal JNK has been implicated in memory for an inhibitory avoidance task (Bevilaqua, Kerr, Medina, Izquierdo, & Cammarota, 2003). Thus, p38 and JNK may also play a role in learning mediated by DA-glutamate interactions.

In neurochemical experiments, we evaluated the effect of injections of amphetamine into the NAc of anesthetized rats on MAPK levels. In behavioral studies, we used CPP (Tzschentke, 1998) to test the hypothesis that NAc DA-mediated learning depends on MAPKs. Rats received NAc amphetamine plus MAPK inhibitors

Todor V. Gerdjikov, Department of Psychology, Queen's University, Kingston, Ontario, Canada; Gregory M. Ross, Department of Physiology, Queen's University; Richard J. Beninger, Department of Psychology and Department of Psychiatry, Queen's University.

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Correspondence concerning this article should be addressed to Richard J. Beninger, Department of Psychology, Queen's University, Kingston, Ontario K7L 3N6, Canada. E-mail: beninger@psyc.queensu.ca

during conditioning. We hypothesized that CPP would be antagonized dose dependently by the p38 inhibitor SB203580, the ERK inhibitor PD98059 (Dudley, Pang, Decker, Bridges, & Saltiel, 1995; Lee et al., 1994), or the JNK inhibitor SP600125 (Bennett et al., 2001). Part of this research was presented in abstract form (Gerdjikov & Beninger, 2002).

Method

Subjects and Surgery

Male Wistar rats (Charles River, St. Constant, Quebec; $N = 204$), weighing between 200–250 g on arrival, were housed in pairs on a 12-hr reversed light–dark cycle (lights on at 1900), at an average temperature of 21 °C (humidity 40%–70%). Water and food (LabDiet 5001; PMI Nutrition International, Brentwood, MO) were freely available. Rats were handled for approximately 1 min per day for 5 consecutive days after arrival. The experimental protocol was approved by the Animal Care Committee at Queen's University. All rats were treated in full compliance with the Animals for Research Act and relevant guidelines set by the Canadian Council on Animal Care.

Approximately 1 week after arrival at the colony, rats used for behavioral studies were anesthetized in an induction chamber by means of an inhalable anesthetic (5% isoflurane; Bimeda, Cambridge, Ontario) mixed with oxygen in a vaporizer system (Benson, Merkham, Ontario) and administered at 1.0 L/min. Anesthetized rats were fitted to a stereotaxic apparatus, and isoflurane was administered at a concentration of 2% or as needed to maintain anesthesia. The head was adjusted so that lambda and bregma were on the same horizontal plane. For analgesia, buprenorphine hydrochloride in solution (0.15 mg/kg; Reckitt & Colman, Richmond, VA) was injected subcutaneously, preoperatively and again immediately on removal from anesthesia. The experimenter shaved the rat's head and applied betadine solution with a cotton tip applicator before incising the skin. Holes were drilled into the skull, and 23 gauge (0.64 mm diameter) stainless steel guide cannulas were chronically implanted bilaterally into the NAc, with coordinates 1.6 mm anterior to bregma, 1.4 mm lateral to the midline, and 6.7 mm ventral from the skull surface (Paxinos & Watson, 1998). The guide cannulas were held in place by four stainless steel screws and dental acrylic. Stainless steel wire stylets (0.31 mm diameter), flush with the end of the guide cannulas, were put in place to prevent occlusion. Rats were allowed approximately 1 week to recover before the start of behavioral testing.

Western Blotting

The extent of ERK activation was determined by Western blotting with protein and phosphoprotein-specific antibodies (Cell Signaling Technology, Beverly, MA). Under isoflurane anesthesia, rats ($n = 4$) were given an injection of amphetamine (20 μg/0.5 μl) into one NAc and saline (0.5 μl) into the other. Injector coordinates and infusion procedures were identical to the ones used in the behavioral experiments. After 15 min, rats were removed from anesthesia and rapidly decapitated. Coronal slices 1 mm thick through the NAc were obtained, and a 16 gauge (1.7 mm) blunted needle was used to take punches of NAc (including both the core and shell regions in a single punch), dorsal striatum, and cingulate cortex, according to the atlas of Paxinos and Watson (1998). Tissues were immediately frozen on dry ice and stored at −80 °C.

Tissues were processed by homogenization in sodium dodecyl sulfate reducing buffer and separated with a 10% polyacrylamide gel. Proteins were transferred to nitrocellulose, and Western blotting was performed according to the manufacturer's directions. For reprobing, blots were stripped by incubation for 30 min at 60 °C in 0.125 M Tris HCl (pH 6.8), 2% SDS, and 0.8% β-mercaptoethanol. Gels were probed first for phosphoprotein and subsequently for protein levels.

Immunoreactive proteins were detected by means of electrochemoluminescence (SuperSignal; Pierce, Rockford, IL) with film exposure, quantified with densitometry performed on a flatbed scanner, and analyzed by Adobe Photoshop. Bands for ERK1 and ERK2 were sampled together. ERK1 and 2 levels were determined together on the same blot for the 4 rats. For the amphetamine-injected side, the level of phosphorylated ERK1 and ERK2 was expressed as a ratio of the level of ERK1 and 2 protein for each rat. Results for the saline-treated side were calculated similarly. These values were then compared in dependent samples t tests for each area sampled.

Drug Infusion

The ERK inhibitor 2′-amino-3′-methoxyflavone (PD98059), the p38 inhibitor 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB203580), and the JNK inhibitor anthra[1,9-cd]pyrazol-6(2H)-one1,9-pyrazoloanthrone (SP600125; Calbiochem, San Diego, CA) were dissolved in dimethyl sulfoxide (DMSO) before the beginning of the experiment and stored at −20 °C; amphetamine sulfate (USP, Rockville, MD) was dissolved in saline daily before each set of injections. Central injections into the NAc were made with a microinfusion pump (Sage Instruments, Cambridge, MA). Injectors were glued to polyethylene tubing (0.75 mm o.d.) filled with distilled water. The tubing was connected to two 10-μl microsyringes (Microliter #701; Hamilton, Reno, NV) mounted on the microinfusion pump. Drugs were backloaded into the injectors by aspiration with the two syringes. Rats were hand-held as the experimenter removed the stylets from the guide cannulas and inserted the two injectors (0.31 mm o.d.). The injectors projected 1.2 mm beyond the guide cannulas.

PD98059 (1.0, 1.7, and 2.5 μg/0.5 μl/side; or 3.7, 6.4, and 9.4 nmol/0.5 μl/side), SB203580 (15, 62, 250, and 500 ng/0.5 μl/side; or 0.04, 0.16, 0.66, and 1.30 nmol/0.5μl/side), and SP600125 (1.0, 2.0, and 2.5 μg/0.5 μl/side; or 4.5, 9 and 11 nmol/0.5 μl/side) were injected bilaterally in DMSO over 30 s. The range for PD98059 included a dose previously reported to impair retention in the water maze task when injected into the hippocampus (Blum et al., 1999). A similar dose range was used for the JNK inhibitor SP600125. We found no behavioral work reporting local administrations of SB203580 and therefore tried a low dose of 500 ng. Because that dose impaired the CPP effect (see below), we tested increasingly lower concentrations until a nonblocking dose was found. After the drug was delivered, the injectors were left in place for another 30 s to allow for diffusion, after which they were slowly retracted from the guide cannulas. Amphetamine (20.0 μg/0.5 μg/side) was injected approximately 10 min later by the same procedure. Place conditioning began immediately after amphetamine injection. DMSO was used for vehicle injections, except for the group that received amphetamine without a kinase inhibitor. This group received saline as the vehicle. Groups were tested drug-free.

Animals tested in a drug-free state may fail to show a CPP for reasons other than kinase inhibition. For example, during conditioning, they may form an association between reward and some internal state putatively caused by the drugs. The CPP effect may be rescued if the animals are tested with the same drug that they received during conditioning. To test for state-dependent learning, we injected two additional groups of rats with PD98059 (2.5 μg/0.5 μl/side) or SB203580 (500 ng/0.5 μl/side) immediately before testing and after conditioning with amphetamine and the same dose of the inhibitor.

Apparatus

The four testing chambers each consisted of two rectangular compartments (38 cm long × 27 cm wide × 34 cm high) connected with a tunnel (8 × 8 × 8 cm). Two different spatial features were varied across the testing chambers. The compartment walls were either urethane-sealed wood or alternating 1-cm-wide black and white vertical stripes covered with clear Plexiglas. The floor was either wire bars 1 cm apart running

perpendicular to the tunnel, or a wire grid with openings of 1 cm^2. This resulted in four possible compartment types, distributed as left or right compartment across the four different testing chambers. Each compartment had a Plexiglas top with a number of circular ventilation holes. The tunnel was fitted with guillotine-type doors, which could be closed to prevent movement from one compartment to the other. Two activity sensors in each compartment and two in the tunnel were used to monitor movement between and within compartments. The number of beam breaks during conditioning sessions was used as a measure of locomotion. Each of the testing chambers was housed in a dimly lit (7.5 W), sound-attenuating and ventilated wooden box. Indirect light reached the rat though the Plexiglas tops of the compartments. Data from the sensors were collected on a 6809 microcontroller with custom-made software and transferred to a Macintosh computer for analyses. For further details of the apparatus, see Brockwell, Ferguson, and Beninger (1996).

Behavioral Procedure

Training and testing occurred during the day (7 a.m.–7 p.m.). Rats were tested in groups of 4, and each rat was tested in a different testing chamber. The experimental protocol consisted of three habituation sessions, eight conditioning sessions, and one test session. Each rat completed one session per day.

Habituation. At the start of each 15-min session, the rat was placed in one compartment of the box—the left compartment for half the rats and the right compartment for the other half. Tunnel doors were open, allowing the rats to move freely between the two compartments. Activity sensors recorded the amount of time spent on each side and in the tunnel.

Conditioning. Over eight consecutive 30-min sessions, MAPK inhibitors and amphetamine were injected into the NAc on Days 1, 3, 5, and 7, and vehicle on Days 2, 4, 6, and 8. Injection of the MAPK inhibitor was followed by an injection of amphetamine approximately 10 min later. Rats received only one injection on vehicle days. Immediately after amphetamine or vehicle injection, depending on conditioning day, the rat was placed in one of the two compartments with the tunnel doors closed, preventing movement into the other compartment or the tunnel. Half the rats were confined to the left side on drug days and to the right side on vehicle days. The other half were confined to the right side on drug days and to the left side on vehicle days. In this way, any given compartment was paired with drug for some rats and with vehicle for others. The number of beam breaks was recorded for each rat to assess locomotion.

Testing. Testing occurred on the day immediately after conditioning. The session lasted 15 min and was identical to habituation sessions. The start side was the same for each rat on test and habituation days. Time spent on each side and in the tunnel was recorded.

Data Analyses for CPP Studies

The amount of time spent on the drug-paired side before conditioning was averaged across the 3 habituation days. Planned dependent samples *t* tests were used to compare that average with the time spent on the same side on the test day. CPP occurred if rats showed a significant increase in time spent on the drug-paired side after conditioning. Similarly, planned dependent samples *t* tests were used to compare tunnel time before and after conditioning. To check for side bias, another set of *t* tests compared time spent on the drug-paired side with time spent on the vehicle-paired side before conditioning. In addition, the number of beam breaks for each rat on each of the 8 conditioning days was totaled and used as an index of motor activity. Activity data were analyzed with a 2 × 2 (Day × Drug) within-subjects analysis of variance (ANOVA).

Histology

After completion of the experiment, rats were placed in an airtight chamber and euthanized with CO_2. Brains were removed and preserved in a 10% formalin solution for at least 72 hr. Coronal sections 60 μm thick from throughout the cannulated region were obtained by slicing the brains on a cryostat at −20 °C. The sections were mounted on gelatin-coated glass slides and stained with cresyl violet. Judgments about the cannula placements in the NAc were made by an observer who was blind to the results for individual rats.

Results

Western Blotting

Microdissection of NAc regions from saline- and amphetamine-treated sides from individual rats were used for Western blotting of protein and phosphoprotein levels of ERK1/2, c-Jun (downstream of JNK), and p38. Levels of ERK1/2 and phosphorylated ERK1/2 were readily detectable. Activated (phosphorylated) products of c-Jun and p38, however, could not be detected with this preparation. Immunoblots from the NAc of each of the 4 rats, showing ERK1/2 (upper panel) and phospho-ERK1/2 (lower panel) determined from reprobing of the same blot are presented in Figure 1A. The ratio of phospho-ERK1/2 to ERK1/2 was calculated for the saline- and amphetamine-treated side of each rat for NAc, dorsal striatum, and cingulate cortex, and *t* tests were used to compare their ratios for each structure. A significant increase in phospho-ERK1/2 to ERK1/2 was observed on the amphetamine-treated side within the NAc, $t(3) = 6.07$, $p < .01$ (Figure 1B).

Histology

A total of 200 rats was tested in CPP. Five rats from the ERK experiment, 3 rats from the p38 experiment, and 2 rats from the amphetamine experiment failed to complete the study because of illness or technical problems. There was no relationship between the type and dose of drug and illness observed in some rats.

Cannula placements were assessed for the remaining rats. A total of 46 rats were excluded: 20 from the ERK experiment, 16 from the p38 experiment, 9 from the JNK experiment, and 1 from the amphetamine experiment, leaving 144 rats for subsequent analyses. Figure 2 shows location of cannula tips for all rats included in the analyses. Placements were classified as hits if the tips of both cannulas were located in the core or shell region of NAc.

Time Spent on Each Side During Preexposure

The interpretation of CPP results is not straightforward if subjects have a natural avoidance of the to-be-drug-paired side. In such a case, an apparent increase in time spent on that side after conditioning may be the result of decreased avoidance of the drug-paired side or simply habituation (Tzschentke, 1998). To check for bias, we averaged time spent on the side that would be paired with drug across the 3 habituation days and compared it with time spent on the side that would be paired with vehicle for each group. Paired samples *t* tests revealed nonsignificant differences for all but one group. However, rats receiving SB203580 both during conditioning and on test day showed a preference for the to-be-saline-paired side, $t(6) = 2.90$, $p < .05$ (see Table 1). Thus, with the exception of one group, rats did not avoid the to-be-drug-paired side during habituation, and the CPP paradigm was unbiased.

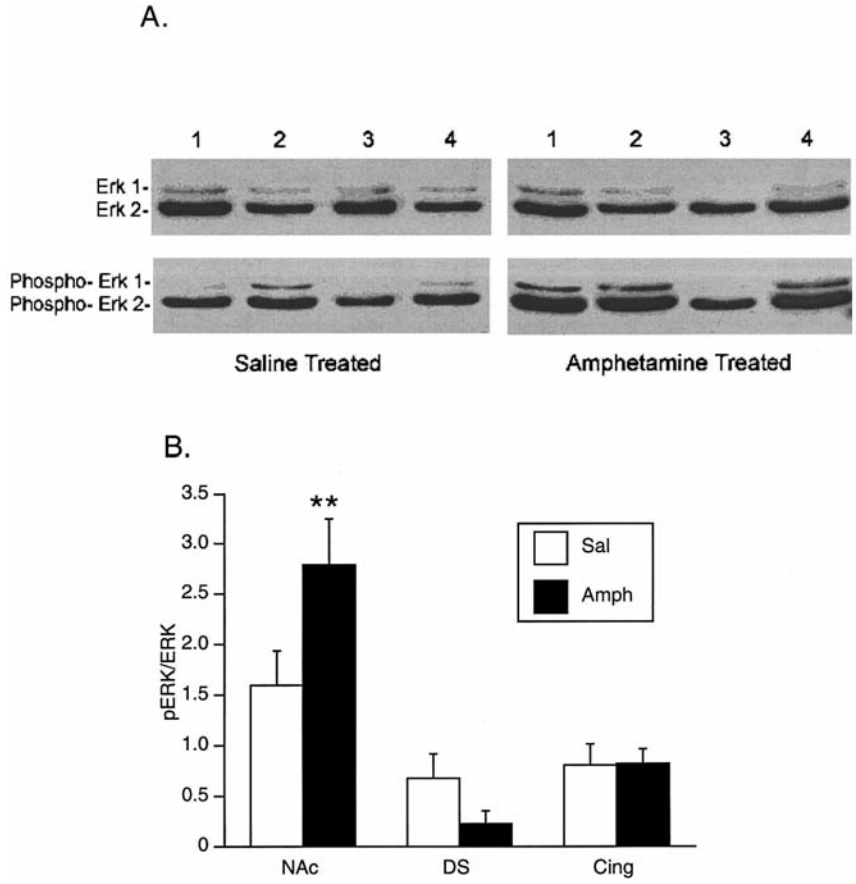

Figure 1. Effects of nucleus accumbens (NAc) amphetamine injection (20 μg/0.5 μl) on extracellular signal-regulated kinase (ERK) protein phosphorylation. Western blotting of both ERK1/2 protein and phosphoprotein was used to characterize relative ERK1/2 activation in the unilateral saline- and amphetamine-treated tissue. Each subject was assayed individually. A: Immunoblots for NAc tissue. The four saline-treated sides and the four amphetamine-treated sides are illustrated. All protein and phosphoprotein values were obtained from a single immunoblot for each structure. Tissues were evaluated for ERK1/2 protein expression (upper panel) and phosphoprotein (Phospho- Erk; lower panel). B: Densitometry analysis of ERK1/2 protein phosphorylation in NAc, dorsal striatum (DS), and cingulate cortex (Cing). Relative activation of protein was calculated by probing, stripping, and reprobing blots with specific antibodies and determining the ratio of phosphorylated (pERK) to nonphosphorylated ERK1/2 protein for each of the indicated regions of each individual. Sal = saline-infused side; Amph = amphetamine-infused side. Error bars represent *SEM.* ** $p < .01$ as compared to saline-injected side (paired samples *t* test).

Tunnel Time

A change in the time spent in the drug-paired side from habituation to test cannot be interpreted unambiguously as a change in place preference if time spent in the tunnel also changes. Thus, additional analyses were performed to compare tunnel time before and after conditioning (see Table 2). Two groups showed a change in time spent in the tunnel. Rats in the amphetamine-only group and in the 1.0 μg PD98059 group spent significantly less time in the tunnel after conditioning, $t(8) = 3.98$, $p < .01$, and $t(10) = 6.53$, $p < .01$, respectively. We analyzed place preference data for these two groups using both raw scores and percentages (reported below). Percentages were based on total time spent in the compartments, disregarding tunnel time, to evaluate the possibility that the change in tunnel time biased the results. Both methods of analyzing the data yielded the same results.

Place Conditioning

Amphetamine alone, PD98059 alone, and SB203580 alone. Rats receiving only amphetamine on drug days and saline on vehicle days showed a place preference for the drug-paired side after conditioning, $t(8) = 2.70$, $p < .05$. Analyzing place preference data as a percentage of total time minus tunnel time yielded a similar result, $t(8) = 2.73$, $p < .05$ (data not shown). Rats receiving PD98059 or SB203580 alone did not show a place preference after conditioning (Figure 3A).

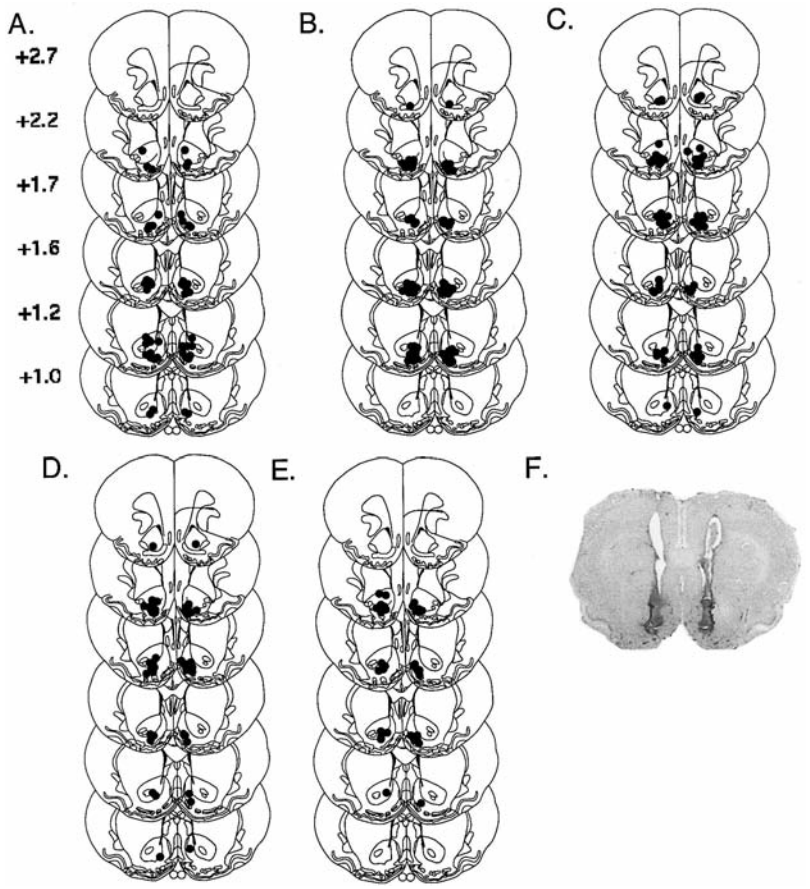

Figure 2. Drawings of coronal sections through the nucleus accumbens (NAc), indicating sites of infusion. Injector sites may appear fewer than the reported number of rats because of overlap of placements. Numbers at the top left indicate distance (in millimeters) from bregma. Reprinted from *The Rat Brain in Stereotaxic Coordinates,* 4th ed., G. Paxinos and C. Watson, Figures 9–14, Copyright 1998, with permission from Elsevier. A: Injector sites for infusion of amphetamine alone (20 μg/0.5 μl), SB203580 alone (500 ng/0.5 μl dimethyl sulfoxide), and PD98059 alone (2.5 μg/0.5 μl). B: Injector sites for PD90859 (1.0, 1.7, 2.5 μg before conditioning, and 2.5 μg after conditioning, per 0.5 μl) and amphetamine (20 μl/0.5 μl) infusion. C: Injector sites for SB203580 (15, 62, 250, and 500 ng/0.5μl) and amphetamine (20 μg/0.5μl) infusion. D: Injector sites for SP600125 (2.5, 2.0, and 1.0 μg before conditioning) and amphetamine (20 μg/0.5 μl) infusion. E: Injector sites for state-dependent control groups: PD98059 (2.5 μg/0.5μl) and SB203580 (500 ng/0.5μl; see text). F: Photomicrograph depicting a representative NAc injector placement.

PD98059. Rats receiving 1.0 or 1.7 μg/0.5 μl/side of PD98059 showed a significant CPP effect, $t(10) = 3.07$, $p < .01$, and $t(8) = 2.57$, $p < .05$, respectively. Analyzing place preference data for the 1.0-μg group as a percentage of total time minus tunnel time yielded a similar result, $t(10) = 2.74$, $p < .05$ (data not shown). In contrast, rats receiving 2.5 μg/0.5 μl/side of PD98059 before or immediately after each conditioning session did not show a significant CPP effect (Figure 3B).

SB203580. Planned *t* tests showed that rats receiving 15 ng/0.5 μl/side of SB203580 showed a significant CPP effect, $t(7) = 3.27$, $p < .05$. In contrast, rats receiving 62, 250, or 500 ng/0.5 μl/side of SB203580 did not show a significant increase in place preference (Figure 3C).

SP600125. Rats receiving amphetamine and 1.0, 2.0, or 2.5 μg/0.5 μl/side of the JNK inhibitor SP600125 showed a significant CPP effect, $t(9) = 2.80$, $p < .05$; $t(7) = 2.40$, $p < .05$; and $t(9) = 2.71$, $p < .05$, respectively (Figure 3D).

State-dependent controls. Rats receiving PD98059 or SB203580 both during conditioning and on test day showed no significant place preference for the amphetamine-paired side (Figures 3B and 3C).

Locomotor Activity

Activity was averaged across the four vehicle sessions and across the four drug sessions on respective conditioning days, yielding measures of vehicle and drug-induced activity.

Amphetamine alone, PD98059 alone, and SB203580 alone. Rats receiving amphetamine alone showed higher activity on drug days compared with saline days, $t(8) = 3.82$, $p < .01$. Rats

Table 1
Mean (± SEM) Times Spent on the Drug- and Vehicle-Paired Sides Before Conditioning

Drug and dose	*n*	Drug-paired	Vehicle-paired
PD98059			
1.0 μg	11	424.2 ± 22.0	419.8 ± 23.6
1.7 μg	9	433.6 ± 24.0	415.3 ± 23.2
2.5 μg	9	438.0 ± 14.0	397.3 ± 13.3
2.5 μg (after)	9	435.1 ± 31.4	402.7 ± 26.0
2.5 μg (stdep)	10	429.2 ± 28.6	418.9 ± 29.5
SB203580			
15 ng	8	426.3 ± 17.0	413.5 ± 17.6
62 ng	7	387.8 ± 26.8	431.1 ± 30.8
250 ng	10	413.6 ± 16.0	434.7 ± 16.9
500 ng	11	402.8 ± 25.6	416.6 ± 22.0
500 ng (stdep)	7	350.5 ± 22.9	482.9 ± 24.4*
SP600125			
1.0 μg	8	443.1 ± 16.9	389.3 ± 16.1
2.0 μg	10	419.1 ± 23.8	410.9 ± 25.3
2.5 μg	10	415.6 ± 17.3	441.4 ± 17.4
Amphetamine alone			
20 μg	9	415.0 ± 12.7	431.8 ± 14.7
PD98059 alone			
2.5 μg	9	403.8 ± 29.6	439.8 ± 29.0
SB203580 alone			
500 ng	7	472.8 ± 31.8	392.4 ± 28.7

Note. All doses are per 5 μg per side. after = group receiving PD98059 after conditioning; stdep = state-dependent group.
* $p < .05$.

receiving either PD98059 or SB203580 alone did not show higher activity on drug days compared with vehicle days (Figure 4A).

PD98059. All groups showed increased activity on drug days compared with saline days. A Dose (1.0, 1.7, 2.5 μg/0.5 μl/side before, and 2.5 μg after) × Drug (amphetamine + inhibitor vs. vehicle) mixed ANOVA revealed only a significant main effect for drug, $F(1, 34) = 90.92$, $p < .001$. Thus, PD98059 did not significantly alter the stimulant effect of amphetamine (Figure 4B).

SB203580. All groups showed increased activity on drug days compared with saline days. A Dose (15, 62, 250, and 500 ng/0.5 μl/side) × Drug (amphetamine + inhibitor vs. vehicle) mixed ANOVA revealed only a significant main effect of drug, $F(1, 32) = 170.92$, $p < .001$. Thus, SB203580 did not affect the stimulant effect of amphetamine (Figure 4C).

SP600125. All groups showed increased activity on drug days compared with saline days. A Dose (1.0, 2.0, and 2.5 μg/0.5 μl/side) × Drug (amphetamine + inhibitor vs. vehicle) mixed ANOVA revealed only a significant main effect of drug, $F(1, 25) = 96.80$, $p < .001$. Thus, SP600125 did not affect the stimulant effect of amphetamine (Figure 4D).

State-dependent controls. Rats receiving PD98059 or SB203580 both during conditioning and on test day showed higher activity on drug days compared with saline days, $t(9) = 4.83$, $p < .001$, and $t(6) = 5.50$, $p < .01$, respectively. (Figures 4B and 4C).

Discussion

The present studies revealed that amphetamine injections into the NAc of awake rats produced a CPP and that similar injections into anesthetized rats stimulated ERK1 and ERK2. The CPP effect was impaired in a dose-dependent manner by inhibition of ERK or p38 MAPK, but not JNK. Control studies revealed that doses of ERK or p38 MAPK that blocked CPP produced by amphetamine had no effect on side preference when injected alone, and that injection of the agents before the testing sessions did not lead to the observation of a CPP effect. Amphetamine stimulated locomotor activity during conditioning, and none of the MAPK inhibitors significantly affected this stimulant effect. Thus, there was a dissociation of the effects of MAPK inhibitors on CPP versus locomotor stimulation produced by NAc injection of amphetamine. A similar dissociation of effects on reward-related learning versus locomotor stimulation has previously been reported with inhibitors to PKA and PKC (Aujla & Beninger, 2003, Beninger et al., 2003). This suggests that amphetamine's ability to endow environmental stimuli with rewarding properties is dissociable from its effects on unconditioned locomotion. Consistent with this, Baldwin et al. (2002) reported that PKA, a molecule functionally related to MAPK, is necessary for the acquisition of conditioned responding for food, but not for the expression of such responding. Thus, PKA seems to be necessary for the establishment of reward-related learning but does not modulate its expression. This suggests that reward-related learning with amphetamine requires the activation of second messenger molecules (PKA, PKC, MAPK, etc.) in the NAc, whereas locomotor activity and the expression of reward-related learning based on amphetamine do not (for a review see Beninger & Gerdjikov, in press).

The CPP paradigm was unbiased, with the exception of one group; rats did not show a systematic preference for one side of the apparatus over the other before conditioning. Also, the time rats spent in the tunnel before and after conditioning did not differ for

Table 2
Mean (± SEM) Time Spent in Tunnel Before and After Conditioning

Drug and dose	*n*	Before	After
PD98059			
1.0 μg	11	56.0 ± 4.0	40.4 ± 4.3**
1.7 μg	9	51.1 ± 6.0	64.1 ± 7.4
2.5 μg	9	65.0 ± 5.0	69.4 ± 6.8
2.5 μg (after)	9	62.2 ± 10.0	64.2 ± 7.2
2.5 μg (stdep)	10	51.9 ± 5.4	54.1 ± 6.3
SB203580			
15 ng	8	60.3 ± 6.1	56.8 ± 8.8
62 ng	7	57.4 ± 7.3	81.6 ± 12.8
250 ng	10	51.7 ± 5.2	57.5 ± 7.6
500 ng	11	54.9 ± 2.6	50.6 ± 5.8
500 ng (stdep)	7	66.5 ± 13.0	75.4 ± 16.8
SP600125			
1.0 μg	8	65.3 ± 7.2	53.9 ± 8.2
2.0 μg	10	70.0 ± 6.7	63.5 ± 6.6
2.5 μg	10	43.0 ± 3.2	44.3 ± 6.9
Amphetamine alone			
2.0 μg	9	53.3 ± 6.0	40.9 ± 6.1**
PD98059 alone			
2.5 μg	9	56.4 ± 8.2	64.2 ± 7.5
SB203580 alone			
500 ng	7	45.0 ± 7.3	48.4 ± 7.4

Note. All doses are per 5 μg per side. after = group receiving PD98059 after conditioning; stdep = state-dependent group.
** $p < .01$.

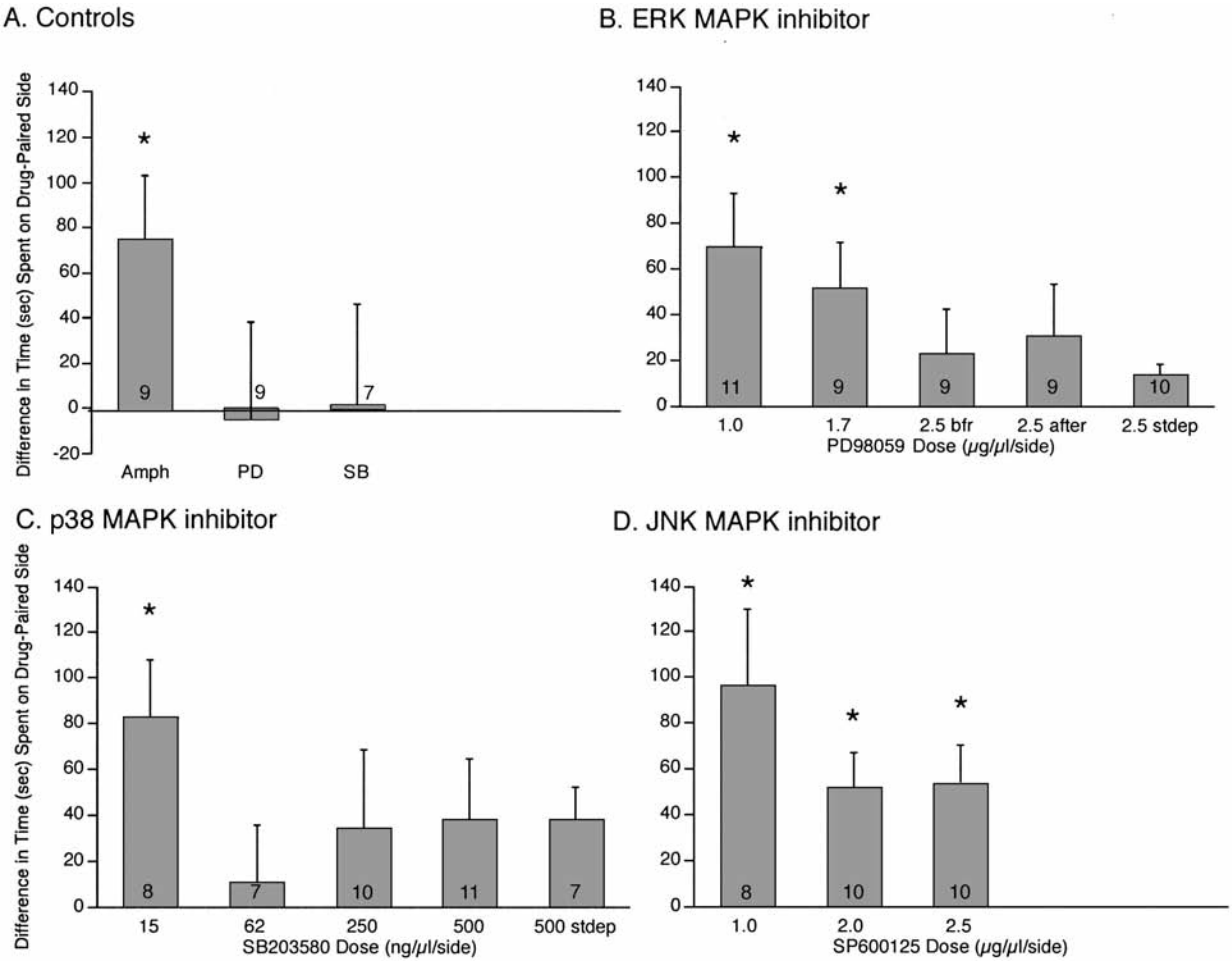

Figure 3. Mean (± *SEM*) difference scores for various doses of the mitogen-activated protein kinase (MAPK) inhibitors SB203580, PD98059, or SP600125, and amphetamine, infused into nucleus accumbens before conditioning sessions. Difference scores are the average of time spent on the drug-paired side across the 3 preconditioning days subtracted from time spent on the drug-paired side recorded on test. A: Control groups. Amph = amphetamine alone (20 μg/0.5 μl); PD = PD98059 alone (2.5 μg/0.5 μl); SB = SB203580 alone (500 ng/0.5 μl). B: Extracellular signal-regulated kinase (ERK) MAPK inhibitor. bfr = inhibitor infused immediately before the conditioning session; after = inhibitor infused immediately after the conditioning session; stdep = state-dependent controls. C: p38 MAPK inhibitor. D: c-Jun-N-terminal kinase (JNK) MAPK inhibitor. Numbers in or above each bar indicate the number of rats tested. * $p < .05$, significant increase in time spent on the drug paired side on test day compared with preconditioning.

most groups, suggesting that changes in time spent on the drug-paired side were in fact due to changes in preference. For the two groups that showed a decrease in time spent in the tunnel after conditioning, we analyzed time spent on the drug-paired side as a percentage of the total time minus tunnel time. The results were the same as the results obtained using raw scores; therefore, tunnel times did not significantly affect the present results. These results, combined with the multiple replications of the amphetamine CPP for lower doses of the ERK and p38 inhibitors and all doses of the JNK inhibitor, emphasize the reliability of the CPP methodology.

The NAc can be subdivided into core and shell regions (Zahm, 2000), and a number of studies have shown that these subregions may be differentially involved in reward-related learning (Di Chiara, 2002; Parkinson, Olmstead, Burns, Robbins, & Everitt, 1999). Recently, Sellings and Clarke (2003) implicated the medial shell region in amphetamine reward. Our data did not permit a direct investigation of the relative contribution of these regions. An attempt was made to assess the possible role of the core and shell by separating the groups into core and shell placements and performing post hoc analyses. However only 10 rats had both cannulas located within the core subregion. These rats were distributed unevenly among the groups, making it impossible to statistically analyze the effect of region. Because of this, no further attempts were made to differentiate between these structures. A more thorough investigation of the role of NAc subregions in reward produced by local injections of amphetamine is awaited.

ERK and p38 inhibitors impaired place conditioning. One possibility is that the agents produced aversive conditioning on their

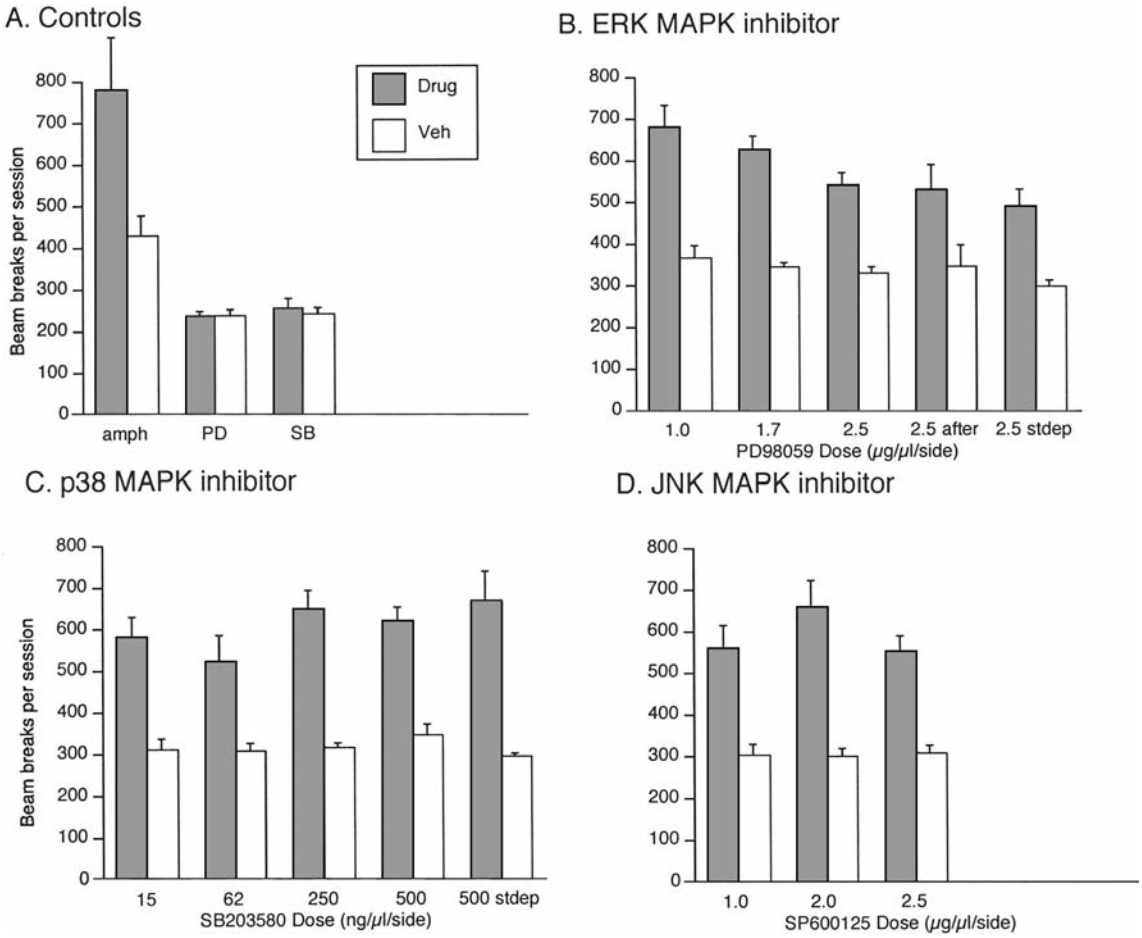

Figure 4. Locomotor activity during conditioning for amphetamine and various doses of mitogen-activated protein kinase (MAPK) inhibitors. Activity counts represent mean (± *SEM*) number of beam breaks per conditioning session averaged across the 4 drug-paired and the 4 vehicle-paired days. A: Control groups. amph = amphetamine alone (20 μg/0.5 μl); PD = PD98059 alone (2.5 μg/0.5 μl); SB = SB203580 alone (500 ng/0.5 μl). B: Extracellular signal-regulated kinase (ERK) MAPK inhibitor. after = inhibitor infused immediately after the conditioning session; stdep = state-dependent controls. C: p38 MAPK inhibitor. D: c-Jun-N-terminal kinase (JNK) MAPK inhibitor. Whenever amphetamine was injected, whether alone or with a MAPK inhibitor, increased activity was seen.

own and that simple additivity of this putative effect plus the positive effect of amphetamine led to the loss of the CPP. To test this, we conditioned some rats with inhibitors to p38 and ERK, but without amphetamine treatment. On test day, these rats did not show a preference or avoidance for the inhibitor-paired side. Thus, simple additivity of effects could not account for the observation that ERK and p38 inhibition impaired CPP.

It has been suggested that some CPP results may be explained by state-dependent learning (Overton, 1985). According to this argument, putative stimulus properties of a MAPK inhibitor, along with the stimulus configuration of the drug-paired side, become associated with the rewarding properties of amphetamine. Rats tested in a drug-free state would fail to show a CPP because part of the stimulus complex that was associated with reward was missing. If this were the case, testing after injection of the MAPK inhibitor would replace the missing stimuli and CPP would be seen. To test this possibility, on test day, we injected some rats with the same MAPK inhibitor they had received during conditioning. These rats failed to show a CPP, suggesting that state-dependent learning cannot account for our results.

A significant activation of ERK1/2 (Figure 1) was detected in NAc of anesthetized rats injected with amphetamine into NAc, but not in the other brain regions examined. Whether anesthesia influenced this result cannot be determined from the present experiments. Other proteins examined by this technique included c-Jun and p38 MAPK. In both instances, detection limits for the phosphorylated species were above the levels obtained using the microdissection technique. As a result, relative levels of protein activation could not be obtained for either of these signaling pathways. The ERK results confirm that NAc injections of amphetamine led to activation of this protein (Choe et al., 2002).

Inhibition of ERK and p38, but not JNK, MAPK impaired CPP produced by NAc amphetamine. These findings support a growing body of research implicating MAPKs in memory and plasticity in

a variety of systems and brain regions (for recent reviews, see Adams & Sweatt, 2002; Impey, Obrietan, & Storm, 1999). Mutant mice deficient in Ras, an ERK activator, had impaired LTP in the amygdala and impaired memory for an aversive event (Curtis & Finkbeiner, 1999). ERK was also necessary for LTP in the CA1 region of the hippocampus and for spatial memory in the Morris water maze (Blum et al., 1999; Roberson et al., 1999). ERK was activated in insular cortex after the presentation of an unfamiliar taste, and inhibition of ERK in this area impaired conditioned taste aversion (Berman, Hazvi, Rosenblum, Seger, & Dudai, 1998). In one study, systemic cocaine-produced CPP in mice was blocked with systemic injections of the ERK inhibitor SL327 (Valjent et al., 2000). Unlike Valjent et al. (2000), we did not observe a decreased locomotor effect after administration of the inhibitor, possibly because we applied our drugs directly into NAc and because a different ERK inhibitor was used. In general, our results are in excellent agreement with Valjent et al. (2000). Thus, previous studies have shown that ERK is involved in plasticity that underlies learning and agree with the present findings that NAc ERK was necessary for amphetamine-induced CPP.

In the present studies, inhibition of ERK impaired amphetamine-produced CPP even when injected after conditioning sessions. This finding suggests that ERK may be involved in the acquisition of CPP, its consolidation, or both. These results are consistent with other studies reporting an impairment of cocaine CPP with posttraining inhibition of PKA or PKC (Cervo, Mukherjee, Bertaglia, & Samanin, 1997), and with studies reporting impairment with posttraining PKA inhibition in other reward-related tasks (Baldwin et al., 2002). They are also consistent with work implicating ERK in the consolidation of memory for tasks mediated by other brain structures (e.g., Walz et al., 2000). Thus, inhibition of NAc ERK either during or after injections of amphetamine blocks reward-related learning.

Amphetamine up-regulates striatal ERK (Choe et al., 2002). Amphetamine also leads to activation of PKA through enhanced DA release and stimulation of D_1-like receptors (Civelli, Bunzow, & Grandy, 1993). Because NAc PKA is necessary for CPP (Beninger et al., 2003), perhaps this learning occurs through an ERK-dependent mechanism. ERK is also downstream from PKC, and PKC has been implicated in synaptic plasticity (Choe & Wang, 2002) and shown to be necessary for CPP based on NAc amphetamine (Aujla & Beninger, 2003). Thus, PKC-dependent learning similarly may depend on ERK. Alternatively, PKA, PKC, and ERK pathways may converge on common nuclear targets through parallel signaling pathways.

The relationship between ERK1 and ERK2 appears to be complex. Thus (Mazzucchelli et al., 2002) reported that $ERK1^{-/-}$ mice showed enhanced learning in striatal tasks and enhanced LTP in the striatum. These mice had up-regulation in stimulus-induced ERK2 in the striatum. These results contrast with the majority of the literature showing memory impairment as a result of ERK inhibition, but are, to our knowledge, the only ones to evaluate ERK1 and 2 separately in behavioral studies.

The current findings with SB203580 are in agreement with an emerging literature implicating the p38 MAPK in learning and plasticity. p38 has been implicated in long term depression in the CA3–CA1 region of the hippocampus (Bolshakov, Carboni, Cobb, Siegelbaum, & Belardetti, 2000). p38 MAPK also plays a role in eyeblink conditioning in the cerebellum (Zhen et al., 2001). Striatal p38 has been shown to activate CREB, ERK, and Elk-1 (Choe et al., 2002). Thus, the role of NAc ERK in CPP may be secondary to p38 activation. Future research is needed to elucidate this point.

Our results do not support a major role for JNK in the CPP paradigm; thus, the JNK inhibitor SP600125 failed to block CPP at molar concentrations similar to those of the ERK inhibitor PD98059 and much higher than those of the p38 inhibitor SB203580 that did block the effect. Hippocampal JNK was implicated in memory for step-down avoidance in one recent paper (Bevilaqua et al., 2003), but its role in the CPP paradigm had not been explored. Nuclear targets downstream from MAPK activation that are responsible for learning in the NAc have also not been sufficiently investigated. Both ERK and p38, as well as PKA, and PKC, have been shown to activate CREB, a protein widely believed to mediate long-term memory, through the transcription of new mRNA (Silva et al., 1998). Although reward-related learning in the NAc has been shown to involve DNA transcription, the exact late gene targets and their products must await further investigation (Hernandez, Sadeghian, & Kelley, 2002).

We investigated the role of MAPKs in reward-related learning using the CPP paradigm. NAc amphetamine-produced CPP was impaired by inhibitors of ERK and p38 MAPK, but not by an inhibitor of JNK kinase. These results are consistent with previous research showing a role for MAPK in memory and plasticity in hippocampus and amygdala, and they fit the role of MAPKs in cellular cascades in the striatum or NAc initiated by the activation of DA receptors. The significance of NAc kinase–kinase interactions in the context of reward-related learning and the downstream role of MAPK need further investigation.

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