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Environmental Enrichment During Early Stages of Life Reduces the Behavioral, Neurochemical, and Molecular Effects of Cocaine

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It is known that negative environmental conditions increase vulnerability to drugs, whereas little is known on whether positive environmental conditions such as enriched environments (EE) have protective effects against addiction. We have previously found that EE consisting of bigger cages containing several toys that were changed once per week reduce cocaine-induced increases in locomotor activity. Here, we also show that the rewarding effects of cocaine are blunted in mice reared from weaning to adulthood in EE compared to mice reared in standard environments (SE). In addition, although both EE and SE mice develop behavioral sensitization to cocaine, EE mice show less activation in response to repeated administration of cocaine injections and reduced responses to cocaine challenges. *In vivo* microdialysis experiments demonstrate that the protective effects of EE do not depend on reduced cocaine-induced increases in the dopamine levels in the ventral or dorsal striatum. On the other hand, they were associated with reduced cocaine-induced expression of the immediate early gene zif-268 in the nucleus accumbens (shell and core) of EE mice. Finally, basal levels of Delta-Fos B, a transcription factor known to be increased by sustained activation of striatal neurons, are higher in the striatum of EE compared to SE mice and repeated administration of cocaine increases Delta-Fos B levels in SE mice but decreases them in EE mice. Altogether our results demonstrate that exposure to complex environments during early stages of life produce dramatic changes in the striatum that result in reduced reactivity to drugs of abuse.
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INTRODUCTION

Of the many people who experience psychoactive drugs, only a small percentage develops drug addiction. It is believed that environmental factors play a central role in determining sensitivity to the rewarding effects of drugs and vulnerability to develop addiction (Sinha, 2001; Goeders, 2002; Marinelli and Piazza, 2002; Kreek *et al*, 2005). For example, several studies have demonstrated that environmental stressors increase the unconditioned, conditioned, and reinforcing effects of drugs such as amphetamine, cocaine, and heroin (Sinha, 2001; Goeders, 2002; Marinelli and Piazza, 2002; Kreek *et al*, 2005). Preclinical studies have also provided important information on the mechanisms underlying this enhanced response to the effects of drugs and have shown that the dopamine system is implicated in this excessive reaction to drugs (Rouge-Pont *et al*, 1995; Goeders, 2002; Marinelli and Piazza, 2002).

The investigation of negative factors exacerbating addiction has been extensive, whereas considerably less attention has been dedicated to environmental manipulations that may mimic positive life experiences such as enrichment environments (EE) and may provide protection against drugs' effects. EE have been shown to increase learning and memory (van Praag *et al*, 2000), to facilitate recovery from brain injuries (Will *et al*, 2004), to reduce the insurgence of neurodegenerative diseases (Nithianantharajah and Hannan, 2006) and some authors have suggested that EE represent more natural and healthy environment than current standard housing conditions (Wurbel, 2001). In addition, some studies have shown that the reinforcing effects of amphetamine and nicotine are reduced in rats reared in EE compared to rats reared in social isolation (Bowling *et al*, 1993; Bardo *et al*, 2001; Green *et al*, 2002, 2003).

In a previous study, we have shown that mice reared in EE show less locomotor activity and a different pattern of c-fos activation in the striatum in response to an acute

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injection of cocaine compared to mice reared in a standard environments (SE) (Bezard *et al*, 2003). We also found that EE mice had less dopaminergic neurons in the substantia nigra pars compacta, lower levels of the dopamine transporter (DAT), the molecular target of cocaine, and higher levels of neurotrophin BDNF in the striatum (Bezard *et al*, 2003). These findings provided an initial demonstration that EE may be crucial in determining resistance to drugs of abuse such as cocaine. However, little information is available on the effects of EE on cocaine's rewarding properties, which are considered directly responsible for the development of drug addiction and on the behavioral and molecular consequences of repeated administration of cocaine.

In this study, we housed mice either in EE or SE from weaning to adulthood and we used (1) conditioned place preferences (Cunningham *et al*, 2006) to assess the rewarding effects of cocaine; (2) behavioral sensitization, an animal model for the intensification of drug craving and relapse believed to underlie addiction in humans (Vanderschuren and Kalivas, 2000; Robinson and Berridge, 2003; Bradberry, 2007), to assess the consequences of repeated exposure to cocaine; (3) *in vivo* microdialysis techniques to assess the response to cocaine of the dopamine mesolimbic system at the presynaptic level (Di Chiara and Bassareo, 2007); (4) *in situ* hybridization techniques to assess the reactivity to cocaine of the dopamine mesolimbic system at the postsynaptic level by measuring expression of immediate early genes (Hope, 1998; Berke and Hyman, 2000; Gerfen, 2000) and (5) western blot techniques to assess whether the differences observed in the behavioral effects of cocaine could be due to changes in the levels of Delta-Fos B, a transcription factor that is implicated in long-term neuroadaptations associated with drug addiction (McClung *et al*, 2004).

MATERIALS AND METHODS

Subjects

Male C57Bl/6J mice (Janvier, France) were housed in a temperature-controlled environment on a 12-h light/dark cycle with the lights on from 0700 to 1900 hours and had ad libitum access to food and water. All experimentation was conducted during the light period. Experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) for the care of laboratory animals.

Housing Environmental Conditions

After weaning (3 weeks of age), mice were randomly divided in two different housing environmental conditions: SE or EE. SE consisted of common cage housing ($25 \times 20 \times 15$ cm). The EE consisted of larger ($60 \times 38 \times 20$ cm) cages containing a constantly running wheel and a small house and four-five toys that were changed once a week with new toys of different shape and color. For both SE and EE conditions, mice were housed in groups of four (each mouse from a different sibling) for at least 2 months and maximum 3 months before the start of the experiments. All the experiments were conducted at adulthood.

Conditioned Place Preference

Conditioned place preference experiments were performed in four identical boxes (IMETRONIC, Pessac, France) formed by two lateral chambers ($15 \times 15 \times 20$ cm) connected by a central alley ($5 \times 15 \times 20$ cm). Two sliding doors separated the alley from the chambers. In each chamber, two Plexiglas prisms with triangular bases ($5 \times 7 \times 19$ cm) were arranged to form different patterns (always covering the same surface of the chamber) and were used as conditioned stimuli. Two different metallic grids, one with large (1 cm) squares and the other with small (0.5 cm) circles were also used as conditioned stimuli. Behavioral data were collected by an Imetronic interface connected to a PC. Two infrared photocells were present in each compartment and detected the presence and movements of mice. Conditioned place preference procedure consisted of three phases: preconditioning, conditioning, and test. For each manipulation, mice were brought to the experimental room 60 min before the start of the experiment to allow for habituation and to reduce stress. Preconditioning was performed on day 1. Mice were placed in the central compartment with the doors closed. After 15 s, the doors were opened and mice were free to explore the entire two-compartment apparatus for 20 min. The time spent in each compartment was recorded and was considered a measure of spontaneous preference. Conditioning sessions were performed on days 2, 3, and 4, twice per day with morning and afternoon sessions separated by at least 6 h. During these sessions, mice were injected with either saline or cocaine (10 and 20 mg/kg) and immediately confined to one of the pairing compartments for 30 min. The order of treatments (saline or cocaine), the time of cocaine injection (morning or afternoon), and the compartment (right or left) were counterbalanced in a semirandom order in both SE and EE mice. Control mice received saline on both sides of the cage. A total of three cocaine- and three saline-conditioned sessions were performed. Test sessions for conditioned place preference were conducted on day 6 and were similar to preconditioning sessions with animals placed in the central compartment for 15 s and then left free to choose a compartment for 20 min. The time spent in each compartment was measured and compared to the time spent in the same compartment during the preconditioning session. Preference scores, which served as a measure of rewarding effects of cocaine, were calculated by subtracting the time in seconds spent during the pretest from the time spent during the test day in the compartment paired to cocaine injections. For saline–saline groups, given that there was not a natural preference for either compartment, we arbitrarily chose to calculate preference score in the left compartment. We also verified that results would not have been different if we had chosen to calculate preference scores for the right compartment.

Locomotor Activity and Behavioral Sensitization Procedure

Motor activity was measured in Plexiglas cages ($19 \times 11 \times 14$ cm) placed in frames mounted with computer-monitored photocell beams (IMETRONIC). Horizontal locomotion was measured by the number of cage crossings. Behavioral data

were collected by an Imetronic interface connected to a PC. Mice were first habituated to locomotor chambers for 60 min. Then, they were injected intraperitoneally with drug or saline and immediately placed back into the chamber.

Behavioral sensitization consisted of two phases: development and expression. For development of behavioral sensitization six injections of cocaine (15 mg/kg intraperitoneally (i.p.)) or saline were administered every second day. Expression of behavioral sensitization was measured 30 days after the last injection (withdrawal day 30). A total of eight groups were obtained ($N=8$ per group): SE sal–sal; sal–coc; coc–sal and coc–coc and EE sal–sal; sal–coc; coc–sal and coc–coc. Sal–sal mice received saline both during development and expression of behavioral sensitization; sal–coc mice received saline during development and cocaine for the expression of behavioral sensitization; coc–sal mice received cocaine during development and saline for the expression of behavioral sensitization; coc–coc mice received cocaine both during development and expression of behavioral sensitization. Locomotor activity was measured for 90 min for cocaine during the development of behavioral sensitization. On withdrawal day 30, locomotor activity was measured for only 45 min to allow the killing of mice and for obtaining brains to perform *in situ* hybridization studies.

In Vivo Microdialysis

Two groups of SE and two groups of EE mice ($N=5$–6 per group) were used for *in vivo* microdialysis experiments. Concentric dialysis probes design was similar to the one previously described for rats (Solinas *et al*, 2006), but sizes were adapted for mice. The exposed dialyzing surface of the probes was limited to the lowest 1.0 mm portion of the probes. Probe diameter was 0.250 mm. Mice were deeply anaesthetized with Avertin (0.4–0.75 mg/g) and placed in a stereotaxic apparatus (Stoelting Co., Illinois, USA). Coordinates for the nucleus accumbens (NAc) ($A=1.5$; $L=0.6$; $V=5.0$) and caudate putamen ($A=0.0$; $L=1.8$; $V=4.0$) were in accordance with the mouse brain atlas (Paxinos and Franklin, 2001). Each mouse was implanted with a single probe in the left hemisphere. About 24 h after probe implant, experiments were performed in freely moving mice. Ringer's solution (147.0 mM NaCl, 2.2 mM $CaCl_2$, and 4.0 mM KCl) was delivered through the dialysis probes at a constant flow rate of 1 μl/min. Dialysate samples (20 μl) were collected every 20 min and 10 μl were immediately injected for analysis by high-performance liquid chromatography (5200a Coulochem III, ESA, Chelmsford, MA, USA). The mobile phase contained 100 mM NaH_2PO_4, 0.1 mM Na_2EDTA, 0.5 mM n-octyl sulfate, and 18% (v/v) methanol (pH adjusted to 5.5 with Na_2HPO_4). After stable dopamine level values (less than 10% variability) were obtained for at least three consecutive samples (typically after about 3 h), treatments started. Mice were first injected with saline and dopamine levels monitored for 60 min, then with 10 mg/kg of cocaine and dopamine levels monitored for 120 min and, finally, with 20 mg/kg of cocaine and dopamine levels monitored for 120 min. Assay sensitivity for dopamine was 2 fmoles per sample. At the end of the microdialysis experiments, mice were euthanized by dislocation and brains were removed and left to fix in 4% formaldehyde in saline solution. Brains were then frozen and cut on a cryostat in serial coronal slices. Slices were colored with cresyl violet to identify the location of the probes (see Supplementary Figures S1 and S2). Only data from mice with correct probe location were considered for statistical analysis. Dopamine levels are expressed as a percentage of basal dopamine values. Basal dopamine values were calculated as the mean of three consecutive samples (differing no more than 10%) immediately preceding the first drug or vehicle injection.

In Situ Hybridization

In situ hybridization experiments were performed on the brain of mice sensitized as described above. The same eight experimental groups were analyzed: SE sal–sal; sal–coc; coc–sal and coc–coc and EE sal–sal; sal–coc; coc–sal and coc–coc ($N=6$ per group). Mice were killed 45 min after the last injection of cocaine at day 30 of withdrawal. This postmortem time was chosen because levels of immediate early genes peak within an hour after cocaine injections (Yano and Steiner, 2005). Brains were removed, frozen in isopentane at -40°C, and then stored at -80°C. Coronal tissue sections (14 μm thick) were thaw mounted onto SUPERFROST slides (VWR International, Fontenay sous Bois, France) and stored at -80°C.

In situ hybridization was performed with [^{35}S] (UTP)-labeled RNA for zif-268 (Thiriet *et al*, 2002). Sections were delipidated, acetylated (0.25% acetic anhydride in 0.1 M triethanolamine (pH 8)), prehybridized for 10 min at 60°C in 50% formamide/SSC 1× (150 mM NaC1 and 15 mM sodium citrate, pH 7), dehydrated and air-dried. Thirty microliters of the labeled probes diluted to 25 000 cpm/μl with hybridization buffer (50% formamide, 10% dextran sulfate, SSC 4×, 10 mM dithiothreitol) was placed on tissue sections and covered with coverslips and left overnight at 52°C. Post-hybridization was then carried out as previously described (Thiriet *et al*, 2002). The sections were exposed to X-ray film (Kodak, biomax-MR) for 4 days, then 7 days to Storm phosphorimager system for quantitative analysis using Image Quant software. Optical densities for each area (see Supplementary Figure S3) were calculated by subtracting to all areas the background level determined between sections of each slide. A total of two slides per animal (four sections per slide) were used for each hybridization experiment. All experiments were duplicated.

Western Blot

To measure Delta-Fos B (35–37 kDa) protein levels in the striatum, mice were exposed to the same regimen for the development of cocaine sensitization used for behavioral and *in situ* hybridization experiments. A total of four groups were used: SE sal, SE coc, EE sal and EE coc ($N=12$ per group). Sal groups received six injections of saline, whereas coc groups received six injections of 15 mg/kg of cocaine every second day. Mice were killed by decapitation 18 h after the last injection because the levels of Delta-Fos B are maximal, whereas other Fos proteins are absent at this time point (McClung *et al*, 2004). Brains were immediately removed and striata (NAc and caudate putamen) were immediately dissected. Proteins were extracted using a

slightly modified version of the protocol described by Brenhouse and Stellar (2006). Briefly, brain tissue was homogenized in boiling lysis buffer (350 µl for 25 mg) containing 50 mM Tris–HCl pH 6.8, 100 mM DTT, 2% SDS, with 0.05U aprotinin A, 1 µM pepstatin, 1 µM leupeptin, 0.1 mM benzamidine, 10 nM chloroquin, 10 nM Soybean Trypsin inhibitor, 0.1 mM N_α-Tosyl-L-lysine chloromethyl ketone hydrochloride, 100 µg/ml N_α-*p*-Tosyl-L-arginine methyl ester hydrochloride, and 0.1 mM PMSF protease inhibitors. After homogenization using ultra-turrax for 15 s (IMLAB Laboratories technology, France), samples were then boiled for 3 min and centrifuged at full speed for 10 min. Supernatant were collected and then stored at −20°C until analysis. Protein samples (about 30 µg) were subjected to SDS–polyacrylamide gel electrophoresis (10% acrylamide/0.27% *N*,*N*′-methylenebisacrylamide resolving gel) and proteins were transferred to PVDF membrane. Blots were blocked for 1 h in 5% nonfat dry milk in TBS-Tween 0.1%. Primary antibody incubation was then performed in blocking buffer for 120 min at room temperature, using rabbit Fos-related antigen (FRA, dilution 1:500), which recognizes the Delta-Fos B isoforms (kindly provided by Dr Iadarola, National Institute of Dental and Craniofacial Research, National Institute of Health, USA). The blots were then washed 3 × 10 min in blocking buffer and were subsequently incubated for 60 min in a 1 : 1000 dilution of goat anti-rabbit antibody conjugated to horseradish peroxidase (Bio-Rad Laboratories, France) in blocking buffer. The blots were then washed again 2 × 10 min in blocking buffer and then for 10 min in TBS, and developed with an enhanced chemiluminescence system (Pierce, France) before being exposed to Kodak MS Film (VWR, France) for 1 min. The blots were subsequently incubated with an anti-α-tubulin antibody (1/2000, Sigma-Aldrich, France). Levels of band density were quantified by densitometry with the VisioLab 2000 image analyser (Biocom, Paris, FRANCE), and for each sample the results are expressed as level of delta-Fos B over level of α-tubulin. The experiment was repeated twice (each time with $N = 6$ for each group). Care was taken to assure that quantification was performed within a linear range (Jaber *et al*, 1999). As relative values among experimental groups did not vary, data were pooled together by normalizing data to the mean value of each SE saline-treated group.

Drugs

Cocaine HCl (15 mg/kg, 10 mg/kg) was obtained from the Research Triangle Institute (Research Triangle Park, NC, USA). It was dissolved in sterile saline solutions (0.9%) and administered i.p. in a volume of 1 ml/100 g.

Statistics

All results are presented as group means ± SEM. Differences in behavioral scores, in the levels of extracellular dopamine, mRNA and protein expression between groups were assessed by one, two or three way analysis of variance ANOVA. Results showing significant overall changes were subjected to Student–Newman–Keuls *post-hoc* test. Differences were considered significant when $p < 0.05$.

RESULTS

EE Mice are Less Sensitive than SE Mice to the Reinforcing Effects of Cocaine

Neither SE nor EE mice showed significant natural preferences for either conditioned place preference compartments during pretests or developed preferences for one or the other compartment when saline was given in both compartments (Figure 1a and b). On the other hand, SE mice developed significant place preferences to cocaine at the dose of 10 and 20 mg/kg, whereas EE mice did not develop significant preferences at either doses (Figure 1b) (two-way ANOVA, environment effect, $F(1, 67) = 5.93$, $p < 0.05$; dose effect, $F(2, 67) = 8.27$, $p < 0.01$; environment effect × dose interaction, $F(2, 67) = 2.18$, $p > 0.05$).

EE Mice are Less Sensitive than SE Mice to the Stimulant Effects of Repeated Administration of Cocaine

As previously reported (Bezard *et al*, 2003), the first injection of cocaine (15 mg/kg i.p.) produces a higher activation

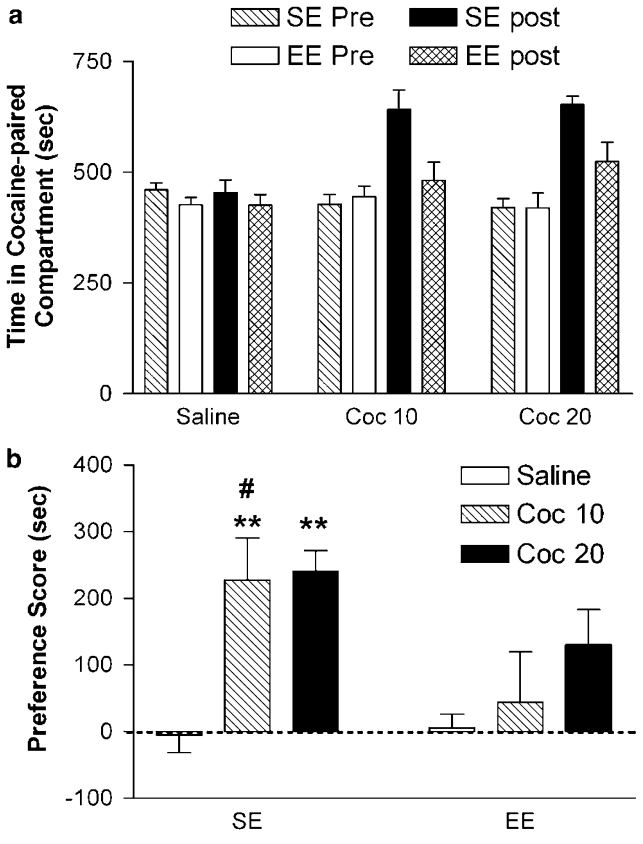


Figure 1 Rewarding effects of cocaine in mice reared in standard (SE) or enriched (EE) environments. EE and SE mice were tested in the conditioned place preference paradigm after conditioning to cocaine (10 and 20 mg/kg i.p.) or saline. EE showed less conditioned place preference to cocaine than SE mice. (a) Shows time in sec spent in the cocaine-paired compartments before and after conditioning, whereas (b) shows preference scores. Preference score was calculated by the time spent in the cocaine-paired compartment during the test session minus the time spent in the same compartment during the pretest session. *Post-hoc* Student–Newman–Keuls's test: $^{**}p < 0.01$ different from saline control; $^{\#}p < 0.05$ EE different from SE mice. Results represent means ± SEM from 11 to 13 mice.

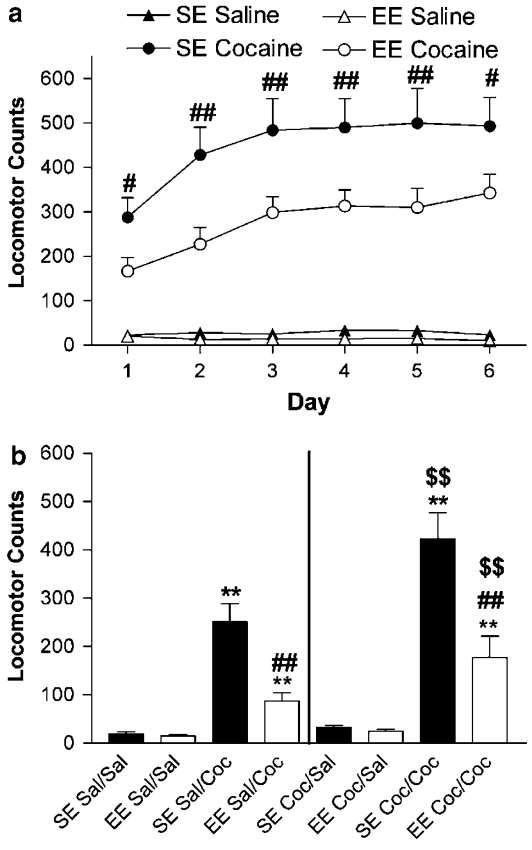


Figure 2 Behavioral sensitization to cocaine in mice reared in standard (SE) or enriched (EE) environments. (a) Development of behavioral sensitization to cocaine (15 mg/kg i.p.). Both SE and EE mice develop behavioral sensitization i.e. cocaine-induce locomotor activity increases over time, but EE consistently show reduced locomotor response to cocaine. (b) Expression of behavioral sensitization to cocaine (10 mg/kg i.p.) after 30 days of withdrawal. Both SE and EE mice show a sensitized response to cocaine (coc-coc *vs* sal-coc groups) but EE show reduced locomotor response to cocaine compared to SE mice. *Post-hoc* Student–Newman–Keuls's test: * and $^{**}p<0.05$ and $p<0.01$ different from saline control; $^{\#\#}p<0.01$ different from SE control; $^{\$\$}p<0.01$ different from chronic saline control. Results represent means ± SEM from 15 to 16 mice for panel a and 7–8 mice for panel b. Note that, for clarity, although all data point for the development of cocaine sensitization (panel a) are significantly different from saline controls in both SE and EE mice, symbols are not shown.

in SE mice as compared to EE mice (Figure 2a and b). With repeated administrations, both groups developed sensitization, ie, for both groups the effects of cocaine on day 6 were higher than on day 1 (Figure 2a). However, the effects of cocaine were consistently lower (about 50%) in EE compared to SE mice (Figure 2a) (three-way ANOVA, environment effect, $F(1,59)=3772$, $p<0.0001$; treatment effect, $F(1,59)=519.61$, $p<0.0001$; day effect, $F(5,354)=13.73$, $p<0.0001$; environment × treatment interaction, $F(1,59)=27.79$, $p<0.0001$; environment × day interaction, $F(5,354)=0.22$, $p>0.05$; treatment × day interaction, $F(5,354)=4.04$, $p<0.01$; environment × treatment × day interaction, $F(5,354)=0.12$, $p>0.05$). In addition, sensitization was maintained in the two groups after 30 days of withdrawal from cocaine as demonstrated by the sensitized response to 10 mg/kg of cocaine (Figure 2b). However, once again, the amplitude of cocaine's effects was higher in SE than in EE mice (Figure 2b) (three-way ANOVA, environment effect, $F(1,55)=25.55$, $p<0.0001$; treatment effect, $F(1,55)=11.64$, $p<0.01$; challenge effect, $F(1,55)=103.82$, $p<0.0001$; environment × treatment interaction, $F(1,55)=1.02$, $p>0.05$; environment × challenge interaction, $F(1,55)=22.71$, $p<0.0001$; treatment × challenge interaction $F(1,55)=8.24$, $p<0.01$; environment × treatment × challenge interaction, $F(1,55)=0.87$, $p>0.05$), indicating that environmental effects on repeated cocaine-induced behaviors and neuroadaptations are due to reduced effects of each acute cocaine administration. Motor impairment could not account for the reduced reactivity to cocaine of EE mice, as EE mice perform actually better than SE on a rotarod paradigm (data not shown).

Cocaine-induced Elevation in Dopamine Levels in the NAc do not Differ Between EE and SE Mice

Dopamine basal levels did not differ between EE and SE mice neither in the NAc (SE = 46.59 ± 4.85; EE = 47.90 ± 6.71 fmol/10 µl) nor in the Caudate Putamen (SE = 51.55 ± 4.87; EE = 52.98 ± 6.03 fmol/10 µl). Saline injections did not alter extracellular levels of dopamine, whereas cocaine produced dose-dependent and time-dependent increases in dopamine levels both in the NAc and in the Caudate Putamen (three-way ANOVA for repeated measures, NAc: dose effect, $F(1,40)=21.28$, $p<0.0001$; time effect $F(5,40)=37.77$, $p<0.0001$; dose × time interaction $F(5,40)=7.23$, $p<0.0001$; Caudate Putamen, dose effect, $F(1,40)=6.31$, $p<0.05$; time effect $F(5,40)=16.88$, $p<0.0001$; dose × time interaction $F(5,40)=2.07$, $p>0.05$) (Figure 3). Increases in the NAc were more pronounced than those in the Caudate Putamen at both 10 mg/kg (300 *vs* 200% of basal levels) and 20 mg/kg (450 *vs* 300% of basal levels) of cocaine. Importantly, cocaine-induced elevations in extracellular levels of dopamine did not differ in EE and SE mice (three-way ANOVA for repeated measures, NAc: environment effect, $F(1,40)=0.34$, $p>0.05$; environment × dose interaction, $F(5,40)=0.002$, $p>0.05$; environment × time interaction, $F(5,40)=0.35$, $p>0.05$; environment × dose × time interaction, $F(5,40)=1.68$, $p>0.05$; CPu: environment effect, $F(1,40)=0.01$, $p>0.05$; environment × dose interaction, $F(5,40)=0.01$, $p>0.05$; environment × time interaction, $F(5,40)=0.48$, $p>0.05$; environment × dose × time interaction, $F(5,40)=0.59$, $p>0.05$).

Cocaine Increases the Expression of the Immediate Early Gene zif-268 in the Striatum of SE but not of EE Mice

As the differences in behavioral responses to cocaine could not be explained by different effects of cocaine at the presynaptic levels, we monitored the expression of Zif-268 (Beckmann and Wilce, 1997) by *in situ* hybridization in the striatum as a measure of reactivity to cocaine at the postsynaptic levels. Basal levels of zif-268 did not differ between SE and EE in any region examined. In addition, zif-268 mRNA levels did not differ between mice chronically pretreated with cocaine (15 mg/kg i.p) and saline pretreated controls (groups coc–sal *vs* sal–sal). Cocaine challenge (10 mg/kg) increased zif-268 mRNA levels (about 25%) in the NAc shell and the core (but not in the caudate putamen)

of SE mice similarly in chronically cocaine- (coc–coc) and saline-treated (sal–coc) mice, indicating that these effects of cocaine do not undergo sensitization. In contrast, in EE mice, cocaine (10 mg/kg i.p.) did not increase expression of zif-268 in any condition (three-way ANOVA: Shell, environment effect, $F(1,37)=7.72$, $p<0.01$; treatment effect, $F(1,37)=2.32$, $p>0.05$; challenge effect, $F(1,37)=5.66$, $p<0.05$; environment × treatment, $F(1,37)=1.06$, $p>0.05$; environment × challenge, $F(1,37)=6.94$, $p<0.05$; treatment × challenge, $F(1,37)=0.71$, $p>0.05$; environment × treatment × challenge, $F(1,37)=0.10$, $p>0.05$; Core; challenge effect, $F(1,37)=16.70$, $p<0.01$; Caudate Putamen, Challenge effect, $F(1,37)=5.28$, $p<0.05$) (Figure 4).

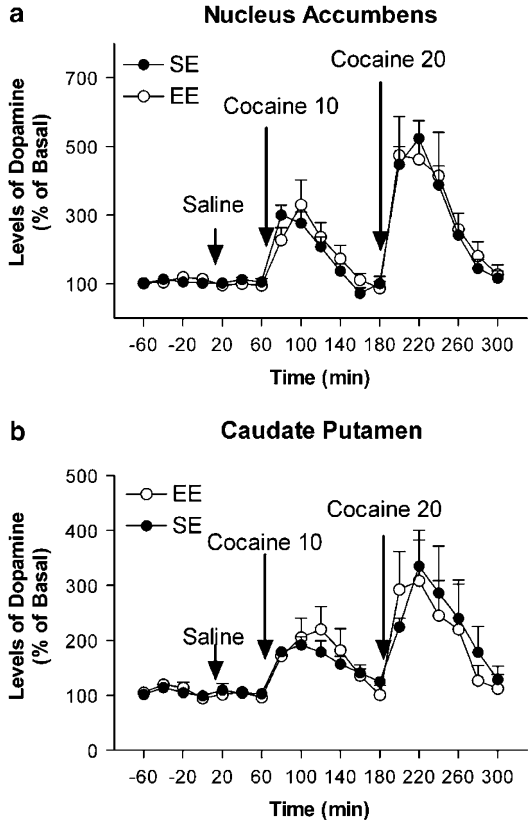
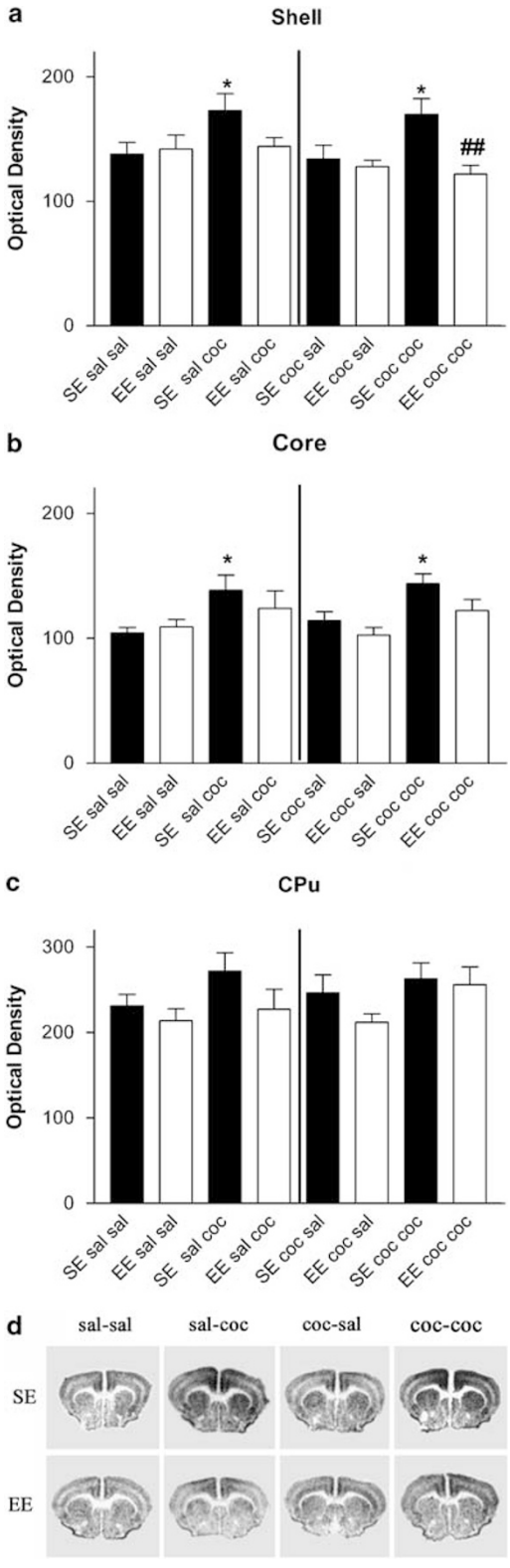


Figure 3 Cocaine-induced elevations of dopamine levels in mice reared in standard (SE) or enriched (EE) environments. Injections of 10 and 20 mg/kg of cocaine increase the extracellular levels of dopamine (a) in the nucleus accumbens and (b) in the caudate putamen to similar extent in SE and EE mice. Arrows indicate the time of injections. Results represent means ± SEM from five to six mice.

Figure 4 Expression of zif-268 in mice reared in standard (SE) or enriched (EE) environments sensitized to cocaine. For induction of behavioral sensitization mice were injected with cocaine (15 mg/kg) or saline as in Figure 2 and injected with cocaine (10 mg/kg) or saline 30 days after the last cocaine or saline injection. Challenge injections of cocaine increase zif-268 expression in the shell (a) and core (b) of the nucleus accumbens in SE but not in EE mice. Importantly, zif-268 expression was not enhanced by sensitization. No significant change was found in the caudate putamen in SE or EE mice (c). *Post-hoc* Student–Newman–Keuls's test: $^{*}p<0.05$ different from saline control; $^{\#\#}p<0.01$ different from SE control. Results represent means ± SEM from six mice.

EE and SE Have Opposite Effects on the Levels of Delta-Fos B Following Cocaine Administration

By western blot, we measured Delta-Fos B levels in the striatum of SE and EE mice sensitized to cocaine (15 mg/kg) and in saline controls. As previously reported (Hiroi *et al*, 1997), repeated administration of cocaine produced a



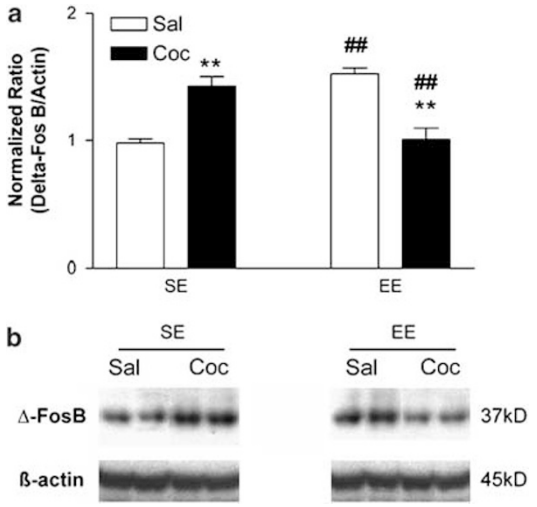


Figure 5 Levels of Delta-Fos B protein in the striatum of mice reared in standard (SE) or enriched (EE) environments sensitized to cocaine. Mice underwent the same protocol for the development of cocaine sensitization as in Figure 2a and were killed 18 h after the last cocaine or saline injection. EE mice show higher basal levels of Delta-Fos B than SE mice. Importantly, repeated administration of cocaine induced an increase of Delta-Fos B levels in SE and a decrease in EE mice. (a) Graphical representation of group means and (b) representative immunoblots. *Post-hoc* Student–Newman–Keuls's test: $^{**}p<0.01$ different from saline control; $^{\#\#}p<0.01$ different from SE control. Results represent means ± SEM from eight mice.

significant increase in Delta-Fos B levels in the striatum of SE mice (about 40%). Interestingly, levels of Delta-Fos B in the striatum of saline-treated EE mice were elevated compared to saline-treated SE mice (about 50%) and were comparable to cocaine-treated SE mice. Surprisingly, in EE mice cocaine did not produce an increase but rather a decrease in Delta-Fos B levels (two-way ANOVA, environment effect, $F(1,44)=2.15$, $p>0.05$; treatment effect, $F(1,44)=0.28$, $p>0.05$; environment × treatment effect, $F(1,44)=21.97$, $p<0.0001$) (Figure 5).

DISCUSSION

In this study, we found that EE mice are protected against the effects of cocaine. EE mice develop less conditioned place preferences for compartments associated with cocaine injections and are less sensitive to the activating effects of cocaine in a sensitization paradigm than SE mice. In addition, cocaine-induced increases in dopamine levels in the striatum do not differ between EE and SE mice, whereas ventral striatal neurons are activated by cocaine in SE but not in EE mice, as measured by expression of the immediate early gene zif-268. Finally, we found that saline-treated EE mice have higher striatal levels of Delta-Fos B than saline-treated SE mice and that, whereas repeated administration of cocaine increases Delta-Fos B levels in the striatum of SE mice, it decreases Delta-Fos B levels in the striatum of EE mice. These results suggest that exposure to positive environmental conditions during early stages of life can greatly influence brain functioning and can provide protection against the effects of addictive drugs.

In a previous study, we found that the activating effects of an acute administration of cocaine were reduced by exposure to EE (Bezard *et al*, 2003). Here, we show that the rewarding effects of cocaine are also diminished in EE mice. In addition, EE reduce the activating effects of repeated administrations of cocaine. Indeed, in a behavioral sensitization paradigm, EE mice show reduced locomotion both during the development and the expression of behavioral sensitization. Although the validity of behavioral sensitization models to study drug addiction in humans is debated (Lecca *et al*, 2007), behavioral sensitization is considered an important preclinical tool to study the increases in the effects of drugs and the neuroadaptations that take place in the brain upon repeated administration of cocaine (Vanderschuren and Kalivas, 2000; Berridge, 2007; Bradberry, 2007). In our study, the rate of sensitization was similar between SE and EE mice with cocaine-treated mice having an average increase of 100% in locomotor counts as compared to the first day of cocaine exposure, whereas the sustained reduction of the effects of cocaine indicates that EE mice are protected against the effects of this drug. In a previous study, it was shown that rats reared in EE are less sensitive to the discriminative effects of cocaine (Fowler *et al*, 1993). In addition, in rats, EE decrease the rewarding effects of amphetamine in a self-administration paradigm (but not in a conditioned place preference paradigm) (Bowling and Bardo, 1994; Bardo *et al*, 2001; Green *et al*, 2002) and the activating effects of amphetamine (Bowling *et al*, 1993; Bardo *et al*, 1995) or nicotine (Green *et al*, 2003). However, in those studies, control groups for the effects of environmental enrichment were animals reared and housed in impoverished/isolated conditions. Whereas such controls may have helped magnifying the effects of EE, in our opinion, they do not represent a proper control to extrapolate the effects of environmental enrichment because of the known important behavioral, neurochemical, and molecular alterations produced by social isolation (Lu *et al*, 2003; Van den Buuse *et al*, 2003). Thus, our model, which compares enriched to standard housing conditions, has the advantage to provide direct and uncontroversial information of the influences of enrichment on the effects of cocaine. A recent study comparing mice reared in EE to mice reared in SE has found that EE mice show less activation and less place preference to morphine than SE mice (Xu *et al*, 2007).

Cocaine elevates dopamine levels by binding to the dopamine membrane transporter (DAT) and preventing dopamine reuptake by presynaptic terminals (Jaber *et al*, 1997) and these dopamine elevations in the NAc are considered central in the mediation of the reinforcing effects of psychostimulants (Di Chiara and Bassareo, 2007). Given that previous studies have shown reduced DAT levels in mice reared in EE (Bezard *et al*, 2003), we expected that the reduced effects of cocaine were related to a deficit in its ability to elevate dopamine in the NAc. In contrast, we found that the levels of dopamine under basal conditions and after cocaine administration did not differ between EE and SE. It is possible that, although significant, reductions of about 20% in DAT levels (Bezard *et al*, 2003) were not sufficient to alter cocaine-induced effects. It is also possible that given the chronic nature of environmental enrichment, neuronal adaptations occur in EE mice that compensate the decrease in DAT. For example, it has been shown that in DAT knockout mice, cocaine induces normal increases in

dopamine accumbal levels through an action on noradrenaline transporter (Carboni *et al*, 2001).

We have previously shown that acute cocaine (20 mg/kg) increases c-fos levels in dorsolateral striatum in EE mice and in the ventromedial striatum in SE mice (Bezard *et al*, 2003). Here, in the same cocaine sensitization paradigm used for our behavioral experiments, we have measured mRNA levels of zif-268, another immediate early gene, whose expression is tightly correlated with cellular activation and synaptic plasticity (Beckmann and Wilce, 1997). Acute administration of cocaine increases the expression of zif-268 in the ventral and dorsal striatum in rats (Hope *et al*, 1992; Moratalla *et al*, 1992; Thiriet *et al*, 2002) and in mice (Brami-Cherrier *et al*, 2005). In addition, in mice genetically lacking zif-268 behavioral sensitization and conditioned place preferences are dramatically reduced (Valjent *et al*, 2006). Finally, injections of zif-268 antisense oligodeoxynucleotides in the basolateral amygdala disrupt the reconsolidation of drug-related memories and reduces cocaine-seeking behavior (Lee *et al*, 2005). Consistent with those studies, we found that zif-268 expression in the shell and core of the NAc was induced by cocaine administration in SE mice to the same extent in sensitized and nonsensitized mice. In contrast, in EE mice cocaine did not induce zif-268 expression in the NAc. Thus, the reduced expression of zif-268 in response to cocaine found in EE mice may participate to the reduced behavioral effects of cocaine in these mice. Several mechanisms could explain how similar levels of extracellular dopamine induced differential reactivity to cocaine in striatal neurons. For example, changes in dopamine receptors availability or functionality might account for the reduced response to cocaine. However, previous studies have failed to show a significant effect of environmental enrichment on D1 and D2 binding (Bardo and Hammer, 1991) and mRNA expression (Bezard *et al*, 2003). Future studies are needed to investigate whether other systems such as the glutamate system (Beckmann and Wilce, 1997), at the presynaptic or postsynaptic level, are involved in the protective effects of environmental enrichment.

Delta-Fos B is a member of the Fos family of transcription factors. In contrast to the other Fos proteins, Delta-Fos B is induced to only a small degree in response to acute drug administration but because of its unique stability, after repeated drug administration, Delta-Fos B gradually accumulates in the striatum and stays elevated for weeks or months after discontinuation of drug exposure (Hope *et al*, 1994; Chen *et al*, 1995; Moratalla *et al*, 1996; Hiroi *et al*, 1997). Thus, it has been hypothesized that Delta-Fos B functions as a sustained molecular switch that mediates some of the more persistent adaptations of the brain that underlie addiction (McClung *et al*, 2004). Importantly, striatal Delta-Fos B has been shown to be increased not only by drugs of addiction but also by nonaddictive drugs, such as antipsychotics as well as environmental manipulations such as exposure to stress (McClung *et al*, 2004). In our study, environmental stimulation *per se* increased striatal levels of Delta-Fos B in saline-treated mice. This increase is likely due to the constant activation of striatal neurons produced by environmental enrichment. A previous study has shown that wheel running increases striatal levels of Delta-Fos B in rats (Werme *et al*, 2002). Thus, it is possible that wheel running, which is a constant part of our EE, was, at least in part, responsible for increases in Delta-Fos B. Interestingly, a recent study has found that Delta-Fos B activation parallels and may be responsible for the increases in spine density subsequent to chronic cocaine administration (Lee *et al*, 2006). Given that EE increase striatal dendritic arborization (Kolb *et al*, 2003), Delta-Fos B may be involved in these structural modifications.

Elevations in Delta-Fos B levels are generally believed to be one of the mechanisms responsible to the sensitized reactions to cocaine (McClung and Nestler, 2003), whereas in our study, EE mice that had higher basal levels of Delta-Fos B showed reduced responses to cocaine. Although somewhat unexpected, these results are in agreement with the work of Hiroi *et al.* (1997) who found that the constitutive Fos B knock-out mice are more sensitive to the effects of cocaine than wild-type control. This supports the view that the influences of Delta-Fos B on subsequent behavioral, neurochemical, and molecular effects of addictive drugs depend on several factors, such as the duration of the increase in Delta-Fos B levels (McClung and Nestler, 2003) and the striatal type neurons where Delta-Fos B is actually increased (McClung *et al*, 2004).

Surprising findings of this study were that when cocaine was administered to EE mice, it decreased, instead of increasing, Delta-Fos B striatal levels indicating that EE induces a form of neuronal plasticity that dramatically alters the effects of subsequent cocaine administration. To our knowledge, this is the first study showing that *in vivo* manipulations can induce decreases in striatal levels of Delta-Fos B. Interestingly, *in vitro* experiments have recently shown that the exceptional resistance of Delta-Fos B to metabolic degradation appears to depend on the phosphorylation of a serine residue (Ulery *et al*, 2006). Although we could not test this hypothesis, it is intriguing to speculate that in EE mice repeated administration of cocaine alters the activity of kinases or phosphatases and results in the dephosphorylation of Delta-Fos B.

In conclusion, in this study we demonstrate that environmental stimulation during early stages of life provides protection against the abuse-related effects of cocaine. This protection appears to be mediated by a reduced activation of striatal neurons by cocaine and by dramatic changes in the neuronal adaptations usually observed after repeated cocaine administration. These results support the hypothesis that positive life experiences during critical periods, such as the adolescence decrease the sensitivity to drugs of abuse and the vulnerability to addiction.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare that except for finance received from their primary employer no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interests.

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