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NMDA antagonist AP-5 increase environmentally induced cocaine-conditioned locomotion within the nucleus accumbens

Enrique Rodríguez-Borrero, Alexandra Bernardo Colón, Melissa A. Burgos-Mártir, José E. Álvarez Carillo, Yaniré Estrada del Campo, Cynthia Abella-Ramírez, Carmen S. Maldonado-Vlaar *

University of Puerto Rico Río Piedras Campus Biology Department P.O. Box 23360 San Juan, 00931-3360, Puerto Rico

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Abstract

An environment previously associated with cocaine use can elicit cravings, even in the absence of the drug, which may be due to the formation of strong associations between the environment and the drug. These associations can result from motor learning and reinforcing effects of cocaine, and may be mediated in part by ionotropic glutamate receptors in the nucleus accumbens (N.Acc.). To determine whether NMDA receptor activity in the N.Acc. affects the expression of conditioned locomotion, rats were trained using an environment-elicited cocaine-conditioning paradigm. Rats trained to pair a cocaine injection with an environment showed an increased locomotor activity when tested in the drug-paired environment, whereas rats injected with cocaine in their home cages did not exhibit greater locomotion. Significantly greater locomotor activity occurred in trained animals that received an infusion of AP-5, a NMDA receptor antagonist, into the N.Acc. These results suggest that animals trained to associate environmental cues with cocaine become conditioned to this environment. Furthermore, our finding demonstrates that NMDA receptor activation within the N.Acc. modulates cocaine-induced conditioning.
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Keywords: Drug addiction; Conditioned stimulus; NMDA receptors; Nucleus accumbens; Reward; Locomotion

Pairing psychostimulants with specific environmental stimuli is a form of classical conditioning long recognized as an important aspect of addiction (Davis and Smith, 1976). Because cocaine triggers reward centers in the brain, its use often leads to addiction. Studies using cocaine conditioning have shown that cocaine-associated stimuli can mimic the drug's locomotor-activating effects induced by repeated pairing of cocaine injections with a specific environment and reinforce instrumental acts directed toward procuring the drug (Koob et al., 1998; Everitt and Wolf, 2002, for review). Brain-imaging studies in humans suggest a role for dopamine-rich regions, including the N.Acc. and the amygdala in cue-induced cocaine craving, (Grant et al., 1996; Breiter et al., 1997).

The N.Acc. forms an integral part of the striatum and has been proposed as a key substrate for psychostimulants. It is commonly divided into two components, the shell and the core, which can be distinguished both anatomically and functionally. The core has strong connections to basal ganglia and motor output structures, whereas the shell is intimately connected to limbic circuits (Zahm and Brog, 1992, Zahm and Heimer, 1993; Brog et al., 1993; Zahm, 1999). The N.Acc. receives glutamatergic neural afferents that originate mostly within the amygdaloid complex, the hippocampal formation and the frontal cortex (Groenewegen et al., 1999). These projections have been implicated in the regulation of locomotor activity and evidence suggests that they may produce their effects by interacting with dopaminergic neurotransmission (Pulvirenti et al., 1994; Kim and Vezina, 1997; Swanson and Kalivas, 2000). Previous studies have demonstrated that blockade of NMDA glutamate receptors with AP-5 induces pronounced locomotor stimulation when injected into the N.Acc. of monoamine depleted mice. These findings suggest that glutamatergic neurons projecting to the accumbens can affect motoric functions depending on the

* Corresponding author. Tel.: +1787 764 0000x2712; fax: +1787 764 3875.
E-mail address: cmalvlar@caribe.net (C.S. Maldonado-Vlaar).

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dopaminergic tone and that these two neurotransmitter system are closely related within the N.Acc. region (Svensson and Carlsson, 1992; Svensson et al., 1994).

Glutamate has been associated not only with natural rewards but also with the rewarding effects of addictive drugs such as cocaine. Electrophysiological, neurochemical and behavioral evidence suggest that glutamate neurotransmission in the N. Acc. may be necessary for the complete development of the rewarding and activating properties of psychostimulant drugs (for review, Koob et al., 1998; Wolf, 1998; Hyman and Malenka, 2001). Several studies have reported that acute administration of high doses of cocaine elevates extracellular glutamate levels in the N.Acc. (Kalivas and Duffy, 1995), and this effect is augmented in rats pretreated with daily cocaine injections (Pierce et al., 1996; Kalivas and Duffy, 1998). In contrast, there is a decrease in extracellular glutamate levels in the N.Acc. after chronic exposure to cocaine (Bell et al., 2000; Hotsenpiller et al., 2001). Furthermore, blockade of ionotropic glutamate receptors into the N.Acc. showed that NMDA and AMPA/KA receptors are involved in cocaine-seeking behavior controlled in part by drug-associated cues (Di Ciano and Everitt, 2001). These results suggest that glutamate within the N.Acc. plays an important role of cocaine addictive effects.

Based on the previous evidence suggesting that the neural pathways through N.Acc. may be responsible for producing the reinforcing effects of cocaine and that NMDA receptors within this brain region play a role in responses to rewarding stimuli such as cocaine, the present study is aimed at testing the role of NMDA receptors within N.Acc. on environment-elicited cocaine-conditioned behavior. It is hypothesized that blockade of NMDA receptors within the N.Acc. will alter locomotor activity triggered by repeated pairing of cocaine injections and multimodal environmental stimuli.

1. Materials and methods

1.1. Subjects

Male Sprague Dawley rats (Charles River, MA, USA) weighing 275–300 g were used in all experiments. Rats were housed in pairs using plastic cages in a controlled humidity and temperature (22 °C) vivarium of the Department of Biology, University of Puerto Rico. Food and water was available at all times. Animals were kept on a 12-h on–off light/dark cycle (lights on 7:00–19:00). Rats were handled immediately upon arrival in order to minimize stress during behavioral testing. All procedures were conducted in strict adherence to the National Institutes of Health Guide for the care and use of laboratory animals.

1.2. Chronic cannulae implantation surgery

At the beginning of surgery, animals were anesthetized with sodium pentobarbital (i.p. 50 mg/kg, Sigma Chemical, MO, USA) and given atropine (0.54 mg/kg, s.c. Phoenix Pharmaceutical, MO, USA). Standard stereotaxic procedures, in conjunction with the atlas of Paxinos and Watson (1998), were used to implant bilateral indwelling 10 mm guide cannulae (23 gauge, Small Parts, FL, USA) aimed at the N.Acc. shell and N.Acc. core. The coordinates in mm for N.Acc. shell were A–P +3.5, M–L ±1.0, D–V −5.3, and for N.Acc. core were A–P +3.5, M–L ±2.0, D–V −5.3. The cannulae were secured with stainless steel screws (Plastic One, VA, USA) and dental cement and light-curable resin (Sepulveda Multidental, San Juan, Puerto Rico). In order to ensure clean and functional cannulae, wire stylets were inserted into the guides. Antibiotic ointment was placed on the incision and then sutured. Animals were allowed to recover for 7 days before the start of behavioral testing.

1.3. Microinfusions

For microinjections, wire stylets were removed and bilateral intracerebral microinjections of the drugs or vehicle were given using 12.5 mm injector cannulae (30 gauge, Small Parts, FL, USA). The injectors were then lowered until they reached the desired injection site. Usually, this site is between 1.0 and 3.0 mm below the tip of the guide cannula. For the N.Acc. shell and N.Acc. core this site is −7.8 mm from the skull. Animals were hand-held during infusion. Before any experimental microinfusions were performed, all animals were given a preliminary saline infusion in order to adapt them to the injection procedure (on day 11). Using a microdrive pump (Harvard Apparatus, CA, USA), drug microinfusions of AP-5 1 μg/0.5 μl (Tocris Cookson, MO, USA) diluted in 0.9% sodium chloride solution (Abbott Laboratories, IL, USA) or vehicle solution (0.9% saline solution) 0.5 μl/site were administered to the site via polyethylene tubing (PE-10, Harvard Apparatus, CA, USA). The selection of the present dose of AP-5 was based on our previous data that has shown that it can significantly disrupt learning mediated by the N.Acc. Furthermore several published results have also demonstrated that treatment with this single dose within the N.Acc. has the capability to significantly impair instrumental learning (Hernandez et al., 2005, for review Kelley et al., 2003). The total infusion time for 0.5 μl volumes was 1 min 33 s followed by a 1 min diffusion period. After the drug infusion, the injectors were removed and the stylets were replaced.

1.4. Conditioning methods

1.4.1. Activity chamber apparatus

We adapted our conditioning protocol from a similar protocol developed by Brown et al. (1992). Specifically, our protocol involves the introduction of olfactory and visuals cues specific for each study group (i.e. control, paired, and unpaired group). In particular, the olfactory cues were included in the protocol to enhance the saliency of the environment paired with the drug, as previous studies have shown that the use of these cues can elicit a similar conditioned response as the compound stimulus (Ferger and Kuschinsky, 1995). The experiments were conducted in Sixteen TruScan Photobeam Scanning System activity boxes designed by Coulbourn Instruments (PA, USA). The dimensions of the cages are 41 cm × 41 cm × 41 cm inside while the base plate

is 53 cm × 53 cm. The activity boxes have 16 photobeams that are 2.54 cm apart. These beams track the horizontal and vertical position of the animal thus allowing the experimenter to analyze many aspects of an animals' general motor activity. The dependent measure for these studies was ambulatory distance traveled by the animals during the testing period. All chambers were located in a test room with distinctive environmental cues. Cocaine-paired chambers were surrounded by a 61 cm × 61 cm × 61 cm black plexi glass box (visual cues) and receive odor infusions of cold-pressed California orange oil (inner cues) (Sigma-Aldrich, MO, USA). These visual cues and the inner chambers' cues became the environmental stimuli for the cocaine-paired animals. Control and cocaine-unpaired subject boxes were surrounded by a 61 cm × 61 cm × 61 cm black and white checkers square (visual cues) and received odor infusions of Nutmeg East Indian Oil (inner cues) (Sigma-Aldrich, MO, USA). These external visual cues and the inner chambers cues became the environmental stimuli for the saline-paired and cocaine-unpaired animals. The animals' home cages were housed in a different room in the animal facility building distant from the testing room.

1.4.2. Training sessions

Animals underwent one daily conditioning training session of 90 min, for 10 consecutive days. Rats were trained during the light phase of their cycle at approximately the same time each day (09:00 a.m.). Rats were assigned to one of three groups: cocaine-paired, cocaine unpaired or control and were placed in a specific activity chamber throughout all experimental sessions. The paired groups received 10-mg/kg i.p. injection of cocaine–hydrochloride (Sigma-Aldrich, MO, USA) diluted in 0.9% sodium chloride solution prior to being placed in the chamber and a saline injection just before returning to their home cage. The control groups were exposed to saline i.p. injections prior to both training and home environments. In addition the cocaine unpaired groups were injected with saline before being placed in the activity chambers and following the end of the sessions were returned to their home cage and given a 10-mg/kg i.p. injection of cocaine.

1.4.3. Testing session

Two days following the last training session (Day 10), trained animals were once again returned to the activity chambers and tested for 120 min. No cocaine or saline i.p. injections were given on this test day. About 10 min prior to testing, animals of each subgroup received either AP-5 or saline i.c. microinfusions and then they were placed in the activity chambers. The same procedures were followed for NAS groups.

1.4.4. Validation of cocaine conditioning protocol

In order to validate our conditioning protocol, 12 animals ($N=4$ in each group) were conditioned as stated above, except that these animals were not submitted to surgery or i.c. injection.

1.5. Measurements of cocaine levels in conditioned animals

1.5.1. ELISA procedure

ELISA assay of cocaine/benzoylecgonine concentration during training and testing session in paired and unpaired

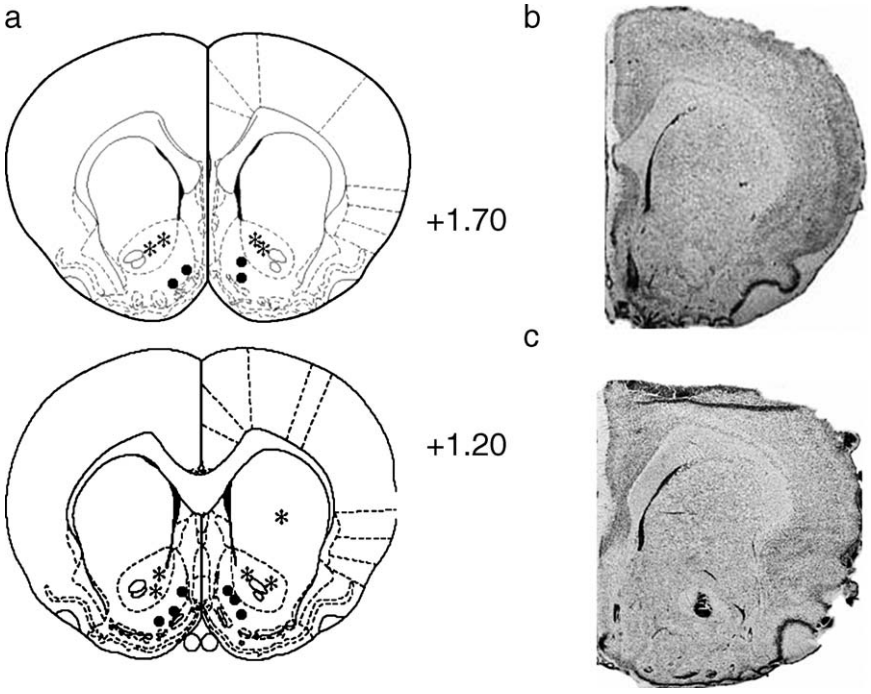

Fig. 1. (a) Drawing of serial coronal sections from representative animals used in the experiments. Bilateral symbols show approximate cannulae placement for the N.Acc. shell (●) and N.Acc. core (∗). Drawings were made from a hand-drawn atlas from Paxinos and Watson (1998). Value indicates the distance in mm from bregma. (b and c) Representative photomicrograph of the injection site for the N.Acc. shell and N.Acc. core, respectively.

animals were performed to show that the behavioral effects measured in the conditioning session were not due to cocaine levels present in the subjects during testing. The antibody used recognizes cocaine and its metabolite, benzoylecgonine. Therefore both were measured. For this experiment, all animals received a sham surgery where the animals were subject to surgical procedure but not cannulated prior to testing. All animals were injected with i.p. cocaine (paired animals, 10 mg/kg) or saline injection (unpaired animals, 1 ml/kg) and placed on the activity chambers. After 60 min, tail blood collection (0.2 cm^3) was taken on days 1, 5 and 10 of the training session, and during the test session (Day 12). The ELISA protocol was performed as described in the manufacturers' guide (NeoGen Corp. KY, USA) with minor modifications. Briefly, the sample was diluted 1:50 in EIA buffer, the reaction was halted by the addition of 1 N HCl and the O.D. was measured in a microplate reader with a 450 nm filter. The extent of color development was inversely proportional to the amount of drug in the sample or control. The concentration was determined by the inclusion of a standard curve in every plate used.

1.6. Statistical analysis

All data are expressed as mean±S.E.M. Repeated measures ANOVA was utilized for the behavioral validation data followed by Newman–Keuls post-hoc analysis. As multiple groups were used for the AP-5 effect, statistical comparisons were made by a three-way analysis of variance (ANOVA) followed by post hoc analysis using Tukey's multiple comparison test ($p<0.05$). For this analysis the data was transformed using the square root. For the biochemical analysis an unpaired *t*-test was performed.

1.7. Histological analysis

After the end of each experiment, animals were deeply anesthetized with chloroform (Fisher Scientific, Cayey, Puerto Rico) and brains were collected. Brains were frozen and cut into 60 μm sections using a Leica Cryocut 1900 cryostat. Each section was mounted to a gelatin-coated slide, defatted, stained with cresyl violet stain (Sigma-Aldrich, St. Louis) and cannula placement verified. Those animals with cannulas outside the N. Acc. subregions were not included in the study.

2. Results

2.1. Histological analysis

The brain sites of the micro injector tips of representative animals utilized in the study are shown in Fig. 1a. Fig. 1b and c shows a representative photomicrograph of the injections site for the N.Acc. shell and the N.Acc. core, respectively. Most placements were found between +1.70 and 1.20 from bregma. Placements for the N.Acc. shell and N.Acc. core were considered accurate if they were located within the structure of interest in both hemispheres. Animals were also excluded if histological analyses revealed evidence of severe infection at the injection site or due to poor cannula placement.

2.2. Behavioral validation of the conditioning protocol

Fig. 2 shows the effect of environmentally-induced cocaine conditioning on locomotor activity. First, cocaine-paired subjects as depicted in Fig. 2a traveled a greater ambulatory distance than control and cocaine unpaired subjects during the training

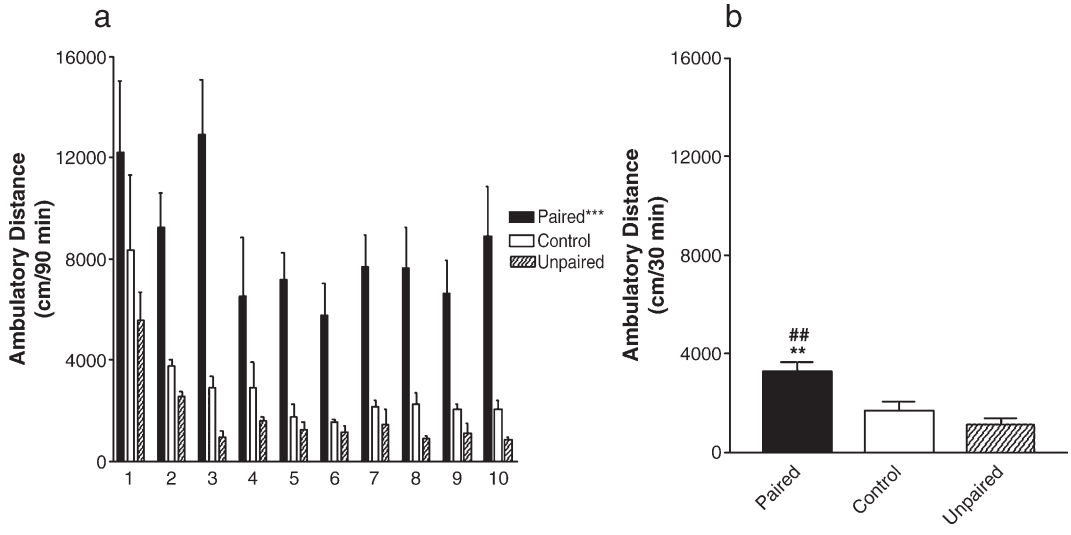

Fig. 2. The present results demonstrate the training and testing sessions of the cocaine-induced environmental conditioning protocol. (a) Ambulatory distance traveled during the training session. Paired subjects (black bars) traveled a greater ambulatory distance (***$p<0.001$; comparison between paired group and control group) than control group (open bars). This effect was also found between the paired group and the unpaired group (black hatched bars; ***$p<0.001$; comparison between paired group and unpaired group). (b) Ambulatory distance traveled during the first 30 min of the drug expression session. Paired group (black bars) traveled a greater ambulatory distance than control group (open bars, **$p<0.01$, comparison between paired group and control group) and unpaired group (black hatched bars, $^{\#\#}p<0.01$, comparison between paired group and unpaired group). The results shown are the mean±S.E.M. of the behavioral parameter studied. All groups of animals were $n=7$–10.

sessions [$F(2,9)=93.70$; $p<0.001$] (days 1 to 10). Second, in the testing session (day 12, Fig. 2b), paired subjects traveled significantly greater ambulatory distance than control and unpaired subjects in the first 30 min [$F(2,9)=13.04$; $p<0.01$]. All animals tended to habituate to the surroundings starting at 40 min after being placed in the chambers. No statistical difference was found between control and unpaired subjects in the locomotor activity pattern studied (Newman–Keuls post-hoc analysis; treatment factor $p>0.05$).

2.3. AP-5 effects within N.Acc. in cocaine conditioning

Three-way ANOVA analysis of effects of AP-5 into both N.Acc. core and N.Acc. shell subregions on cocaine conditioning revealed no significant interaction between brain area (core or shell), conditioning (control, paired and unpaired) and treatment (saline or AP-5) [$F(2,76)=0.16$; $p=.851$]. Therefore, it can be concluded that there are no differences between N.Acc. core and N.Acc. shell data following AP-5 treatment. Moreover, no interaction was found between brain area and conditioning neither brain area nor treatment ($p>0.05$). However an interaction between conditioning and treatment was found [$F(2,76)=3.25$; $p<0.05$]. Fig. 3 shows the effect of AP-5 within the N.Acc. in cocaine conditioned locomotion. Specifically, post hoc analysis (Tukey's test) demonstrate that cocaine-paired subject injected with AP-5 significantly traveled greater ambulatory distance than cocaine-paired subjects that received saline microinfusions ($p<0.01$). In addition, cocaine-paired injected with AP-5 showed greater locomotor activity than the control and unpaired group both saline and AP-5 injected ($p<0.001$). Furthermore, cocaine-paired subject injected with saline traveled greater ambulatory distance than the control and unpaired group both saline and AP-5 injected ($p<0.001$). No significant difference was found between control and unpaired subjects. ($p=0.997$)

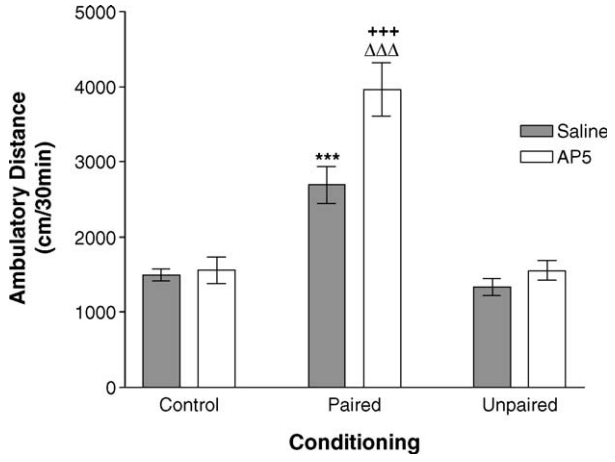

Fig. 3. Effects of AP-5 microinfusion within the N.Acc. subregions during the first 30 min of the testing session. Figure shows that cocaine-paired AP-5-injected subjects traveled a greater ambulatory distance than control–saline, control–AP-5, unpaired saline, unpaired–AP-5 ($^{+++}p<0.001$, comparison between cocaine-paired AP-5 and each control and unpaired groups) and cocaine-paired saline subjects ($^{\Delta\Delta\Delta}p<0.001$, comparison between cocaine-paired AP-5 vs. cocaine-paired saline group). Also, cocaine-paired saline-injected subjects traveled greater ambulatory distance than control–saline, control–AP-5, unpaired saline and unpaired AP-5 subjects (***$p<0.001$, comparison between cocaine-paired saline and each control and unpaired group). The results shown are the mean±S.E.M. of the behavioral parameter studied. All groups of animals were $n=7$–10.

Table 1
Cocaine/benzoylecgonine concentration in blood during training and testing session

Treatment	Training	Testing
Paired	2.36±0.21*	0.140±0.03
Unpaired	0.058±0.036	0.210±0.04

The values are expressed as mean±S.E.M. $N=4$ for each group.
An ELISA assay revealed that cocaine/benzoylecgonine levels in paired subjects were higher during the training session than in unpaired subjects (***$p<0.001$). Unpaired subjects show minimum levels of cocaine/benzoylecgonine during the training phase. During the testing session no statistical difference was shown between paired and unpaired subjects.
* $p<0.001$, unpaired t-test.

2.4. Biochemical analysis of cocaine blood levels

Using the enzyme-linked immunosorbent assay (ELISA) techniques, cocaine concentration was measured from the blood of cocaine and unpaired group during days 1, 5, 10, and the test day. Table 1 shows an average of these data during the training session days. Statistical analysis (unpaired t-test) revealed that animals in the cocaine-paired group, their cocaine blood levels were significantly higher than the unpaired group ($p<0.001$). In contrast, on the test session day, analysis of the data demonstrated no significant differences observed between cocaine-paired and unpaired subjects ($p>0.05$) (Table 1, test session panel).

3. Discussion

We investigated the effects of intra-accumbens shell and core infusions of the NMDA receptor antagonist AP-5 on the expression of a locomotor conditioned response induced by successive pairings of cocaine with specific environmental visual and olfactory cues. Infusion of AP-5 in both N.Acc. subregions potentiated conditioned locomotor activity induced by the environmental context previously paired with cocaine. This overall increase of motor activity was not found in control or unpaired subjects that received AP-5 microinjection. Furthermore, AP-5 main effect resulted in significant interaction between treatment (AP-5 or saline) and conditioning (control, paired or unpaired). These results suggest that there is not a behavioral dissociation of NMDA receptors within N.Acc. subregions and that the enhancement in locomotor activity is caused by the interaction of cocaine treatment and NMDA blockade.

The cocaine conditioning effects presented in Fig. 2 show that only the animals from the paired group that received systemic cocaine injections prior to being placed in the activity cages showed increased locomotion when placed in the cocaine-associated environment. Neither the control nor the unpaired groups showed an increase in locomotor activity. These results suggest that the animals in the paired group established an

association between the environment and the cocaine injections such that the presentation of the environmental cues alone induces behavioral response not observed in the control neither unpaired groups. This notion is supported by previous studies that have shown that the psychostimulant reinforcing properties of conditioned stimuli elicit neural events that are similar to those produced by the drug itself (Martin-Iverson and McManus, 1990; Brown and Fibiger, 1992; Brown et al., 1992). We propose that the conditioned response observed in the cocaine-paired group after exposure to the cocaine-associated environment may be due to triggered memories related to the previous drug experience. Since only the paired group had a greater locomotor activity when tested, it appears that only this group formed an association between cocaine and the environmental cues during the conditioning phase.

Previous studies have shown that cocaine-paired environment not only enhances locomotor activity but also affects gene expression and several neurotransmitters system. Several studies have shown that a cocaine-paired environment can enhance Fos-related antigen expression (Franklin and Druhan, 2000), increase dopamine levels (Duvauchelle et al., 2000) and regulate glutamate levels within the N.Acc. (Bell et al., 2000). Specifically, Bell and colleagues (2000) showed that a cocaine challenge in the training environment increases N.Acc. glutamate levels in rats for which the environment has previously been paired with the drug. Moreover, during a drug-free microdialysis test session, discrete cocaine-associated stimuli increased N.Acc. glutamate levels (Hotsenpiller et al., 2001), demonstrating the importance of this neurotransmitter in the association between environment and drug experience. A striking finding in the study of Hotsenpiller and colleagues (Hotsenpiller et al., 2001) was that basal extracellular glutamate levels in the N.Acc., measured before the introduction of cocaine cue, were markedly reduced in the paired group; showing that not only cocaine, but also a cocaine-paired environment can have a marked effect on glutamate neurotransmission.

Our data also suggests that NMDA receptors within this N.Acc. subregions are involved in eliciting a multimodal environmental conditioned locomotor response associated with cocaine treatment possibly due to modulation of synaptic changes by cocaine conditioning. This response may be selective for NMDA–cocaine interactions, since blockade of NMDA receptors in both N.Acc. shell and N.Acc. core did not significantly affect behavioral response in control–saline groups during the test session. More specifically, our data shows that the NMDA receptors within N.Acc. are sensitive to specific cocaine reward associations allowing the formation of certain memories that can trigger a conditioned locomotor response. We suggest that the increases in locomotor activity caused by AP-5 in the cocaine-paired subjects are a combination of several factors. The blockade of NMDA receptors combined with the fact that a cocaine-paired environment enhances glutamate release can facilitate the activation of other glutamate receptors such as AMPA receptors involved in cocaine behavioral effects. It has been demonstrated that AMPA receptor activation produced more selective responding toward a drug paired lever than NMDA activation (Cornish et al., 1999). Moreover, microinjections of an AMPA receptor antagonist block the reinstatement of cocaine-seeking behavior and an intra-accumbal microinjection of AP-5 alone dose-dependently reinstated cocaine seeking (Park et al., 2002). Recently, it has been demonstrated that AP-5 microinfusions within the N.Acc. shell reinstated cocaine-seeking behavior (Famous and Pierce, 2005). Our data supports the hypothesis that glutamate innervations in the N.Acc. are needed for the expression of associative learning related to the cocaine-conditioning effects. Therefore, based on the present data and the published evidence thus far, it is possible to suggest that a combination of the synaptic changes caused by the drug use and the disruption of glutamate action within the N.Acc. after several experiences with the drug can account for the conditioned stimuli that results in the enhanced response reported. Thus, the present results support the notion of a role of NMDA receptors within the N. Acc. in mediating environmentally induced cocaine locomotor activation.

However, more experiments are needed to further characterize the role of other glutamatergic receptors within N.Acc. in the enhancement of environmentally induced cocaine locomotor activation in order to contribute to a more clear understanding of the neuronal substrates of cocaine conditioning.

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