Contributions of the Mitogen-Activated Protein Kinase and Protein Kinase C Cascades in Spatial Learning and Memory Mediated by the Nucleus Accumbens

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ABSTRACT

Several studies have reported a role for the nucleus accumbens (NAcc) in learning and memory. Specifically, NAcc seems to function as a neural bridge for the translation of corticolimbic information to the motor system mediating locomotor learning, but the signaling mechanisms involved in this striatal learning await further investigation. The present experiments investigated the role of the mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) cascades within the NAcc of Long-Evans rats in a food-search spatial learning task (FSSLT). First, we used immunoblotting to examine changes in MAPK p42/p44 phosphorylation within the NAcc in the acquisition phase of the FSSLT. Second, we examined the effect on the acquisition and retention phases in the FSSLT of pretraining intra-accumbal microinjections of the MAPK [U0126; 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene, 1 μg/side] or PKC [GF109203X; bisindolylmaleimide or 1-(3-dimethylaminopropyl)-indol-3-yl]-3-(indol-3-yl) maleimide, 0.5 ng/side] inhibitors (four training sessions; one session/day). Third, the potential coupling of PKC and MAPK signaling pathways in the NAcc during spatial learning was studied using microinjections of GF109203X, radioactive activity assays, and immunoblotting. Results showed that 1) MAPK p42/p44 phosphorylation is augmented within the NAcc after spatial learning, 2) MAPK and PKC inhibition caused differential deficits in the acquisition and formation of spatial memories, and 3) inhibition of PKC activity by GF109203X caused a reduction in MAPKs phosphorylation in the NAcc in an early stage of the acquisition phase. Overall, these findings suggest that NAcc-PKC and -MAPK play important roles in spatial learning and that MAPKs phosphorylation seems to be mediated through the activation of the PKC signaling pathway.

The nucleus accumbens (NAcc) is considered a neural bridge between corticolimbic structures and the motor system (Mogenson et al., 1980). The hippocampus and prefrontal cortex have been implicated in spatial learning and memory processes (Morris et al., 1982; Chiba et al., 1994), and it is known that these brain areas send anatomical projections to the NAcc (Kelley and Domesick, 1982). Neural communications between the hippocampus, prefrontal cortex, and NAcc have led to hypothesize a role for the NAcc in certain aspects of place navigation. For example, investigations conducted thus far suggest that the NAcc is involved in the process of converting sensory information into appropriate spatial learning responses (Schacter et al., 1989; Floresco et al., 1997). Pharmacological evidence has suggested that NAcc-dependent spatial learning is mediated by excitatory amino acid receptors (Maldonado-Irizarry and Kelley, 1995). More recently, it was demonstrated that protein kinase C-γ within the NAcc plays an important role in the acquisition and memory of spatial information (Alvarez-Jaimes et al., 2004). Research focused on the examination of the molecular substrates and mechanisms underlying spatial learning mediators.
ated by the NAcc is limited. We wanted to evaluate the role of two protein kinases systems in spatial learning mediated by the NAcc because evidence has demonstrated that protein kinase activation is required for the induction and expression of long-term potentiation (LTP) in brain regions implicated in learning and memory processes (Asztely et al., 1990; Finch and Jackson, 1990; English and Sweatt, 1996).

The mitogen-activated protein kinase (MAPK) cascade has been associated with the control of synaptic plasticity in the brain (English and Sweatt, 1997; Huang et al., 2000) and a variety of learning and memory processes such as fear conditioning, spatial memory, and avoidance learning (Atkins et al., 1998; Blum et al., 1999; Selcher et al., 1999; Cammarota et al., 2000; Schafe et al., 2000). Protein kinase C (PKC) has been implicated in the initiation and maintenance of LTP (Asztely et al., 1990; Finch and Jackson, 1990). Evidence has also demonstrated that the PKC-γ isoform is required in spatial learning in the Morris water maze (Abelevich et al., 1993) and that training in a spatial discrimination task causes increases in PKC-γ immunoreactivity in the hippocampus (Van der Zee et al., 1992). Recently, it was reported that NAcc-PKC is activated after spatial training in a food search spatial learning task (FSSLT) (Alvarez-Jaimes et al., 2004).

In the present study, we used pharmacological and molecular strategies to characterize the role of NAcc-MAPK and -PKC cascades in spatial learning and memory and to determine a potential coupling between them. We hypothesize that MAPK and PKC within the NAcc are needed for spatial learning and memory and that both cascades might be interacting during spatial learning.

Materials and Methods

Subjects

Male Long-Evans rats weighing between 275 and 300 g (Harlan, Indianapolis, IN) were housed in pairs in plastic cages with controlled humidity and temperature (22°C) and maintained on a 12/12-h light/dark cycle. Food and water were provided at all times except during the food deprivation protocol that required maintaining animals at 85% of free-feeding weight. The food deprivation protocol started 5 days after the arrival of the animals. Animals were food restricted for 7 to 10 days, with access to only 15 g of food per day until they reached 85% of their initial body weight. During the food deprivation period, animals were also given 45 mg of Kellogs Fruit Loop pellets in addition to the regular laboratory chow. This food deprivation protocol was designed to habituate the animals before training to the sugar pellets used as bait in the maze. Throughout the experimental procedures, all animals were handled to acclimate them and minimize stress during behavioral testing. All procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Surgery

Animals were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and given atropine (0.54 mg/kg s.c.) for the implantation of bilateral indwelling guide cannulae within the NAcc core. The surgery was performed using a stereotaxic instrument with the nose bar 3.5 mm above interaural zero and coordinates A-P, +3.5 mm from bregma; M-L, ±2.0 mm from the midline; and D-V, -5.3 mm from skull. The cannulae (10 mm in length) were secured to stainless steel screws with dental cement and light-curable resin. Wire stylets were inserted in the guides until the microinjections were made. After surgery, animals were allowed to recover for a period of 5 to 7 days.

Inhibitors and Microinjection Procedure

GF109203X (Calbiochem, San Diego, CA) is a highly selective cell-permeable PKC inhibitor. This drug acts as a competitive inhibitor for the ATP binding site of PKC. In vitro studies have shown that GF109203X exhibits high selectivity for PKC-α, PKC-β, PKC-βIII, and PKC-γ (Toulec et al., 1991). Dimethyl sulfoxide (DMSO) was used to dissolve GF109203X. No published data until now have shown that DMSO that is frequently used as a solvent has a modulator side effect on the PKC and MAPK signaling pathways (for review, see Santos et al., 2003). GF109203X was dissolved in 100% DMSO to a final stock concentration of 1.65 μg/g. For the dose-response experiment, GF109203X was diluted with saline (sterilized 0.9% sodium chloride solution, pH 7.0), and the following doses were injected: 0.01, 0.02, 0.04, 0.08, and 0.5 ng/side. GF109203X was microinjected 30 min before training.

U0126 is a specific inhibitor of MAPK kinase (MAPKK) that is the upstream regulator of MAPKs/extracellular signal-regulated protein kinases (Pavata et al., 1998). U0126 (Promega, Madison, WI) was dissolved in 100% DMSO to a final stock concentration of 16 μg/mL. For the dose-response experiment, U0126 was diluted with saline to the following doses: 0.1, 1, and 2 μg/side. In the acquisition experiment, UO126 was always microinjected 20 min before training. The vehicle for both experiments was a dilution 1:1 of DMSO and saline. Bilateral microinjections into the NAcc core were done using a micropump (Harvard Apparatus Inc., Holliston, MA). A volume of 0.5 μL/side was always administered with an infusion time of 1 min and 33 s followed by 1 min of diffusion. After the microinjection procedure, injectors were removed, stylets were replaced, and animals were placed back in their cages.

Histological Analyses

Animals were deeply anesthetized and sacrificed by decapitation. Brains were taken out and frozen at −20°C. Brain tissue was cut into 45-μm sections using a cryostat (Leica Microsystems, Bannockburn, IL). Each section was mounted to a gelatin-coated slide, defatted, and stained with cresyl violet stain. Verification of the cannulae placements was done with a light microscope. Reconstructions of microinjection sites were made using a hand-drawn atlas, originally derived from serial Nissl-stained sections of a normal rat brain (head orientation, nose bar 5 mm above interaural zero). Each section from the atlas is 0.12 mm apart. Also, representative photomicrographs were obtained. Animals with cannulae placements outside the NAcc core were not included in the data analysis (n = 1–2/group).

Behavioral Methods

Holeboard Apparatus. A slightly modified hole poke activity meter (Columbus Instruments, Columbus, OH) was used as the food search apparatus. A square field (1 m × 1 m × 46 cm in height) of black Plexiglas with 16 equidistant holes (2.5 cm in diameter, 1.5 cm in depth) was present, in the floor plate was used. Several extramaze cues surrounded the apparatus to allow spatial orientation (i.e., posters, videocamera tripod, computer, and experimenter). This apparatus was used to examine performance, acquisition, retention, and locomotor activity during spatial learning behavior.

FSSLT. Originally, the food search spatial learning task was used to test deficits in attention disorders (Oades, 1982). However, it has been demonstrated that this paradigm is a hippocampal-dependent spatial discrimination task (Oades and Isaacson, 1978; Van der Staay et al., 1990; Van der Staay, 1999; Van der Zee et al., 1992, 1995; Peña de Ortiz et al., 2000; Vázquez et al., 2000) as are the radial arm and water maze (Olton and Papas, 1979; Morris et al., 1982). In the present experiment, we used an adapted version of the spatial discrimination task described by Oades and Isaacson (1978). In the food search task, animals learn to discriminate between baited (relevant) and nonbaited (irrelevant) holes within a 16-hole arena using spatial information cues. The FSSLT is acquired after four sessions of training (Maldonado-Irizarry and Kelley, 1995). In addi-
Behavioral treatments in the spatial training experiment

**TABLE 1**

Behavioral treatments in the spatial training experiment

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<th>Food Deprivation</th>
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*Naive rats were sacrificed after food deprivation, when they reached 85% of their body weight.

*Untrained rats were sacrificed 48 after habituation.*
Experiment 2: MAPKK Inhibitor Microinjections within the NAcc during Performance and Acquisition Phases of the FSSLT

Behavioral Testing. Two independent experiments were conducted to study the effect on spatial behavior of MAPK inhibition within the NAcc core. The first experiment evaluated the effect on the performance phase in the FSSLT (n = 8) of MAPKK inhibitor (U0126) microinjections at the following doses: 0, 0.1, 1, and 2 μg/side) within the NAcc core. This experiment was conducted to determine a dose-response curve for U0126. After behavioral testing, animals were sacrificed, and histological analysis was performed.

The second experiment tested the effect of MAPK inhibition within the NAcc core on the acquisition and retention phases in the FSSLT. A specific concentration of U0126 (1 μg/side) (n = 7) or vehicle (n = 7) was microinjected within the NAcc core before each training session of the acquisition phase. Also, spatial behavior during the retention test was evaluated. Animals were sacrificed after retention, and histological analysis was performed.

Molecular Procedure. A new group of animals underwent bilateral indwelling cannulae implantation into the NAcc core. After recovery period, animals were food-deprived and habituated for 2 days. Forty-eight hours after habituation, animals began spatial training in the food search task. Animals were microinjected with vehicle or the MAPKK inhibitor (U0126) 20 min before the beginning of each training session. In this experiment, animals were sacrificed at two different times. One group of control (n = 3) and experimental (n = 6) animals was sacrificed 5 min after the end of the last trial of the first session. The second group of control (n = 3) and experimental (n = 6) animals was sacrificed immediately after the third trial of the fourth session. Both time points were selected to verify the MAPKs phosphorylation inhibitory effect expected with the MAPKK inhibitor U0126. Also, confirmation of MAPKs phosphorylation reduction could be associated with spatial learning deficits observed during acquisition in the FSSLT.

Experiment 3: PKC Inhibitor Microinjections within the NAcc during Performance and Acquisition Phases of the FSSLT

Behavioral Testing. The effect on spatial behavior of PKC inhibition within the NAcc core was investigated in two independent experiments. The first experiment was conducted to investigate the effect on the performance phase of the FSSLT (n = 8) of PKC inhibitor GF109203X microinjections at several doses (0, 0.01, 0.02, 0.04, 0.08, and 0.05 ng/gside). Also, a dose-response curve for GF109203X was determined. Animals were sacrificed, and histological analysis was performed.

The second experiment studied the effect on the acquisition and retention phases in the FSSLT of PKC inhibition within the NAcc core. A specific GF109203X dose (0.5 ng/gside) (n = 7) or vehicle (n = 7) was microinfused within the NAcc core before each training session of the acquisition phase. Also, the effect on retention of PKC inhibition during acquisition was tested. Animals were sacrificed after the retention test, and histological analysis was performed.

Experiment 4: Coupling of PKC Activity and MAPKs Phosphorylation within the NAcc during Early Acquisition of Spatial Information

Biochemical and Molecular Procedure. Another group of animals underwent bilateral indwelling cannulae implantation into the NAcc core. After the recovery period, animals were food-deprived and habituated for 2 days as described previously. Forty-eight hours after habituation, animals began the one-session acquisition training in the food search task. Animals were microinjected with the vehicle (n = 7) or the PKC inhibitor GF109203X (n = 7) 30 min before the beginning of spatial training. Animals were sacrificed 5 min after the end of the last trial of this session. This time point was selected for two reasons. First, to verify the inhibitory effect of GF109203X on PKC activity and to establish an association between spatial learning deficits observed during acquisition session 1 and inhibition of PKC activity. Second, taking into consideration previous studies in our laboratory showing that PKC is activated 5 min after first acquisition session in the FSSLT (Alvarez-Jaimes et al., 2004), animals sacrificed at this time point were also used to examine a potential coupling between PKC activity and MAPKs phosphorylation. PKC activity was determined using the PKC activity assay and MAPKs phosphorylation with Western blotting.

Biochemical and Molecular Methods

Protein Extraction. Since animals were trained in pairs, they were sacrificed in groups of two. Brains were taken out and punches of the whole NAcc were done and stored at −80°C until the next day. NAcc punches were obtained with a 1-mm punch tool (Fine Science Tools, Foster City, CA) from 1-mm-thick sections taken on a brain matrix (Plastics One, Roanoke, VA). The whole NAcc was used for the biochemical and molecular studies to compare the results with previously published data suggesting an increase in NAcc-PKC activity associated with spatial training in the FSSLT (Alvarez-Jaimes et al., 2004). Tissue homogenates for each condition were prepared from a pool of four NAcc. The NAcc were homogenized with a precooled Polytron homogenizer (Brinkmann Instruments, Westbury, NY) in extraction buffer. Sodium orthovanadate and p-nitrophenolphosphate are inhibitors of phosphotyrosine-specific phosphatases and are required for maintaining the phosphorylation state of activated MAPKs. The homogenates were centrifuged at 100,000g for 1 h at 4°C. The supernatants obtained from this procedure were saved and used as the cytosolic fraction for MAPK immunoblotting and PKC activity assays. The remaining pellets were resuspended with the extraction buffer and sonicated at 4°C. The sonicated pellets were incubated with 0.2% Triton X-100 for 30 min at 4°C. Upon completion of this; the samples were centrifuged at 100,000g for 1 h at 4°C. The resulting supernatants were saved and used as the membrane-associated fraction for PKC activity assays. Protein concentration was measured by the Bradford method. Certain volumes from cytosolic and membrane-associated fractions were maintained at 4°C to perform PKC activity assays. These assays were conducted the same day as tissue homogenization. The remaining fraction volumes were diluted using glycerol, aliquoted, and stored at −80°C to conduct Western blotting later.

Immunoblotting. Protein electrophoresis was performed using 10% sodium dodecyl sulfate-polyacrylamide mini gels loaded with 40 μg of total protein. A biotinylated protein molecular weight standard of high range is included in a gel lane. The trans-blot electrophoresis apparatus (Bio-Rad, Hercules, CA) was used to transfer proteins electrophoretically to nitrocellulose membranes in Towbin’s buffer (39 mM glycine, 48 mM Tris base, pH 8.3, 0.037% SDS, and 20% methanol) at 4°C and 10 V overnight. Gels were stained with Coomassie Brilliant Blue to verify transfer efficiency. Blots were incubated with blocking solution [phosphate-buffered saline (PBS), 0.1% (v/v) Tween 20, and 5% nonfat dried milk] for 1 h at room temperature. Membranes were then washed once for 15 min and twice for 5 min with washing solution [PBS and 0.1% (v/v) Tween 20]. Membranes were incubated with a polyclonal antibody for phosphorylated MAPK p44/p42 (T-MAPKs) (1:1000) (Cell Signaling Technology Inc., Beverly, MA) at 4°C overnight. Next, blots were washed as described previously and incubated with a sheep anti-rabbit horseradish peroxidase-linked antibody (1:2000) (Amersham Biosciences, Inc., Piscataway, NJ) for 1 h and 15 min at room temperature. Then, washing was repeated, and the ECL Western blotting analysis system (Amersham Biosciences, Inc.) was used for detection of the luminescent signal. Membrane stripping was done with stripping solution (0.5 M acetic acid and 0.5 M NaCl, pH 2.5) and agitation for 15 min at room temperature. Then, they were rinsed in PBS for 25 min. Western blotting was repeated using a polyclonal antibody for total MAPK p44/p42 (T-MAPKs) (1:1000) (Cell Signaling Technology Inc.). To
normalize P-MAPKs and T-MAPKs immunoblotting data, membranes were stripped and reprobed using a monoclonal antibody for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein (1:10,000) (Advanced Immunochemical, Long Beach, CA). The films were scanned using the GS-800 densitometer system (Bio-Rad), and the densitometry analysis was carried out with Quantity One software version 4.2.1 (Bio-Rad). P-MAPKs and T-MAPKs levels were normalized using the housekeeping gene GAPDH, which has shown no significant changes in expression under some experiment conditions (Zablocka et al., 1998). Specifically, normalization of the MAPK immunoblotting data was done as follows: a ratio was calculated between the optical density (OD) value for the T-MAPKs band and the OD value for the GAPDH band. Then, OD values for P-MAPKs were divided by normalized T-MAPKs OD values. Thus, immunoblotting data values were presented as a ratio between P-MAPKs and T-MAPKs immunoreactivity.

PKC Activity Assay. PKC activity was quantified using the protein kinase C assay system (Promega). This assay measures the activity of all the Ca2+/phospholipid-dependent kinase Cs (α, β, βII, and γ). Biochemical reactions were performed and analyzed as described by Vázquez et al. (2000). Briefly, two master mixes were prepared: a reaction mix with the presence of phospholipids that is used for the activation of PKC and a control mix with the absence of phospholipids that corresponds to the control reaction. The control mix is similar to the reaction mix, except that the activation buffer (100 mM Tris-HCl, pH 7.5, and 50 mM MgCl2). The activated and control reactions for both the treatment were performed in triplicate. Radioactive wastes were disposed of in accordance with the regulations of the Nuclear Regulatory Commission and the University of Puerto Rico.

Data Analysis

Statistical analysis was performed using GraphPad Software Inc. (San Diego, CA) Prism version 3.02. Behavioral data were analyzed using one- or two-factor analysis of variance (ANOVA) with repeated measures (one-factor RM ANOVA or two-factor RM ANOVA). Trials were considered as the repeated factor. Statistical analysis of the PKC assays and immunoblotting data were done using Student’s t test or one-factor ANOVA. Post hoc analysis using the student Newman-Keuls was performed when necessary.

Results

Experiment 1: MAPKs Phosphorylation and Expression within the NAcc Associated with Acquisition Phase of the FSSLT

One-factor RM ANOVA of the behavioral parameters measured in the first session of spatial training showed significant differences throughout the trials. Specifically, a decrease in the mean total time spent searching for food was observed [trial factor: F(4,60) = 5.450; p < 0.001]. Also, statistical analysis showed a significant reduction in the mean reference errors [trial factor: F(4,60) = 4.174; p < 0.01] when animals were spatially trained in the FSSLT. In contrast, no significant difference was found in the working error parameter [trial factor: F(4,60) = 2.282; p > 0.05] (data not shown).

Normalized values of MAPKs phosphorylation within the NAcc associated with early acquisition in the FSSLT are presented in Fig. 1A. Overall, one-factor ANOVA showed that MAPKs were differentially phosphorylated between behavioral treatments [MAPK p42: F(3,23) = 12.65; p < 0.001 and MAPK p44: F(3,23) = 10.00; p < 0.01]. Specifically, post hoc analysis revealed that MAPK p42 and p44 phosphorylation was significantly increased 5 min after training session 1 in comparison with naive animals that showed basal levels of phosphorylation (MAPK p42: p < 0.001 and MAPK p44: p < 0.001). In addition, the spatially trained animals showed a significant increase in MAPK p42 and p44 phosphorylation compared with untrained animals that were only habituated (MAPK p42: p < 0.001 and MAPK p44: p < 0.001). No significant changes were observed between naive and untrained animals (MAPK p42: p > 0.05 and MAPK p44: p > 0.05) or between naive and spatially trained animals sacrificed 1 h after training (MAPK p42: p > 0.05 and MAPK p44: p > 0.05). As a representative Western blot for the phosphorylated levels of MAPKs is presented in Fig. 1B. The statistical analysis revealed no significant differences in T-MAPKs levels between behavioral conditions [MAPK p42: F(3,23) = 1.642; p > 0.05 and MAPK p44: F(3,23) = 0.5953; p > 0.05]. A representative Western blot for the total levels of MAPKs is presented in Fig. 1C.

Experiment 2: Effects of MAPKK Inhibitor U0126 Microinjections within the NAcc on the Performance, Acquisition, and Retention Phases of the FSSLT

Histological Analyses. Figure 2 shows a schematic drawing of brain slices and the reconstruction of some representative cannulae placements from animals microinjected with the vehicle (Fig. 2A) or the MAPKK inhibitor U0126 (Fig. 2B). Placements of cannulae within the NAcc were verified by examination of brain slices under a light microscope. Animals with incorrect cannulae localization were eliminated. Figure 2 shows two representative animals microinjected with the vehicle (Fig. 2D) or U0126 (Fig. 2E) in the acquisition experiment. Overall, no significant tissue damage at the microinjection site was observed.

Acquisition and Retention. Based on the data obtained in the performance experiment (dose-response curve) (data not shown), we determined to use the intermediate dose of U0126 (1 μg/side) for the acquisition experiment. Figure 3 shows the effect on behavior of U0126 microinjections within the NAcc during the acquisition phase. Overall, two-factor RM ANOVA revealed significant increases in the mean reference and working errors during the acquisition phase in animals microin fused with U0126. Analysis by the trial factor showed significant differences throughout trials for the total time, reference, and working errors: mean total time [trial factor: F(19,120) = 1.756; p < 0.05], mean reference errors [treatment factor: F(1,120) = 41.10; p < 0.001 and trial factor: F(19,120) = 4.838; p < 0.001] (Fig. 3A), and mean working errors [treatment factor: F(1,120) = 30.10; p < 0.001] and trial factor: F(19,120) = 1.739; p < 0.05] (Fig. 3B). Post hoc analyses for the reference and working errors data showed that there are significant differences between the vehicle and the U0126-treated rats at specific trials [reference errors: trials 1, 2, 3, and 15 (p < 0.05); working errors: trials 2, 14, 15, 16, and 17 (p < 0.05)]. Representative searching strategies of animals microinjected within the NAcc core with the vehicle or U0126 (1 μg/side) during the first acquisition session are presented in Fig. 4. Data showed a more complex searching strategy across the five trials in animals microinjected with U0126 compared with the vehicle group.

The effect on retention of U0126 microinjections (1 μg/side) administered during acquisition was also analyzed using the two-factor RM ANOVA (Fig. 5). Statistical significant differ-
ences between treatments (vehicle versus U0126) were found in the mean total time [treatment factor: $F(1,30) = 4.573; p < 0.05$] (Fig. 5A) and mean working errors [treatment factor: $F(1,30) = 9.087; p < 0.01$] (Fig. 5B). Significant differences were detected between the vehicle and the U0126-treated groups in the following trials: total time: trial 24 ($p < 0.05$); working errors: trials 23 and 24 ($p < 0.05$). No significant differences were detected for the mean reference errors [treatment factor: $F(1,30) = 1.364; p > 0.05$]. In addition, statistical analysis showed no significant differences throughout trials in the mean total time [trial factor: $F(4,30) = 0.8952; p > 0.05$], mean reference errors [trial factor: $F(4,30) = 1.697; p > 0.05$], and mean working errors [trial factor: $F(4,30) = 0.7102; p > 0.05$].

Statistical analysis of locomotor activity found significant differences across the trials but not between treatments in the acquisition phase [trial factor: $F(19,120) = 2.625; p < 0.001$ and treatment factor: $F(1,120) = 0.7178; p > 0.05$] (data not shown). In addition, differences in locomotor activity during the retention test (session 5) were not observed [treatment factor: $F(1,30) = 0.2040; p > 0.05$ and trial factor: $F(4,30) = 0.4730; p > 0.05$] (data not shown).

**Molecular Analyses.** Two new groups of subjects were spatially trained in the FSSLT. One of them was sacrificed 5 min after session 1 showed a significant increase in MAPK p42 (***, $p < 0.001$) and MAPK p44 (***, $p < 0.001$) phosphorylation compared with naive animals. No differences in MAPK p42/p44 phosphorylation between naive and habituated (untrained-48 h) animals were detected ($p > 0.05$). Also, MAPK p42/p44 phosphorylation levels were not different in naive and spatially trained animals sacrificed 1 h after training ($p > 0.05$). Representative Western blots of phosphorylation (B) and total expression (C) levels of MAPK p42/p44 in naive, untrained animals sacrificed after 48 h (untrained-48 h), and spatially trained animals sacrificed 5 min (trained-5 min) or 1 h (trained-1 h) after spatial training.
min after the first acquisition session and the other was sacrificed after the third trial of the fourth acquisition session. Statistical analysis revealed that animals microinfused with U0126 and sacrificed after the first acquisition session showed a significant reduction in MAPK p42 and p44 phosphorylation compared with the control group.

Fig. 2. Reconstructions of cannulae placements and photomicrographs of representative animals microinjected within the nucleus accumbens. Bilateral symbols show the approximate cannulae placement within the nucleus accumbens core in rats microinjected with vehicle (A) (circles) (n = 7), U0126 (B) (squares) (n = 7), and GF109203X (C) (triangles) (n = 7). Drawings were made from a hand-drawn atlas with nose bar 5 mm above the interaural zero. Values indicate the distance from bregma. CPu, caudate-putamen; NAC, accumbens core; NAS, accumbens shell; LS, lateral septum; ac, anterior commissure. Photomicrographs show three half-brain sections with the microinjection site of animals microinjected with vehicle (D), U0126 (E), and GF109203X (F).

Fig. 3. Effects of MAPKK inhibitor (U0126) microinjections into the nucleus accumbens during acquisition phase in the food search spatial learning task. Graphs represent the effect of U0126 microinjections (1 μg/side) on the number of reference errors (A) and the number of working errors (B). Means (±S.E.M.) of the errors were calculated as the average of the number of errors per trial for all the animals in the group. Two-factor RM ANOVA of the complete acquisition phase found significant differences between vehicle- (white circles) (n = 7) and U0126-microinjected (black circles) (n = 7) animals. Significant differences were detected in the number of reference (treatment factor: ***p < 0.001; trial factor: +++p < 0.001) and working (treatment factor: ***, p < 0.001; trial factor: ++, p < 0.01) errors. Post hoc analyses showed that significant differences between groups were in the following trials: reference errors: 1, 2, 3, and 15 (*, p < 0.05) and working errors: 2, 14, 15, 16, and 17 (**, p < 0.05). Sessions 1 to 4 were abbreviated as S1, S2, S3, and S4, respectively.
t(7) = 4.567; p < 0.01 and MAPK p44: t(7) = 4.479; p < 0.01] (Fig. 6A). No significant differences were observed between treatments in the total levels of MAPK p42 [t(7) = 0.752; p > 0.05] and MAPK p44 [t(7) = 1.052; p > 0.05]. Representative Western blots for the phosphorylated and total levels of MAPKs are presented in Fig. 6, B and C, respectively.

Animals microinfused with U0126 and sacrificed after the third trial of the fourth acquisition session showed a significant decrease in MAPK p42 and p44 phosphorylation compared with animals microinfused with the vehicle [MAPK p42: t(3) = 4.194; p < 0.05 and MAPK p44: t(7) = 4.076; p < 0.01] (Fig. 7A). Statistical analysis did not detect significant differences between treatments in the total levels of MAPK p42 [t(7) = 0.305; p > 0.05] and p44 [t(7) = 2.108; p > 0.05]. Representative Western blots for the phosphorylated and total levels of MAPKs are shown in Fig. 8, B and C, respectively.

**Experiment 3: PKC Inhibitor GF109203X Microinjections within the NAcc during Performance, Acquisition, and Retention Phases of the FSSLT**

**Histological Analyses.** A schematic drawing of brain sections and the reconstruction of representative cannulae placements from animals microinfused within the NAcc with the vehicle or the PKC inhibitor GF109203X are presented in Fig. 2, A and C, respectively. Verification of cannulae place-
ments were performed through the examination of brain slices under a light microscope. Animals with incorrect cannula placements were eliminated (n = 1). Figure 2 presents photomicrographs from two representative animals microinfused with the vehicle (Fig. 2D) or GF109203X (Fig. 2F) in the acquisition phase of the FSSLT. No significant tissue damage at the microinjection site was found.

**Acquisition and Retention.** The dose-response curve obtained in the performance experiment showed significant behavioral differences between animals microinfused with the highest concentration of the PKC inhibitor GF109203X and the vehicle (data not shown). Therefore, the 0.5 ng/side GF109203X dose was used to determine the effect of PKC inhibition on the acquisition and retention of the FSSLT. Figure 8 shows the behavioral data for the acquisition phase of the FSSLT. Overall, two-factor RM ANOVA detected significant differences between treatments and throughout trials. Specifically, significant increases in the mean reference errors [treatment factor: F(1,120) = 38.98; p < 0.001] (Fig. 9A) and mean working errors [treatment factor: F(1,120) = 10.12; p < 0.01] (Fig. 9B) were found in animals microinfused with GF109203X compared with control animals. Specifically, these differences were found in the following trials: reference errors: trials 13 and 19 (p < 0.01) and 20 (p < 0.05) and working errors: trials 11 and 19 (p < 0.05). No significant differences between treatments were observed for the total time spent searching for food [treatment factor: F(1,120) = 0.4459; p > 0.05]. In addition, significant differences throughout trials were found in the mean total time [trial factor: F(19,120) = 3.073; p < 0.001] and mean reference errors [trial factor: F(19,120) = 3.635; p < 0.001]. The test did not find differences in the mean working errors [trial factor: F(19,120) = 1.558; p > 0.05]. Figure 10 shows a representation of the food searching strategies of animals microinjected within the NAcc core with the vehicle or GF109203X (0.5 ng/side) during the first acquisition session. Animals injected with the PKC inhibitor showed more complex searching trajectories in comparison with animals injected with the vehicle.

The effect on the retention test of GF109203X microinjections (0.5 ng/side) during acquisition is shown in Fig. 10. One-factor RM ANOVA revealed significant differences in the mean reference errors [treatment factor: F(1,30) = 38.94; p < 0.001] (Fig. 10A) and the mean working errors [treatment factor: F(1,30) = 23.42; p < 0.001] (Fig. 10B). Post hoc analyses showed that there were significant differences at certain trials [reference errors: trials 22 (p < 0.01), 23, and 24 (p < 0.05); working errors: trial 22 (p < 0.01)]. However, no significant differences were detected in mean total time [treatment factor: F(1,30) = 1.984; p < 0.05].

Analysis of locomotor activity during the acquisition phase show significant differences across the trials [treatment factor: F(1,120) = 0.8256; p > 0.05] and trial factor: F(19,120) = 3.187; p < 0.001] (data not shown). Similarly, no significant differences in locomotor activity were observed during the retention test [treatment factor: F(1,30) = 2.474; p > 0.05] and trial factor: F(4,30) = 1.410; p > 0.05] (data not shown).

**Biochemical and Molecular Analyses.** Figure 11A presents the effect of PKC inhibitor microinjection on PKC activity 5 min after the first session of acquisition. Student’s t test showed significant differences between treatments in PKC activity in the cytosolic [t(9) = 3.271; p < 0.01] and membrane-associated [t(10) = 3.449; p < 0.01] fractions. PKC activity was reduced in the cytosolic and membrane-associated fractions from animals treated with GF109203X compared with animals treated with vehicle.

Statistical analysis of the MAPKs immunoblotting data revealed significant differences in MAPKs phosphorylation. Specifically, Student’s t test showed a significant reduction in MAPK p42 [t(9) = 3.398; p < 0.01] and p44 [t(10) = 7.716; p < 0.05] phosphorylation in animals microinfused with the PKC inhibitor versus animals microinfused with the vehicle (Fig. 11B). Representative Western blots for the phosphorylated and total levels of MAPKs are shown in Fig. 11, C and D, respectively.

**Discussion**

The present study demonstrates that NAcc-PKC cascade is involved in the mediation of spatial learning and memory. Furthermore, our results present for the first time evidence for the following: 1) MAPK p42/p44 activation within the
NAcc is needed for spatial learning and 2) an interaction between intra-accumbal PKC and MAPK signal transduction cascades in spatial learning.

**Role of MAPKs Phosphorylation within the NAcc in Spatial Learning and Memory in the FSSLT.** Experiment 1 showed that animals learning the task present a significant increase in the levels of phosphorylated MAPK p42/p44 5 min but not 1 h after acquisition session 1 compared with naive animals. No significant differences in MAPKs phosphorylation were found between naive and untrained (habituated) animals, suggesting that habituation is not accompanied by MAPKs activation. However, we cannot exclude the possibility that habituation per se induces changes in MAPKs phosphorylation because untrained animals were sacrificed 48 h after habituation. In our study, it is demonstrated that at the time of the first acquisition session (48 h after habituation), MAPKs phosphorylation is not different from the levels observed in naive animals. Therefore, the induction in MAPKs phosphorylation observed in trained animals is associated with spatial training and not with a sustained MAPKs phosphorylation induced by habituation.

To our knowledge, this is the first time that changes in MAPKs phosphorylation are evaluated within the NAcc of spatially trained rats. However, our results are in accordance with several experimental data showing MAPK phosphorylation.
lation in association with synaptic plasticity events. Among these are the activation of MAPKs in physiological mechanisms underlying learning and memory (English and Sweatt, 1996) and in behavioral tasks of classical conditioning (Crow et al., 1998) or learning and memory (Atkins et al., 1998).

Experiment 2 focused on the examination of the effect of an MAPKK inhibitor, U0126 (1 μg/side), on the acquisition and retention phases of spatial behavior. Two-factor RM ANOVA detected significant learning impairments in animals microinjected with U0126 compared with the vehicle in the acquisition phase. Analysis of the searching strategies supports the differences observed between groups because more complicated patterns of food searching were observed in U0126-treated animals versus vehicle-treated animals. In contrast, moderate but significant disruptions were observed in spatial memory. These results suggest that MAPKs phosphorylation within the NAcc is notably required for acquisition of spatial information but less crucial for the formation of spatial memory.

These results are in accordance with published data demonstrating that MAPKs phosphorylation is required for several types of learning and in different brain areas. It has been found that MAPK p42/p44 phosphorylation is needed for contextual fear conditioning in the hippocampus (Atkins et al., 1998), auditory fear conditioning in the amygdala (Schafe et al., 2000), and avoidance learning (Cammarota et al., 2000). These behavioral data are supported by in vitro studies in which has been demonstrated that MAPK cascade is required for the induction of LTP in the CA1 of the hippocampus (English and Sweatt, 1997) and LTP maintenance in the amygdala (Huang et al., 2000). Furthermore, it has been demonstrated that MAPKs activation is required for spatial learning, although opposed results have been reported with regards to the learning phase affected by MAPKK inhibitor treatments (Blum et al., 1999; Selcher et al., 1999). Particularly of interest is that there is in vitro evidence showing that MAPKs are activated in striatal neurons and that this activation occurs through the stimulation of Ca²⁺-permeable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (Perkinton et al., 1999). These results and ours might implicate the MAPK cascade as a potential mechanism associated with synaptic plasticity within the striatum.

Based on the behavioral results observed for the acquisition phase, we determined to sacrifice animals 5 min after first acquisition session and after the third trial of the fourth session to determine whether deficits observed in spatial learning were due to a decrease in MAPKs phosphorylation. Western blotting analysis showed significant reductions in the phosphorylation of MAPK p42 and p44 isoforms at both times. These results confirmed that deficits in spatial learning observed during some stages of acquisition were accompanied by a reduction in MAPK p42 and p44 phosphorylation.

Role of PKC Activity within the NAcc in Spatial Learning and Memory in the FSSLT. Published data have reported an increase in PKC activity in rats spatially trained in the FSSLT. The increase in PKC activity was observed 5 min after the first acquisition session (Alvarez-Jaimes et al., 2004). In experiment 3, we evaluated the effect of microinjections of a selective PKC inhibitor, GF109203X (0.5 ng/side), within the NAcc on the acquisition and retention phases in the FSSLT.

Two-factor RM ANOVA revealed that animals microinjected with GF109203X showed significant impairments in learning and more complicate searching strategies compared with vehicle-treated animals. Statistical analysis of the retention test demonstrated significant deficits in spatial memory in animals microinjected with GF109203X during acquisition. Overall, these results suggest that NAcc-PKC activity is a considerable requirement for learning and the efficient
formation of spatial memories. These results are supported by previously published data reporting impairments in spatial learning and memory after microinjections of an antisense oligodeoxynucleotide sequence for the PKC-γ isoform within the NAcc (Alvarez-Jaimes et al., 2004). In addition, PKC has been implicated in the initiation and maintenance of LTP (Asztely et al., 1990; Finch and Jackson, 1990; Wang and Feng, 1992), which also supports the hypothesis that PKC may be underlying synaptic plasticity within the NAcc.

PKC as an Upstream Regulator of MAPKs within the NAcc during an Early Stage of Spatial Learning in the FSSLT. Biochemical analysis of the effect of PKC inhibitor microinjection (GF109203X; 0.5 ng/side) within the NAcc on PKC activity was performed in animals sacrificed 5 min after the first acquisition session. Statistical analysis revealed a significant decrease in PKC activity in the cytosolic and membrane-associated fractions in animals treated with GF109203X compared with those treated with the vehicle. These results confirmed the inhibitory effect of GF109203X on PKC activity and demonstrated that deficits in learning observed in animals microinjected with GF109203X correlated with reductions in PKC activity. Therefore, these findings suggest that PKC activity plays a role in spatial learning mediated by the NAcc during an early stage.

Taking in consideration that our results suggest a role for PKC activity and MAPKs phosphorylation in spatial learning and memory mediated by the NAcc, a potential coupling between them was evaluated. To establish a relationship between PKC and MAPKs activation, animals microinjected with GF109203X were also evaluated for their levels of MAPK p42/p44 phosphorylation. Western blotting results demonstrated that animals treated with GF109203X also presented a significant reduction in MAPK p42 and p44 phosphorylation. Based on the fact that GF109203X is a highly selective PKC inhibitor and that inhibition of PKC activity caused a reduction in MAPK phosphorylation, the data suggest that PKC is working as an upstream regulator of MAPK p42/p44 phosphorylation within the NAcc during an early stage of the acquisition phase in the FSSLT. Previous published data support our results. For example, it has been reported that homologs of vertebrate PKC (PKC1) and MAPK (MPK1) in the yeast Saccharomyces cerevisiae are coupled (Lee et al., 1993). In Hermisenda, it has been found that incubation with a phorbol ester, an activator of PKC, produces increases in MAPK phosphorylation, and it is blocked by treatment with a PKC inhibitor, suggesting that PKC contributes to the phosphorylation of MAPK (Crow et al., 2001). An in vitro study found that stimulation of PKC activation using a phorbol ester triggers MAPK activation in the CA1 area of the hippocampus (English and Sweatt, 1996) and that MAPKK inhibitors blocked the phorbol ester stimulation of MAPK in the same hippocampal area, suggesting that MAPK phosphorylation is regulated by the PKC cascade (Roberson et al., 1999). Recently, Ahi et al. (2004) found that microinjections of a PKC inhibitor within the dorsal hippocampus of mice during context-dependent fear conditioning prevents the phosphorylation of MAPKp42/p44, suggesting that MAPKs is a target of PKC during memory consolidation.

In summary, the present results support the involvement of the NAcc in certain aspects of spatial learning and memory. Our data provide evidence that PKC and MAPKs cascades, which have been widely implicated in the function of the synaptic plasticity cellular mechanism known as LTP and associative learning, also play important roles within the NAcc in spatial learning. In particular, NAcc-MAPK p42/p44 and -PKC activation seems to be differentially required for the acquisition and formation of spatial memories. Furthermore, our molecular studies revealed that PKC activation is working as an upstream regulator of MAPKs phosphorylation within the NAcc during early spatial learning. These
data provide evidence to propose a model in which PKC and MAPKs coupling may be one of several signal transduction interactions within the NAcc that could be underlying spatial learning mediated by this striatal structure.

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