

A Glutamate–Dopamine Interaction in the Persistent Enhanced Response to Amphetamine in Nucleus Accumbens Core but not Shell Following a Single Restraint Stress

Alejandra M Pacchioni^{1,3}, Martine Cador², Claudia Bregonzio¹ and Liliana M Cancela^{*1}

¹Departamento de Farmacología, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, Argentina; ²Laboratoire de Neuropsychobiologie des Désadaptations, CNRS-UMR5541, Université Victor Segalen, Bordeaux II, Bordeaux Cedex, France

The administration of psychostimulant drugs or stress can elicit a sensitized response to the stimulating and reinforcing properties of the drug. We previously demonstrated that a single restraint stress session enhanced d-amphetamine (d-AMPH)-induced locomotion the day after the stress session, which lasted up to 8 days. The present experiments were designed to identify the contribution of major dopamine (DA) brain areas in the short- and long-lasting enhancement of d-AMPH-induced locomotion following a single stress, and to test the involvement of N-methyl-D-aspartate (NMDA) receptors in that phenomena. To achieve our goal, 24 h and 8 days after a 2-h restraint stress session either with or without a NMDA receptor blockade, we measured locomotor activity and DA overflow in nucleus accumbens (NAcc) core and shell and caudate putamen (CPu) following a d-AMPH injection (0.5 mg/kg i.p.). The stimulant effect of d-AMPH on DA overflow was enhanced in all nuclei at 24 h after a single stress, while at 8 days the enhanced responsiveness was maintained only in the NAcc core. When the rats were administered with MK-801 (0.1 mg/kg i.p.) 30 min before restraint stress, the d-AMPH-induced enhancement on locomotor activity and DA neurotransmission was prevented in all studied brain areas at both times. These findings show that a glutamate–dopamine link is underlying the short- and long-term d-AMPH-induced enhancement on DA and locomotor activity following stress. The persistent glutamate-dependent DA enhancement in NAcc core highlights the relevance of this region in the long-term proactive effects of stress on vulnerability to drug abuse.

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INTRODUCTION

Clinical and epidemiological studies point out that stress is positively related to the use and abuse of addictive drugs (O'Doherty, 1991). In laboratory animals, there is evidence for the proactive influence of stress on the behavioral and neurochemical responses to many of abused drugs. It has been shown that exposure to different stressors induces a potentiation of the psychostimulant effects of both amphetamine (AMPH) and cocaine (see Lu *et al*, 2003). As well, their rewarding properties using the place preference (Capriles and Cancela, 1999) or the drug self-administration

paradigms (Piazza and LeMoal, 1998; Shaham *et al*, 2003) have been shown to be enhanced in previously stressed rats.

A preponderant role has been attributed to the dopaminergic mesocorticolimbic system in the rewarding and stimulating effects of drugs of abuse as well as in the response to stress. Both drugs of abuse and stress experiences increase dopamine (DA) release in mesocorticolimbic structures such as the nucleus accumbens (NAcc) core and shell and striatum (Imperato *et al*, 1993; Kalivas and Duffy, 1995; Pontieri *et al*, 1995). Besides an implication in the acute effect of stress and drugs, accumbens and striatal dopaminergic neurotransmission are thought to be involved in the development and expression of long-term locomotor sensitization as well as in relapse to drug seeking (Kalivas and Stewart, 1991; Robinson and Berridge, 2000; Vezina *et al*, 2002). Although behavioral effects and changes in DA are affected in parallel by stress and psychostimulants, a strict relationship between them is not clearly evidenced (see Di Chiara, 1995; Robinson *et al*, 1988; Paulson and Robinson, 1995; Pierce and Kalivas, 1997). In addition to DA, a common mechanism between stress- and drug-induced long-term neuroadaptation might be the

*Correspondence: Dr LM Cancela, Departamento de Farmacología, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria, (5000) Córdoba, Córdoba, Argentina, Tel: +54 351 4334437, Fax: +54 351 4334420, E-mail: lcancela@fcq.unc.edu.ar

³Current address: Department of Neurosciences, Medical University of South Carolina, Charleston, SC, USA

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excitatory amino-acid neurotransmission, which has been shown to be involved in drug-induced neuroadaptations (Wolf, 1998; Cador *et al*, 1999). Consistently, we have shown that MK-801 blocked the restraint stress-induced sensitization of the locomotor psychostimulant effect of d-AMPH (Pacchioni *et al*, 2002) and morphine (Capriles *et al*, 2002). Furthermore, as an enhancement of the d-AMPH-induced striatal DA release seemed to underlie the locomotor sensitization following a single restraint stress (Pacchioni *et al*, 2002), it is highly probable that the influence of MK-801 could also be observed in the releasing effect of the drugs at DA terminals.

As locomotor sensitization to d-AMPH was observed at 24 h and 8 days following a single restraint stress (Diaz-Otañez *et al*, 1997), in the present study we looked into short and long-lasting presynaptic changes in the dopaminergic effects of d-AMPH in the dorsal striatum (CPu) and the two subdivisions 'core' (NAcc core) and 'shell' (NAcc shell) of the NAcc. The NAcc is a heterogeneous structure that can be separated anatomically into core and shell subdivisions (Pennartz *et al*, 1994), where DA transmission is differentially sensitive to drugs of abuse and undergoes different habituation patterns to repeated treatment with drugs or stress (see Di Chiara *et al*, 2004). The effects of the drug on DA release were analyzed by microdialysis from these nuclei at 24 h and 8 days after a single restraint stress. The implication of a glutamatergic mechanism was further studied by MK-801 or saline being peripherally administered before the restraint stress.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (250–330 g) purchased from Facultad de Ciencias Veterinarias (Universidad Nacional de La Plata, Buenos Aires, Argentina) were used. The rats were maintained at 20–24°C under a 12 h light–dark cycle (lights on at 0700 hours) with free access to food and water. Rats were placed in the colony room for at least 7 days before experimental tests and housed two individuals per cage during the experiment.

Surgery

Rats were anesthetized with chloral hydrate (400 mg/kg i.p.) and placed in a Stoelting stereotaxic instrument with the incisor bar at –3.3 mm above the interaural line. The skull was exposed and a hole was drilled to allow implantation of a CMA/12 microdialysis guide cannulae in NAcc core, NAcc shell and CPu. The coordinates were (in mm): for NAcc core AP +1.4, ML ±1.5 and DV –7.8; for NAcc shell AP +1.4, ML ±0.7 and DV –8.0; and, for CPu AP +1.2, ML ±2.5 and DV –6.0. The cannulae were secured in place with two stainless steel screws and dental cement. The rats were allowed to recover for 48–72 h.

Drugs

d-AMPH sulphate was purchased from Sigma Chemical Co. (St Louis, MO); and (+)-MK-801 hydrogen maleate from Research Biochemical International (Natick, MA). Drugs

were dissolved in saline immediately before use. The doses were calculated on the basis of the weight of the salt of each drug, and were chosen considering our previous works (Diaz-Otañez *et al*, 1997; Pacchioni *et al*, 2002).

Stress

Rats were immobilized for 2 h in a Plexiglas restraining device. The Plexiglas cylinders were devised so that the rats' head and tail emerged from the front and the rear, respectively. The stress phase was conducted between 1000 hours and 1400 hours. All procedures were handled in accordance with the NIH Guide for the Care and Use of Laboratory Animals as approved by Animal Care and Use Committee of the Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Argentina.

Locomotor Activity

The testing apparatus consisted of rectangular cages (30.5 × 19.5 × 46.5 cm) equipped with two parallel infrared photocell beams located 3 cm above the floor. Interruption of either beam resulted in a photocell count. The testing apparatus was placed in a different room from the one where restraint was applied to avoid any conditioning effects. All rats were tested once between 0900 hours and 1800 hours, under white light in a quiet room. They were placed individually in the testing apparatus for a 1-h habituation period, before being injected with d-AMPH (0.5 mg/kg i.p.), and motor activity counts were monitored at 10 min intervals for 2 h following the injection.

Microdialysis Procedure

In the morning of the experimental day, a CMA/12 (2 mm for NAcc and 4 mm for CPu) dialysis probe was inserted through the guide cannulae and the rats were transferred to the dialysis chamber (40 × 34 cm). The probes were connected to the Bioanalytical Systems syringe pump via peek tubing connected to two-channel swivel. The perfusion fluid was composed of 145 mM NaCl, 2.2 mM CaCl₂, and 4 mM KCl. Samples were collected and immediately injected onto an HPLC column. Twenty-four hours or 8 days after restraint, a dialysis probe was inserted through the guide cannula and the perfusion fluid started immediately (1 µl/min). After 1 h, baseline samples were collected. Once the basal dopamine levels were stable, the rats received an injection of d-AMPH (0.5 mg/kg i.p.) or saline and samples were collected for 180 min.

Measurement of DA Levels in Microdialysis Samples

The perfusate was assayed for DA by reverse-phase HPLC coupled with electrochemical detection. The mobile phase was composed of 50 mM NaH₂PO₄/5 mM Na₂HPO₄, 0.1 mM EDTA-Na₂, 0.5 mM *n*-octyl sodium sulphate, and 12% methanol; pH was adjusted to 5.5. The mobile phase was delivered at a flow of 1 ml/min (Model 582, solvent delivery model; ESA, Chelmsford, MA) through a RP 18 column (C18, 125 × 4.6 mm, 5 µm). Samples were injected via a 20 µl injection loop. DA was detected using coulometric detection (ESA Coulochem II). Three electrodes were used: a guard

cell (+350 mV), an oxidation analytical electrode (+175 mV), and a reduction analytical electrode (-175 mV). Peaks were recorded, and the height was measured by a computer using an ESA Chromatography Data System. These values were quantified by comparison with an external standard curve.

Data Analyses

All values from microdialysis experiments are expressed as percentage of baseline \pm SEM. The basal DA values are included in the text and expressed as fmol/20 μ l \pm SEM. The average concentration of the four samples before drug injection was defined as the baseline. Data were included for analysis if the four baseline samples varied less than 15% from each other and if, within group, they did not exceed the mean value plus or minus twice the SD. All data was statistically evaluated using a two-way ANOVA for repeated measures analysis, with the following factors: treatment (0 or 1 restraint stress session), drug (MK-801 or saline) and

time as the repeated measure (Figures 1, 3–5); or treatment (0 or 1 restraint stress session), AMPH dose (d-AMPH or saline) and time as the repeated measure (Figure 2). All ANOVA were followed by a Newman–Keul's test for *post-hoc* comparisons. The level of significance for all procedures was $p < 0.05$.

Histology

At the end of the experiments, in order to assess probe placements, rats were deeply anesthetized with chloral hydrate (400 mg/kg i.p.) and perfused transcardially with a solution of formaldehyde (10% v/v). The brains were stored in formaldehyde (10% v/v) until the time of sectioning. Following fixation, coronal sections of 60 μ m thicknesses were cut on a cryostat, and each section was stained with Thionin. The cannula placements were determined according to the atlas of Paxinos and Watson (1997) with a light microscope.

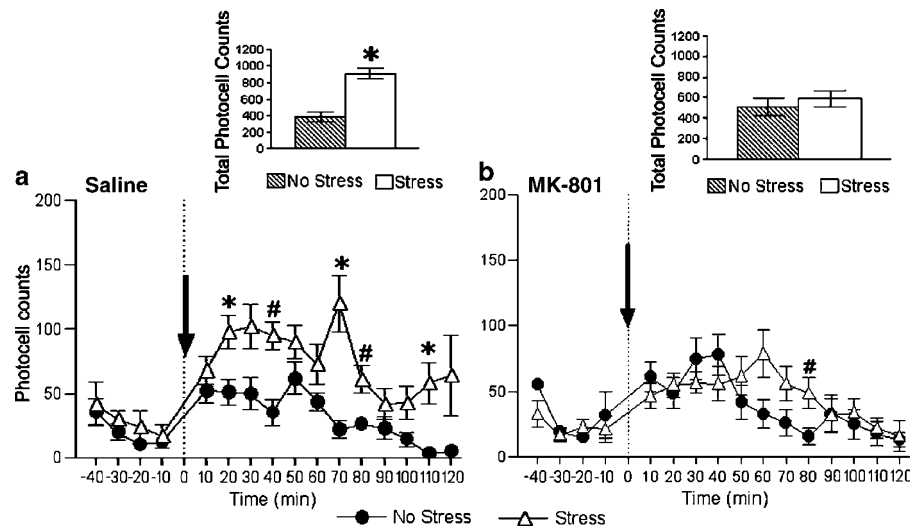


Figure 1 Reversal by MK-801 of the restraint stress-induced sensitization to locomotor activating effects of d-AMPH. Rats were injected with (a) saline or (b) MK-801 (0.1 mg/kg i.p.) 30 min before 2 h of restraint stress. Rats from the no-stress group after receiving drug or saline, were returned to their home cages. Twenty-four hours after, the stress-induced sensitization at locomotor response to d-AMPH was blocked in MK-801 stress group ($n = 8-11$ for each group). Values represent the means (\pm SEM) of photocell counts over 10-min periods. * indicates $p < 0.005$ compared with all the remaining groups (Saline No Stress, MK-801 No Stress and MK-801 Stress). A # sign indicates $p < 0.05$ compared with Saline No Stress group. Comparisons between groups were at the same time point by the Newman–Keuls test. Arrow indicates 0.5 mg/kg d-AMPH i.p. injection. At the top of both figures (a and b) the total photocell counts over 120 min were shown as means (\pm SEM), * indicates $p < 0.005$ compared with all the remaining groups.

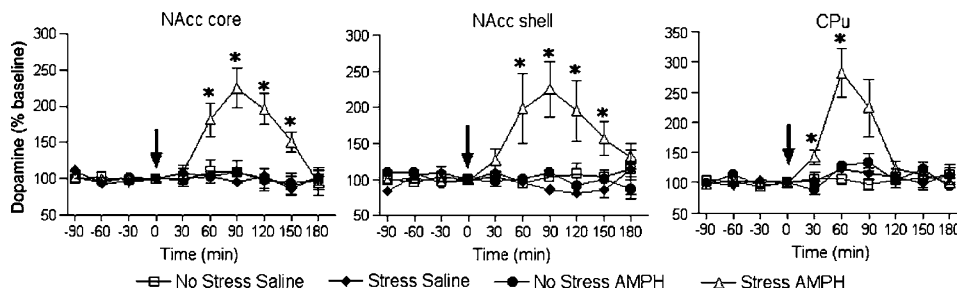


Figure 2 Twenty-four hours after a restraint stress DA release from NAcc core, NAcc shell, and CPu is enhanced following an AMPH injection. Rats were restrained for two hours or stayed in their home cages. Twenty-four hours later, DA release from NAcc core, NAcc shell, and CPu was measured following a saline or d-AMPH (0.5 mg/kg i.p.) injection ($n = 5-8$ for each group). Values represent percentage of increase of DA release from baseline (means \pm SEM). * indicates $p < 0.05$ compared with No Stress (Saline and d-AMPH), and Stress Saline. Arrow indicates a saline or d-AMPH (0.5 mg/kg i.p.) injection. Comparisons between groups were at the same time point by the Newman–Keuls test.

Experimental Design

Rats were randomly assigned to one of four conditions defined by treatment (0 or 1 restraint stress session) and drug: saline (1 ml/kg i.p.) and MK-801 (0.1 mg/kg i.p.) or by treatment (0 or 1 restraint stress session) and AMPH dose: saline (1 ml/kg i.p.) and d-AMPH (0.5 mg/kg i.p.). Thirty minutes after MK-801 or saline administration, the 'stressed' rats were submitted to a restraint stress session, whereas the 'control' rats were returned to their home cages

and left undisturbed. Twenty-four hours (Figures 1 and 3) or 8 days after (Figures 4 and 5), the animals of each group were subdivided for microdialysis and locomotor activity assays to test the effects of a challenge dose of d-AMPH (0.5 mg/kg i.p.). Different no stress and stress groups were used to test the effect of a challenge injection of saline or d-AMPH on DA release 24 h after the restraint stress (Figure 2). Considering that a persistent stress-induced sensitization on DA release after d-AMPH was found only in NAcc core, the influence of MK-801 pretreatment 8 days

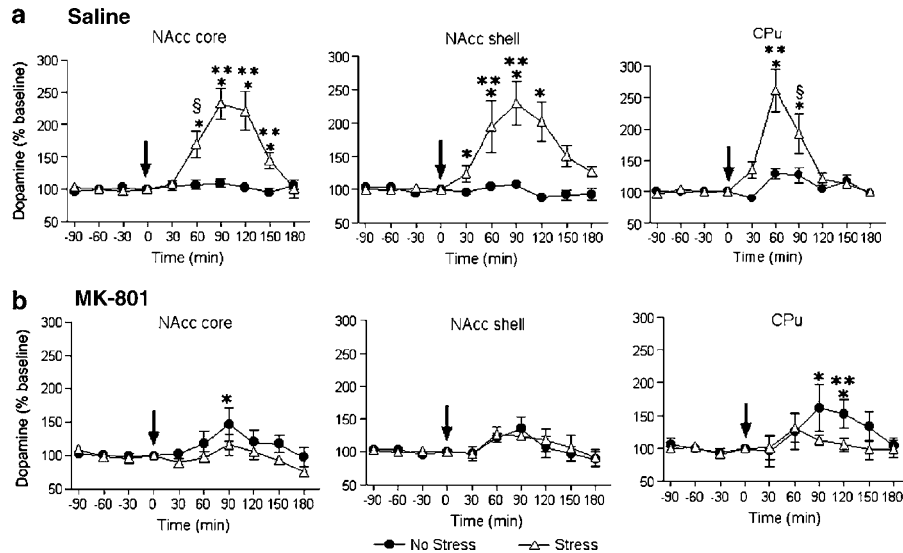


Figure 3 Reversal by MK-801 of the restraint stress-induced sensitization to d-AMPH-induced DA release from NAcc core, NAcc shell, and CPU. Rats were under the same experimental design described in Figure 1. Twenty-four hours later, the stress-induced sensitization on d-AMPH-evoked DA release from NAcc core, NAcc shell, and CPU were blocked in MK-801 Stress group ($n=5-7$ for each group). * indicates $p<0.05$ compared with baseline DA levels. ** indicates $p<0.005$ compared with the values of all the remaining groups (Saline No Stress, MK-801 No Stress and MK-801 Stress). A § sign indicates $p<0.05$ compared with Saline No Stress and MK-801 Stress groups. Comparisons between groups were at the same time points by the Newman-Keuls test. Arrow indicates 0.5 mg/kg d-AMPH i.p. injection.

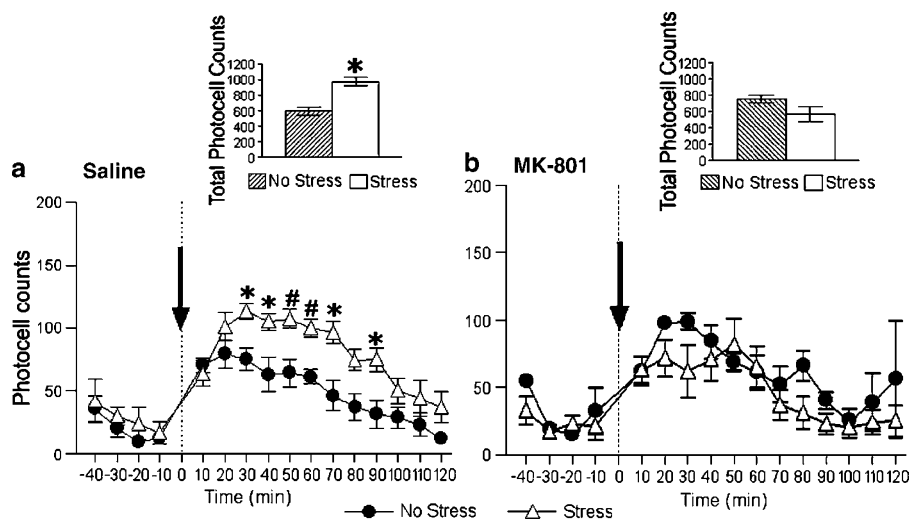


Figure 4 Reversal by MK-801 of the restraint stress-induced sensitization to locomotor activating effects of d-AMPH administered 8 days after stress. Rats were under the same experimental design described in Figure 1. Eight days after, the stress-induced sensitization at locomotor response to d-AMPH was blocked in MK-801 Stress group ($n=6-10$ for each group). Values represent the means (\pm SEM) of photocell counts over 10-min periods. * indicates $p<0.005$ compared with all the remaining groups. A # sign indicates $p<0.05$ compared with Saline No Stress group. Comparisons between groups were at the same time point by the Newman-Keuls test. Arrow indicates 0.5 mg/kg d-AMPH i.p. injection. At the top of both figures the total photocell counts over 120 min were shown as means (\pm SEM), * indicates $p<0.005$ compared with all the groups.

after stress session on this effect was only studied in NAcc core. Each animal was used only once.

RESULTS

A total of 191 rats were used for the microdialysis experiments. The data from 156 rats passed all inclusion criteria, including measurable dopamine levels in both NAcc and CPu, stable baseline DA levels and confirmed probes placements (Figure 6). There was no significant difference in basal dopamine levels among different experimental groups in the NAcc core, NAcc shell, or CPu. Average basal DA levels (not corrected by recovery) were 116.4 ± 13.3 fmol/20 μ l in NAcc core, 115.3 ± 18.3 fmol/20 μ l in NAcc shell, and 115.7 ± 20.3 fmol/20 μ l in CPu.

A Single Restraint Stress Induced an Enhanced Response to d-AMPH on Locomotor Activity and DA Release from NAcc Core, Shell and CPu, Which was Prevented by a MK-801 Pretreatment

We have previously demonstrated that the stimulation of NMDA receptors is necessary for the stress-induced enhancement to the stimulating effect of d-AMPH and

morphine on locomotor activity, 24 h after a restraint stress session (Pacchioni *et al*, 2002; Capriles *et al*, 2002). To draw a parallel between the previous findings and the microdialysis experiments, we ran the locomotor activity assay in a different group of rats, and used a new set of activity monitors. The results (Figure 1a and b) again showed a restraint stress-induced enhanced behavioral response, which was prevented by the MK-801 pretreatment (treatment $F(1,34) = 16.39$, $p < 0.0005$; drug $F(1,34) = 4.77$, $p < 0.05$; and time $F(11,374) = 8.89$, $p < 0.0001$; treatment \times drug $F(1,34) = 6.62$, $p < 0.05$; and treatment \times time $F(11,374) = 2.29$, $p < 0.05$). Newman-Keuls *post-hoc* comparisons indicated that the Saline Stress group shows significantly higher photocell counts at 20, 70, and 110 min compared with MK-801 Stress and all No Stress groups. These comparisons also showed that counts at 40 and 80 min in the Saline Stress group were significantly higher than the Saline No Stress group. The total photocell counts over 120 min after d-AMPH injection (right top corner Figure 1a and b) also showed the higher response of the Saline Stress group with regard to that of the MK-801 Stress and all no-stress groups (treatment $F(1,34) = 15.6$, $p < 0.0005$; and drug $F(1,34) = 4.95$, $p < 0.05$; treatment \times drug $F(1,34) = 6.79$, $p < 0.05$). Newman-Keuls *post-hoc* comparisons indicated that the total cumulative counts in

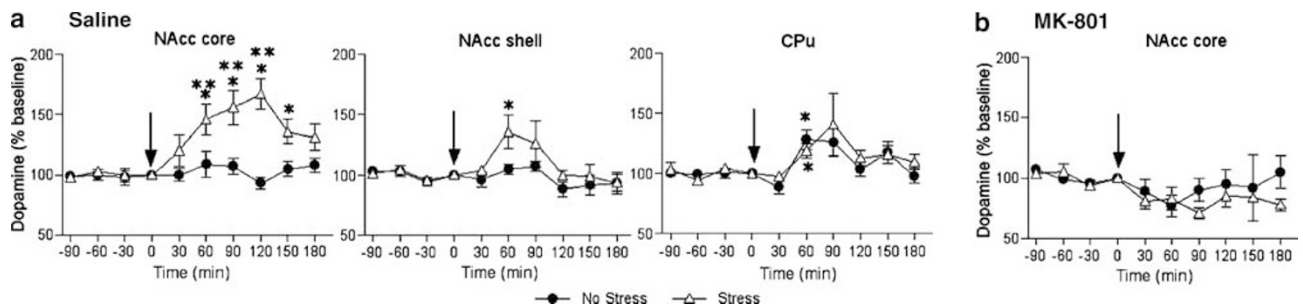


Figure 5 Reversal by MK-801 of the restraint stress-induced sensitization to d-AMPH-induced DA release from NAcc core, 8 days after stress. Rats were under the same experimental design described in Figure 1. Eight days later, the stress-induced sensitization on d-AMPH-evoked DA release from NAcc core was blocked in MK-801 Stress group ($n = 5-7$ for each group). * indicates $p < 0.05$ compared with baseline DA levels. ** indicates $p < 0.005$ compared with the values of all the remaining groups. Comparisons between groups were at the same time points by the Newman-Keuls test. Arrow indicates 0.5 mg/kg d-AMPH i.p. injection.

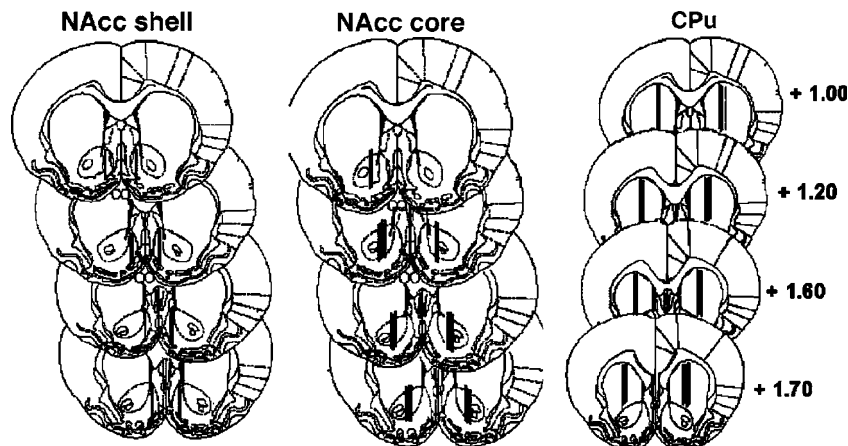


Figure 6 Schematic sections of the rat brain, adapted from the stereotaxic atlas of Paxinos and Watson (1997), showing the location of microdialysis probes in NAcc shell, NAcc core, and CPu. For details and exact coordinates, see Materials and methods.

the Saline Stress group was significantly increased compared to those of the MK-801 Stress and all No Stress groups. It is important to mention that the irregular enhanced response observed in Saline Stress rats (significantly higher than the Saline No Stress group at 20, 40, 70, 80, and 110) does not mimic the sensitized response seen in d-AMPH-exposed animals by other authors. This pattern seems to be a characteristic response to AMPH 24 h after the stress session, as was also shown in our previous studies (Diaz-Otañez et al, 1997; Pacchioni et al, 2002).

Restraint stress induced a higher DA release evoked by d-AMPH in NAcc core and shell subdivisions and CPU compared with those observed after Saline injection (Figure 2). A two-way ANOVA applied on results in Figure 2 showed the following significant effects: for NAcc core data treatment \times AMPH dose \times time interaction $F(6,126) = 8.08$, $p < 0.0001$; for NAcc shell data treatment \times d-AMPH dose \times time interaction $F(6,90) = 4.51$, $p < 0.01$, and for CPU data treatment \times d-AMPH dose \times time interaction $F(6,120) = 3.34$, $p < 0.05$. Newman-Keuls *post-hoc* comparisons for NAcc core and NAcc shell showed a significant increase in the percentage of basal DA output at 60, 90, 120, and 150 min in stressed rats following an d-AMPH injection comparing to baseline levels and to values in No Stress groups (Saline and d-AMPH), and Saline Stress group. The same comparisons for CPU data showed a significant increase of DA release 30 and 60 min after d-AMPH. A Saline injection did not modify DA release either in No Stress or in Stress rats in any of the studied brain areas. It should be mentioned that using a low dose of d-AMPH (0.5 mg/kg i.p.), we confirmed our previous findings on stress-induced effects obtained with a higher dose of d-AMPH. As the low dose of d-AMPH (0.5 mg/kg i.p.) was not effective in evoking DA release in no stressed rats, we injected a few rats from the No Stress group with d-AMPH (1 mg/kg i.p) at the end of saline challenge experiment and we measured the DA release in NAcc core ($n = 4$) and NAcc shell ($n = 3$) only for 60 min. A clear DA increase following d-AMPH was observed in both nuclei from the no-stress group (data not shown), which once again corroborates the well-known DA-releasing effect of this drug in naive rats.

In agreement with the behavioral results, we showed in a separate set of rats that MK-801 pretreatment prevented the stress-induced enhancement in the DA release for all nuclei studied (Figure 3a and b). As saline injection has no effect in No Stress or Stress groups (Figure 2), only the challenge with d-AMPH was tested in these studies on the influence of MK-801 on stress-induced effects. With regard to the NAcc core data (Figure 3a and b), a two-way ANOVA for repeated measures revealed a significant drug \times treatment \times time interaction $F(6,90) = 4.59$, $p < 0.0005$. Newman-Keuls *post-hoc* comparisons showed that only in the Saline Stress group there was a significant increase in the percentage of basal DA output observed following 60, 90, 120, and 150 min of the d-AMPH challenge with regard to baseline levels. These comparisons also showed that this increase was significantly different from the values of all the remaining groups (Saline No Stress, MK-801 No Stress, and MK-801 Stress) at 90, 120, and 150 min. For NAcc shell data, a two-way ANOVA for repeated measures performed on the data in Figure 3a and b, revealed a significant

drug \times treatment \times time interaction $F(6,102) = 2.47$, $p < 0.05$. Newman-Keuls *post-hoc* comparisons indicated a significant increase in the percentage of basal DA at the 60, 90, 120, and 150 min time points post d-AMPH challenge in the saline-stressed group with regard to baseline levels, and at 90 and 120 min it was also different from the values of all the remaining groups.

For CPU data, a two-way ANOVA for repeated measures conducted on data depicted in Figure 3a and b revealed a significant drug \times treatment interactions $F(1,21) = 14.65$, $p < 0.001$; drug \times time interaction $F(6,126) = 2.5$, $p < 0.05$; and treatment \times time interaction $F(6,126) = 2.48$, $p < 0.05$. Newman-Keuls *post-hoc* comparisons showed a significant increase in the percentage of basal DA output following 60 and 90 min of d-AMPH challenge in the Saline Stress group with regard to baseline levels. At 60 min, a significant increase in the percentage of basal DA output was observed in the Saline Stress group compared with values of all the remaining groups.

The Saline No Stress group (Figure 3a) did not show an increase in DA output in response to d-AMPH in none of the nuclei studied, and the MK-801 No Stress group (Figure 3b) showed an increase in DA output from NAcc core and CPU compared to basal levels in response to AMPH. Both of these observations need to be addressed.

The Increase in Locomotor Activity and DA Response to d-AMPH in NAcc Core Persisted 8 days after Stress and was Prevented by a MK-801 Pretreatment

We have previously demonstrated that a single restraint session induced an enhancement of d-AMPH stimulating effect, 8 days after a restraint stress session (Diaz-Otañez et al, 1997). The present results (Figure 4a and b) confirmed these previous findings and demonstrated that glutamate also participates in the persistence of the stress-induced effects. Indeed, locomotor enhancement observed 8 days following a single restraint stress experience is prevented by MK-801 pretreatment (time $F(11,308) = 15.3$, $p < 0.0001$; treatment \times drug interaction $F(1,28) = 16.21$, $p < 0.0001$). Newman-Keuls *post-hoc* comparisons indicated that photocell counts at 20, 30, 70, and 90 min in Saline Stress group were significantly higher than those obtained in MK-801 stress and all no-stress groups. These comparisons also showed that counts at 50 and 60 min in the Saline Stress group were significantly higher than those from the Saline No Stress group. These figures also depicted the total photocell counts over 120 min in saline and MK-801 Stress and No Stress groups challenged with d-AMPH. A two-way ANOVA applied to the total cumulative counts over 120 min showed a significant treatment \times drug interaction $F(1,28) = 16.21$, $p < 0.0001$. Newman-Keuls *post-hoc* comparisons indicated that the total cumulative counts in the saline stress group was significantly increased with regard to those of the MK-801 stress and all no-stress groups. Although it is clear that MK-801 before restraint stress blocked the enhanced response to d-AMPH, it should be mentioned that MK-801 administration induced a non-significant trend of increasing the locomotor responding to d-AMPH in Saline No Stress animals (see explanation of this effect in Discussion).

Eight days after restraint (Figure 5a and b), only NAcc core shows an enhanced DA release after d-AMPH and this effect was blocked by MK-801 pretreatment (drug $F(1,18) = 29.33$, $p < 0.0001$; drug \times treatment $F(1,18) = 12.82$, $p < 0.005$; and time \times drug $F(6,108) = 2.9$, $p < 0.05$). Newman-Keuls *post-hoc* comparisons showed that only in the saline stressed group was a significant increase in the percentage of basal DA output observed at 60, 90, 120, and 150 min following the d-AMPH challenge with regard to baseline levels. These comparisons also showed that this increase was significantly different from the values of all the remaining groups (Saline No stress, MK-801 No Stress, and MK-801 Stress) at 60, 90, and 120 min.

Figure 5a also showed the data from NAcc shell and CPu. One-way ANOVA for repeated measures applied to the data on each area, revealed a main effect of time $F(6,54) = 4.39$, $p < 0.005$ (NAcc shell); and $F(6,90) = 6.903$, $p < 0.0005$ (CPu). Newman-Keuls *post-hoc* comparisons showed a significant increase in the percentage of basal DA output at 60 min following of the d-AMPH challenge compared to baseline levels in NAcc shell Stress group, and in CPu Stress and No Stress groups. However, no difference was observed among all the different groups. Also, saline no-stress animals failed to show an increased DA response in the different nuclei following d-AMPH.

Assessment of Cannula Placements

Animals in which the traces of the probes were located outside the NAcc core, shell or CPu, and animals showing excessive gliosis were discarded from the experiments. Probe placements are illustrated in Figure 6.

DISCUSSION

The current study provides a neural/neurochemical basis for the restraint stress-induced behavioral enhancement to d-AMPH response. The principal findings are that (1) a single restraint stress exposure is a pertinent stimulus to enhance d-AMPH's stimulating effects on DA transmission in the CPu and the two subdivisions shell and core of the NAcc, (2) only the NAcc core shows an enhanced DA release following the longest interval between restraint stress and d-AMPH challenge (ie 1 week vs 1 day), (3) pretreatment with a non-competitive NMDA antagonist, MK-801, abrogated the restraint stress-induced d-AMPH enhancement on DA neurotransmission and locomotor activity (at both times after stress). Our results strongly suggest that NMDA receptors are involved in the stress-induced changes at the dopaminergic pathways underlying the higher response to d-AMPH. The long-term DA-enhanced response observed in the NAcc core (but not in the NAcc shell or CPu), highlights the potential relevance of the NAcc core in the long-lasting proactive stress effects on vulnerability to drug abuse.

The present results are in agreement with our previous reports showing that a single restraint stress is sufficient to induce behavioral enhancement to the stimulating and rewarding properties of d-AMPH (Diaz-Otañez *et al*, 1997; Capriles and Cancela, 1999). The enhancement in psychostimulants effects induced by non-pharmacological stressors

has been observed by several laboratories at behavioral and neurochemical levels (Camp and Robinson, 1988; Rouge-Pont *et al*, 1995; Deroche *et al*, 1995; Kalivas and Stewart, 1991; see Lu *et al*, 2003). Stressors as well as drugs of abuse acutely activate the mesocorticolimbic dopaminergic system (Doherty and Gratton, 1992; Imperato *et al*, 1992; Di Chiara and Imperato, 1988; Pontieri *et al*, 1995) which is known to be involved in the stimulating and rewarding effects of opioid and psychostimulant drugs (Wise and Bozarth, 1987).

In the context of long-lasting effects of stress and drug exposure, the mesocorticolimbic system has been implicated in the development and expression of sensitization to psychostimulants (Kalivas and Stewart, 1991; Pierce and Kalivas, 1997). In agreement, the present results provide evidence of an enhancement in the d-AMPH-induced DA responsiveness in the NAcc in both core and shell subdivisions, and in the CPu, 24 h following a single restraint stress which parallels the behavioral enhancement in terms of locomotor activity. These observations are reminiscent of those of Vanderschuren *et al* (1999), who found that a single exposure to d-AMPH is sufficient to induce an increase in the electrically evoked release of [H^3]dopamine from Nacc, CPu and cortex slices, in parallel to the development of locomotor sensitization at 3 days and 3 weeks after treatment. Notwithstanding, this latter study did not provide any evidence regarding the contribution of NAcc shell and NAcc core to AMPH sensitization. A significant finding of the present study was that the differential contribution of both subdivisions of NAcc to the hyperreactivity of dopaminergic terminals depends on the interval between the stress experience and the drug challenge. The long-term enhanced DA response to d-AMPH in NAcc core resembles that found in a model of opiate- and psychostimulant-induced sensitization (Robinson *et al*, 1988; Wolf *et al*, 1993; Paulson and Robinson, 1995; Cadoni and Di Chiara, 1999; Cadoni *et al*, 2000) as well as that observed in response to different psychostimulants following a chronic schedule of food restriction (Cadoni *et al*, 2003), while that observed in NAcc shell (ie the short lasting one) is consistent with sensitization to morphine following an inescapable shock administered 24 h previously (Bland *et al*, 2004). As contradictory evidence has been found in NAcc shell in terms of DA release in models of drug-induced sensitization, it is difficult to relate our present findings to them. For instance, while cross sensitization to d-AMPH in NAcc shell is observed following a repeated treatment with cocaine (Pierce and Kalivas, 1995) it was not found following an opiate and d-AMPH repeated treatment (Cadoni and Di Chiara, 1999; Cadoni *et al*, 2000). In general terms, the majority of models of drug-induced sensitization mentioned above are based on laboratory animals that have received repeated injections of psychostimulants. Most of them have reported that sensitized DA release is not seen 3–4 days later but rather emerges 1 week after the last pre-exposure injection. Our current findings indicate that restraint stress-induced short-term DA enhancement appears faster (ie at 24 h) than that observed following drugs, and in a similar way to that shown following an inescapable shock (Bland *et al*, 2004). Although caution should be used when comparing stress- and drug-induced sensitization, we can speak of the long-

term nature of the restraint stress-induced enhancement of DA release in NAcc core as the behavioral enhancement seen 1 week after restraint stress resembles that found 1 week or more following drugs. However, Saline Stress rats also showed a significant increase in the DA output from baseline in NAcc shell (Figure 5a). Although this increase was not significant compared to the other groups, it does suggest a trend in NAcc shell which should be considered in future studies. Restraint stress did not affect the DA response to a saline injection in any studied area, since 'no stressed' and 'stressed' animals showed no changes in DA output from basal levels (Figure 2). Therefore, the DA response observed in 'stressed' animals is necessarily d-AMPH-dependent and not due to injection stress. Furthermore, restraint stress-induced enhanced DA release in CPu was also shown by using a higher dose of d-AMPH (1.5 mg/kg i.p.) (Pacchioni *et al*, 2002).

The enhanced behavioral response to d-AMPH after stress had different temporal patterns depending on the interval between stress exposure and drug. The irregular pattern to d-AMPH observed 24 h after the stress session seems to be a characteristic response at this time point, as was shown in our previous studies (Diaz-Otañez *et al*, 1997; Pacchioni *et al*, 2002), while that observed 1 week after stress had a more regular and sustained pattern. The difference in the behavioral responses to d-AMPH after stress might be related to the clearly different d-AMPH-induced DA response underlying both the short- and the long-term stress-induced effects.

It is well known that an AMPH injection increases the behavioral response and DA release in striatum (Carboni *et al*, 2003; Cadoni *et al*, 2003; Kuczenski *et al*, 1997; Lecca *et al*, 2004). However, in the current study, the temporal response to d-AMPH was not significantly different from Saline in No Stress animals. The neurochemical studies also failed to show a d-AMPH-induced DA release in the No Stress group for all nuclei studied. Two factors could help to explain the lack of behavioral and neurochemical effects after d-AMPH (0.5 mg/kg i.p.) in No Stress group: (1) rat strain: Wistar rats were used in the current study *vs* Sprague Dawley or Wistar Kyoto, (2) housing conditions previous to the microdialysis assay: two animals per cage in the present study *vs* individually housed. In addition, our microdialysis conditions (collection time of 30 *vs* 20 min) might dilute the d-AMPH's effect in terms of percentage of change of DA release from baseline.

We have also shown that a pretreatment with MK-801 blocked the restraint stress-induced locomotor and DA enhancement in the studied brain areas at both time points. In a similar way, Wolf *et al* (1994) found that MK-801 prevented behavioral sensitization to d-AMPH and related changes in the mesoaccumbens DA system. Thus, a link between glutamate and DA can be established in a neural circuit underlying the development of restraint stress-induced enhancement and drug-induced sensitization to psychostimulants. Notwithstanding the clear blocking effect of MK-801 in the development of stress-induced enhanced response, it should be mentioned that MK-801 induced a significant increase in DA output compared to basal levels in response to d-AMPH in the No Stress group (Figure 3b). Our behavioral results also showed a non-significant trend to increase the d-AMPH response 8 days following one

injection of MK-801 in the No Stress group. It has been reported that repeated MK-801 treatment increases locomotor activity and can develop sensitization to its own injection (Carey *et al*, 1995; Segal *et al*, 1995; Sripada *et al*, 1998; Vanderschuren *et al*, 1997; Wolf and Khansa, 1991). However, when MK-801 is coadministered with AMPH, the development of sensitization was blocked suggesting that MK-801 itself produces sensitization through a different mechanism than AMPH (Wolf and Khansa, 1991). Furthermore, repeated MK-801 pretreatment prevented the social defeat-induced behavioral sensitization to AMPH (Yap *et al*, 2005). The relationship between the stimulant effect and DA release in NAcc is not clear, some researchers found an increase in DA release after MK-801 (Mathe *et al*, 1999; Yan *et al*, 1997) but others did not (Druhan *et al*, 1996). Regarding the long-term effect of a single MK-801, O'Neill and Sanger (1999) showed that a single MK-801 injection can modify the behavioral response to MK-801 4, 7, and 14 days after, indicating that a single injection of MK-801 produced long-term modifications. In brief, MK-801 alone might modify the behavior and the DA response inducing long-term effects. However, when it is coadministered with drugs or stress it prevents their long-term effects, suggesting that different mechanisms are involved.

There are recent studies focusing on the idea that common mechanisms between drugs and stress underlie their effects at excitatory mesocorticolimbic synapses. Saal *et al* (2003) have provided evidence that either acute administration of drugs of abuse (AMPH, cocaine, alcohol, nicotine, morphine) or stress (cold water swimming) induces similar effects at excitatory synapses on midbrain DA neurons, as measured by electrophysiological *in vitro* experiments. Specifically, an involvement of NMDA receptors has been shown in the enhancement of strength at excitatory synapses on midbrain DA neurons. Furthermore, Ungless *et al* (2003), have found that a key hormone in the stress response, corticotrophin-releasing factor (CRF), potentiates the NMDA receptor component of glutamatergic synaptic currents onto ventral tegmental area (VTA) DA neurons. Although it is difficult to see the relevance of the *in vitro* data outlined above, it has been recently shown that CRF has an important role in stress-induced cocaine relapse. In studies on footshock-induced cocaine relapse, CRF released in VTA caused a glutamate increase, which drove the cocaine seeking behavior. This CRF release in VTA after footshock is also observed in naive animals, but the glutamate increases only happen in cocaine experienced rats (Wang *et al*, 2005). It has also been shown that both repeated administration of cocaine or morphine, as well as repeated restraint stress, increased levels of the α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor subunit GluR1 in the VTA (as well as the NMDA receptor subunit NR1) (Fitzgerald *et al*, 1996). However, this idea of a common mechanism between stress and drugs is challenged by other pharmacological and neuroanatomical studies suggesting that different neuronal systems are involved in drug priming- and footshock stress-induced relapse (see Shaham *et al*, 2003).

Our present findings provide an interesting behavioral/neural/neurochemical perspective into the stress-induced enhanced d-AMPH response. Although there is not always

a strict temporal correlation between presynaptic adaptations on DA systems and behavioral sensitization to psychostimulants (see Di Chiara, 1995), it is worth noting that our observations show a temporal parallel between the restraint stress-induced enhanced response to drugs on DA release in NAcc core and on locomotor activity. In spite of this, it remains likely that DA in NAcc shell and CPU may also be involved in the short-term effects. The distinct pattern of NAcc core and NAcc shell output targets, with the core projections to pallidal structures and the shell, in addition, projecting to more limbic domains (Zahm and Heimer, 1990), suggests that the two regions may mediate different aspects of the same behavioral process. We could speculate that our present findings of DA-enhanced release to d-AMPH can occur as a result of restraint stress effects on excitatory synapses on the midbrain DA neurons, rendering neurons more vulnerable to a subsequent challenge with the drug. Previously described results are consistent with this hypothesis, with CRF potentiating NMDA-mediated transmission in DA neurons on VTA slices (Ungless *et al*, 2003), and controlling glutamate and DA release in VTA during stress-induced cocaine relapse (Wang *et al*, 2005). Another way in which stress could induce changes is through the glutamatergic innervation of the NAcc shell and NAcc core and CPU, arising from structures such as the hippocampus via the subiculum, the amygdala, and the prefrontal cortex (Sesack *et al*, 1989; Christie *et al*, 1987). Wheeler *et al* (1995) demonstrated that MK-801 in the striatum was able to block the tail-pinch induced increase in DA in the striatum. Glutamatergic agonists applied directly to the NAcc cause release, whereas antagonists inhibit DA release (Youngren *et al*, 1993; Svensson *et al*, 1994), although more complex interactions are involved (Feenstra *et al*, 1998; Taber *et al*, 1996). More studies need to be done to determine the glutamate circuit involved in our model.

The phenomenon of behavioral sensitization has been proposed to be an adaptive process in addiction to psychostimulants and other drugs of abuse (Robinson and Berridge, 1993). This sensitization process, which shares many of the characteristics of other forms of neuronal plasticity, has been associated with either the early stages or relapse in the cycle of addiction (Robinson and Berridge, 2000; Vezina *et al*, 2002). Based on data on cross-sensitization of locomotor activity between drug and stressors (see Kalivas and Stewart, 1991), it has also been suggested that sensitization of the stimulant effects of drugs may be of relevance to the understanding of relapse induced by stressors (see Piazza and LeMoal, 1998; Robinson and Berridge, 1993; Shaham and Stewart, 1995). Our present study provides new evidence that the neurobiological mechanisms underlying stress-induced enhanced stimulating responses resemble those observed following drugs, including involvement of NMDA receptor stimulation and augmented DA release.

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