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Effects of Long-Term Acetyl-L-Carnitine Administration in Rats—II: Protection Against the Disrupting Effect of Stress on the Acquisition of Appetitive Behavior

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Long-term acetyl-L-carnitine (ALCAR) administration prevents the development of escape deficit produced by acute exposure to unavoidable stress. However, it does not revert the escape deficit sustained by chronic stress exposure. Rats exposed to chronic stress show a low dopamine (DA) output in the nucleus accumbens shell (NAcS) and do not acquire an appetitive behavior sustained by the earning of vanilla sugar (VS) made contingent on the choice of one of the two divergent arms of a Y-maze (VS-sustained appetitive behavior, VAB), while control rats consistently do. The present study shows that ALCAR treatment in rats exposed to a 7-day stress protocol prevented a decrease in DA output in the NAcS and medial prefrontal cortex (mPFC) of rats, and that it strengthened the DA response to VS consummation in the same two areas. Moreover, rats treated with long-term ALCAR or exposed to chronic stress while treated with ALCAR acquired VAB as efficiently as control rats. Moreover, VAB acquisition in stressed rats treated with ALCAR coincided with the reversal of the deficits in escape and in dopaminergic transmission in the NAcS. Thus, repeated ALCAR treatment preserved the DA response to VS in chronically stressed rats and this effect appeared to be predictive of the rat's competence to acquire VAB.
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INTRODUCTION

L-Carnitine has been described as a conditionally essential nutrient for humans required for the transport of long-chain fatty acids into the mitochondria. It also facilitates the removal, from the mitochondria, of excess short- and medium-chain fatty acids that accumulate during metabolism (Liu *et al*, 2002). Acetyl-L-carnitine (ALCAR) is the acetyl ester of carnitine, and both ALCAR and carnitine play a crucial regulatory role in fatty acid oxidation (Fritz, 1963; Bieber, 1988). Carnitine and ALCAR affect other cellular functions, including maintenance of key proteins and lipids of the mitochondria at sufficient levels and proper membrane orientation, for maximum energy production (Liu *et al*, 2002). ALCAR, like L-carnitine, is present in high concentration in the brain. ALCAR is more widely used than L-carnitine in animal research and clinical trials because, in aging or in conditions of disease, ALCAR is better absorbed and it crosses the blood–brain barrier more efficiently than L-carnitine (Kidd, 1999). Moreover, at the cellular level, ALCAR seems to be the active molecule in some metabolic pathways (Cha and Sachan, 1995; Swamy-Mruthinti and Carter, 1999). Several *in vitro* and *in vivo* studies have indicated that ALCAR is involved in different aspects of neuronal activity and a thorough review of its CNS action has been published (Calvani and Carta, 1991). ALCAR may prevent some of the neurochemical sequelae of stress exposure, as repeated ALCAR treatment prevents the stress-induced decrease in nerve growth factor binding in rat brain (Foreman *et al*, 1995), and it counteracts the increase in β-endorphine induced by repeated exposure to stress (Bidzinska *et al*, 1993). ALCAR improves the feedback control of hypothalamus–pituitary–adrenal axis response to stress, at least in aged rats (Angelucci and Ramacci, 1989; Patacchioli *et al*, 1989).

We have shown that in rats a 7-day ALCAR treatment produces a steady increase in dopamine (DA) and serotonin (5-HT) output in the nucleus accumbens shell (NAcS), and it prevents the development of the avoidance deficit induced by exposure to acute unavoidable stress (Tolu *et al*, 2002). Antidepressant drugs, such as imipramine, fluoxetine, and clomipramine, show a similar preventive effect on the development of stress-induced behavioral sequelae (Gambarana *et al*, 2001). Moreover, they also revert a condition of chronic escape deficit sustained by repeated

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stress exposure (Gambarana *et al*, 2001), and we consider this reversal to be crucial to the definition of antidepressant activity. ALCAR administered daily for 3 weeks to rats exposed to chronic stress failed to modify the escape deficit condition (Tolu *et al*, 2002); thus, it cannot be defined as an antidepressant. The failure of ALCAR to counteract the effect of chronic stress on avoidance indicates that a steadily increased DA and 5-HT output in the NAcS is not sufficient for preventing the development of avoidance deficit.

Chronic stress exposure not only induces an avoidance deficit, but it also disrupts the acquisition of an appetitive behavior sustained by a highly palatable food (vanilla sugar, VS; sustained appetitive behavior, VAB) in rats fed *ad libitum* (Ghiglieri *et al*, 1997). Moreover, it decreases DA and 5-HT output in the medial prefrontal cortex (mPFC) and NAcS (Gambarana *et al*, 1999a; Mangiavacchi *et al*, 2001). However, no apparent correlation exists between the stress-induced behavioral modifications and these neurochemical effects. In fact, rats that have acquired VAB and are then exposed to chronic stress develop an avoidance deficit while retaining VAB (Ghiglieri *et al*, 1997); moreover, they show a DA output in the NAcS similar to that of control rats, and higher than that of chronically stressed rats (Masi *et al*, 2001). Furthermore, long-term treatment with lithium induces an avoidance deficit and a significant decrease in DA output in the NAcS (Gambarana *et al*, 1999b), but it does not interfere with VAB acquisition (Masi *et al*, 2000). Thus, no correlation can be demonstrated between basal DA output and VAB acquisition, as both long-term stress exposure and lithium treatment significantly reduce basal DA output in mesolimbic areas (Gambarana *et al*, 1999a, b; Mangiavacchi *et al*, 2001), but only lithium-treated rats maintain the competence to acquire VAB (Masi *et al*, 2000). However, satiated rats fed a palatable food show an increased DA release in the NAcS (Martel and Fantino, 1996; Ghiglieri *et al*, 1997) and in the mPFC, although rapid habituation to this effect selectively develops in the NAcS (Bassareo and Di Chiara, 1997, 1999a). Thus, we hypothesized that the acute dopaminergic response to VS consumption in the NAcS and/or mPFC could be predictive of the capacity of satiated rats to learn VAB, and ALCAR treatment seemed to be a useful tool for testing this hypothesis. In order to further investigate the relation between DA output in mesolimbic areas and the response to noxious or pleasurable stimuli in rats exposed to unavoidable stress, we studied whether:

- repeated ALCAR administration, which by it self increases DA output, would counteract the effect of repeated exposure to unavoidable stress on DA output in the NAcS and mPFC;
- rats exposed to unavoidable stress and treated or not treated with ALCAR would show a dopaminergic response to palatable food consumption;
- repeated ALCAR administration would sustain the ability to learn an appetitive behavior in stressed rats.

METHODS

Animals

Experiments were carried out on male Sprague–Dawley rats (Charles River, Calco, Italy) weighing 125–150 g at their arrival in the vivarium. Animals were housed five per cage ($59 \times 38.5 \times 20\,cm^3$) for the entire duration of the experiments and they were moved to a different cage or apparatus only for the time required for the behavioral manipulation. They were kept in an environment maintained at a constant temperature and humidity, with free access to food and water. A 12 h inverted light/dark cycle (7:00 am lights off, 7:00 pm on) was used. Experiments were carried out from 9:00 am to 5:00 pm under a red light and controlled noise conditions in a testing room separated from and adjacent to the main animal room, under the same conditions of temperature and humidity. Rats were allowed at least 1 week of habituation to the animal colony and when experimental procedures began they weighed 200–225 g. The procedures used in this study are in strict accordance with the European legislation on the use and care of laboratory animals (EEC no. 86/609), with the guidelines of the National Institutes of Health on the use and care of laboratory animals, and had the approval of the local Ethics Committee.

Chronic Escape Deficit

Apparatus. A dark Plexiglas cage ($30 \times 60 \times 30\,cm^3$) with a floor fitted with stainless-steel rods spaced 1 cm apart was divided into two equal chambers by a dark partition with a $10 \times 10\,cm^2$ sliding door. One compartment was connected to a S48 Grass stimulator (Grass Instrument, Astro-Med Inc., West Warwick, RI, USA) (electrified chamber), while the other was disconnected from it (neutral chamber).

Procedure. The experimental procedure, previously described in detail (Gambarana *et al*, 2001), consisted of exposure to unavoidable stress (pretest) followed by an escape test. Briefly, rats were immobilized with a flexible wire-net, an electrode was applied to the distal third of the tail, and about 80 electric shocks (1 mA × 5 s, 1 every 30 s) were administered; 24 h later, rats were tested in a shock-escape paradigm in the Plexiglas cage. The number of escapes out of 30 trials was recorded. Rats selected on the basis of their failure to escape (0–3 escapes/30 trials), starting 48 h after the escape test: (1) were restrained for 10 min; (2) received 10 min of restraint plus four unavoidable shocks, 48 h after (1); (3) spent 20 min in the cage where the unavoidable shock had previously been administered, 48 h after (2). By repeating this procedure on alternate days, the escape deficit can be maintained in all rats (De Montis *et al*, 1995). No significant differences in the amount of daily food and water consumption or in the curve of body weight increase was ever observed between control and chronically stressed rats (data not shown).

Induction of VS-Sustained Appetitive Behavior (VAB)

Apparatus. Two dark Plexiglas boxes (box 1 and 2) were separated by a $10 \times 10\,cm^2$ sliding door. Box 2 formed the straight arm of a Y-maze ($15 \times 40 \times 20\,cm^3$ for each of the three arms). A VS pellet used as a reinforcer was placed at the end of one of the two divergent arms of the maze. VS pellets were made daily: standard food pellets were crushed by mortar and pestle, the fragments dampened with water

and rolled in VS to obtain regular pellets weighing approximately 150 mg.

Training procedure. The experimental procedure was previously described in detail (Ghiglieri *et al*, 1997). The day before the first training session, rats were allowed a first run in the Y-maze with one of the two arms closed. Each animal was placed in box 1 and 10 s later a 5 s cue-light signaled the opening of the sliding door. Rats were given 2 min to enter box 2 and to reach the end of the open arm where a VS pellet was earned. Training sessions began 24 h later.

1. Training sessions 1–3: The rat was placed in box 1 and the cue-light signaled the opening of the sliding door. If the rat did not enter box 2 within 60 s, it was returned to the home cage for 15 min. If it entered box 2, it was allowed 60 s to reach the end of one of the two diverging arms. Either the right or the left arm was designated correct, balanced among the animals. If the empty arm was chosen, the rat was returned to the home cage for 15 min before the next trial. When the baited arm was chosen, the rat was allowed to consume the VS pellet and then returned to the home cage for 15 min before the next trial.
2. The two time periods (time to leave box 1 and time to reach the end of an arm) were progressively reduced throughout training sessions and at session 10 they were fixed at 10 and 20 s, respectively.

One training session was administered every other day. Each rat underwent a total of 10 complete trials for each session, at 15 min intervals. A trained rat consistently made 6–8 correct runways out of 10 trials at each session, and this ratio of correct responses was reached within 10 sessions. In the remaining 2–4 trials rats did not necessarily reach the end of the nonbaited arm. At the end of the training, when a steady level of correct responding was obtained, rats were tested in the Y-maze according to the protocol described in (2) and their final score (number of correct runways out of 10 trials) was recorded.

Microdialysis Procedure

Anaesthetized rats (pentobarbital 50 mg/kg, scopolamine 0.4 mg/kg, i.p.) were placed in a stereotaxic instrument and two concentric vertical probes were lowered into the NAcS (AP +1.7 mm, L $\pm$ 1.2 mm, V -8.0 mm) and the mPFC (AP +3.7 mm, L $\pm$ 0.7 mm, V -5.0 mm), according to Paxinos and Watson (1986). Concentric microdialysis probes were made from semipermeable dialysis tubing (ID: 0.22 mm; OD: 0.31 mm; AN 69, Hospal, Bologna, Italy). The length of the permeable portion of the membrane was 2.0 mm for the NAcS, and 3.0 mm for the mPFC. The probes were fixed to the skull with stainless-steel screws and dental cement, and the skin was sutured. After surgery, the animals were housed individually in a microdialysis Plexiglas box ($20 \times 30 \times 30\,cm^3$) with a grid floor and an open top, and 24 h of recovery and habituation to the chamber were allowed before the beginning of microdialysis. On the day of the experiment, Ringer solution (147 mM NaCl, 2.2 mM $CaCl_2$, 4 mM KCl) was infused at a flow rate of 1 µl/min through the probe. After a 2 h equilibration period, dialysate samples were collected every 15 min (NAcS) or every 25 min (mPFC). The 4–5 consecutive samples that showed variations in DA concentrations $\leqslant$10% were utilized to estimate basal levels.

Dialysate samples were immediately analyzed by reverse-phase High Performance Liquid Chromatography (HPLC) with electrochemical detection. DA was eluted on a C-18 reverse phase column (Supelco LC18 DB). The detector was an ESA Coulochem II with a 5014 analytical cell. The potential of the first electrode was set at +175 mV, and that of the second electrode at -175 mV. The mobile phase consisted of an aqueous solution containing: 33 mM NaH_2PO_4, 0.1 mM Na_2EDTA, 1 mM sodium dodecyl sulfate, 20% methanol (vol/vol) and 15% acetonitrile (vol/vol), pH 5.7. A flow-rate of 1.0 ml/min was used. Data were taken by PC using EZChrom 6.6 software (Scientific Software Inc., San Ramon, CA, USA) and quantified based on peak area by comparison with a standard curve run before and after each experiment.

At the end of the experiment, rats were killed to verify probe placement. Microdialysis data was utilized only when the correct placement of the probes had been microscopically confirmed on cresyl violet-stained brain sections.

Drugs

ALCAR and cocaine were dissolved in 0.9% saline and injected in a volume of 1 ml/kg rat body weight. Pentobarbital was dissolved in a mixture of 12% ethanol, 38% propylene glycol, 50% deionized/distilled water (vol/vol) and injected in a volume of 4 ml/kg rat body weight. Scopolamine was dissolved in deionized/distilled water. All chemicals were purchased from commercial sources; cocaine was purchased from SALARS (Como, Italy). ALCAR was donated by Sigma-Tau (Pomezia, Italy).

Statistical Analysis

Statistical analyses were performed on commercially available software (Instat 2.01 for Macintosh, GraphPad software Inc., San Diego, CA, USA). All data are expressed as mean $\pm$ SEM. Comparisons were made by one-way analysis of variance (ANOVA), followed by *post hoc* Bonferroni test, when applicable ($p<0.05$). The increases in DA levels within each experimental group after the first and second VS presentation, and the number of escapes before and after Y-maze training were compared by paired *t*-test.

RESULTS

Experiment 1: Extraneuronal DA Output in the NAcS and mPFC in Rats Exposed to Stress for 7 days, with and without ALCAR Treatment

In order to study whether the ALCAR-induced increase in DA output could interfere with the disrupting effect of stress on basal DA output and on the dopaminergic response to palatable food consumption, 40 rats were divided into four groups of 10 animals each (Table 1). Two groups were handled daily for 2 days, and on day 3 they started treatment with saline (1 ml/kg, *Control*) or ALCAR (10 mg/kg, *ALCAR*) i.p. twice a day for 7 days; two groups

were exposed to the pretest and escape test, then to the chronic stress protocol for 7 days while injected with saline (1 ml/kg, *Stress*) or ALCAR (10 mg/kg, *Stress+ALCAR*) i.p. twice a day. At 2 days after the last stress exposure and drug treatment, all rats were implanted with microdialysis probes in the NAcS and mPFC, and they were dialyzed the following day.

ALCAR treatment prevented the stress-induced decrease in DA basal levels in the NAcS. Analysis of data by ANOVA showed a significant difference between basal extraneuronal DA levels in experimental groups ($F_{28,31} = 19.31$, $p < 0.001$). *Post hoc* Bonferroni's test indicated a reduction in DA basal levels in the *Stress* group compared to the *Control* and *Stress+ALCAR* groups ($p < 0.01$), and the *ALCAR* group ($p < 0.001$) (Figure 1a). In the mPFC, analysis of data by ANOVA showed a significant difference between basal extraneuronal DA levels in the different experimental groups ($F_{28,31} = 7.509$, $p < 0.001$). Bonferroni's test indicated significantly lower DA levels in the *Stress* group compared to the *Stress+ALCAR* group ($p < 0.01$, Figure 1b).

After the assessment of DA baseline values, each rat was presented with a small tray containing five VS pellets and given 5 min to consume them. ALCAR treatment prevented the negative effect of unavoidable stress exposure on DA response to palatable food. While rats in the *Control*, *ALCAR*, and *Stress+ALCAR* groups voraciously ate all the pellets in less than 5 min, rats in the *Stress* group showed less interest in the food and only six out of 10 of them ate at least some of the pellets within 5 min. In the *Stress+ALCAR* rats, the consumption of VS pellets was accompanied by an increased motility and stereotypies that lasted about 15 min. Microdialysis sample collection continued during and after food consumption. Extraneuronal DA levels increased after eating VS; when they returned to baseline, a second meal of VS pellets was presented and microdialysis samples continued to be collected. The four groups of rats showed a behavioral response to the second VS pellet presentation similar to that observed after the first presentation. In the *Stress* group, analysis of DA levels was performed only in rats that consumed VS pellets ($n = 6$). Because of the significant differences between the groups in the basal values of extraneuronal DA, variations in DA levels after VS consumption were not calculated as percentage increases compared to basal values. DA output was calculated as the sum of the absolute amounts of the monoamine (measured values minus the mean basal value) in each of the four samples collected following VS consumption. In the NAcS, analysis by ANOVA indicated a significant difference between groups in DA release after the first VS consumption ($F_{26,29} = 117.1$, $p < 0.001$). Bonferroni's test demonstrated that in the *Control*, *ALCAR*, and *Stress+ALCAR* groups, DA output was significantly higher than in the *Stress* group ($p < 0.001$ for all comparisons), with significantly higher DA values in the *Stress+ALCAR* group than in the *Control* and *ALCAR* groups ($p < 0.001$ for both comparisons) (Figure 2a). After the second VS consump-

Table 1 Experimental Design of Experiment 1: Extraneuronal DA Output in the NAcS and mPFC in Rats Exposed to Stress for 7 days, with and without ALCAR Treatment

Group	Day 1	Day 2	Days 3–9	Day 11	Day 12
Control	Handling	Handling	Saline	Surgery	Microdialysis
ALCAR	Handling	Handling	ALCAR	Surgery	Microdialysis
Stress	Pretest	E test	Stress+saline	Surgery	Microdialysis
Stress+ALCAR	Pretest	E test	Stress+ALCAR	Surgery	Microdialysis

E test: escape test.

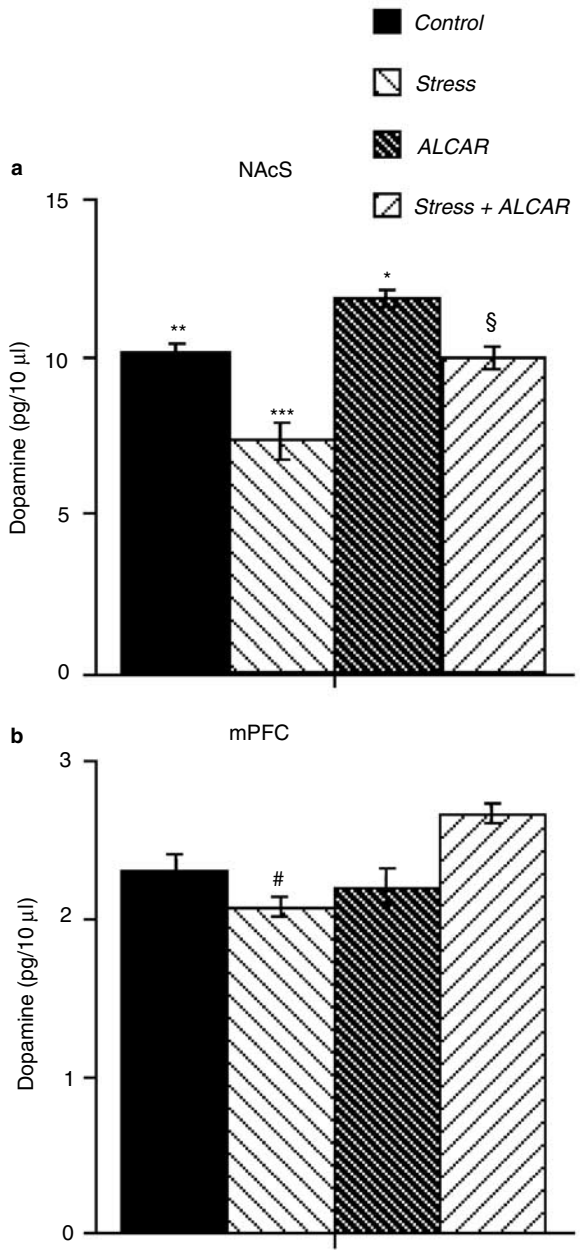

Figure 1 Extraneuronal DA levels in the NAcS (a) and mPFC (b) in rats exposed to chronic stress for 7 days, with or without concomitant ALCAR treatment. Rats were administered the sequence of pretest and escape test and then began treatment with saline (1 ml/kg), or ALCAR (10 mg/kg) i.p. twice a day while exposed to chronic stress procedure for 7 days. Rats were implanted with microdialysis probes in the NAcS and mPFC, 24 h after the last stress exposure and drug treatment and dialyzed the following day. Values represent the mean ± SEM of DA levels. *Significantly different from DA levels in the *Control* group ($p < 0.05$). **Significantly different from DA levels in the *Stress* group ($p < 0.01$). ***Significantly different from DA levels in the *ALCAR* and *Stress+ALCAR* groups ($p < 0.001$). §Significantly different from DA levels in the *ALCAR* group ($p < 0.01$). #Significantly different from DA levels in the *Stress+ALCAR* group ($p < 0.01$).

tion, analysis by ANOVA showed a significant difference in DA release between groups ($F_{26,29} = 126.9$, $p < 0.001$). Bonferroni's test demonstrated that DA release was significantly higher in the *ALCAR* and *Stress+ALCAR* groups than in the Stress group ($p < 0.01$ and $p < 0.001$, respectively), and it was significantly higher in the *Stress+ALCAR* group than in the *Control* and *ALCAR* groups ($p < 0.001$ for both comparisons) (Figure 2c). Habituation to the second presentation of VS pellets was present in the *Control* and *ALCAR* groups, as a comparison of DA release after the first and second VS meal revealed that the second response was significantly lower than the first ($p < 0.001$ for both comparisons, paired *t*-test). In the mPFC, ANOVA indicated a significant difference in DA release between groups after the first VS consumption ($F_{26,29} = 46.45$, $p < 0.001$). Bonferroni's test demonstrated that DA output was significantly lower in the *Stress* group than in the *Control* and *Stress+ALCAR* groups ($p < 0.01$ and $p < 0.001$, respectively); moreover, DA values were also significantly higher in the *Stress+ALCAR* group than in the *Control* and *ALCAR* groups ($p < 0.001$ for both comparisons) (Figure 2b). After the second VS consumption, analysis by ANOVA showed a significant difference in DA release between groups ($F_{26,29} = 65.77$, $p < 0.001$). Bonferroni's test demonstrated that DA output was significantly lower in the *Stress* group than in the *Control*, *ALCAR*, and *Stress+ALCAR* groups ($p < 0.001$, $p < 0.01$, and $p < 0.001$, respectively); in the *Stress+ALCAR* group DA output was significantly higher than in the *Control* and *ALCAR* groups ($p < 0.001$ for both comparisons) (Figure 2d). No statistically significant differences were observed between DA release after the first and second VS meal in any of the groups.

When baseline levels were reached, cocaine (5 mg/kg, i.p.) was administered and four samples were collected. Because of the significant differences between the groups in the basal values of extraneuronal DA, variations in DA levels after cocaine administration were not calculated as percentage

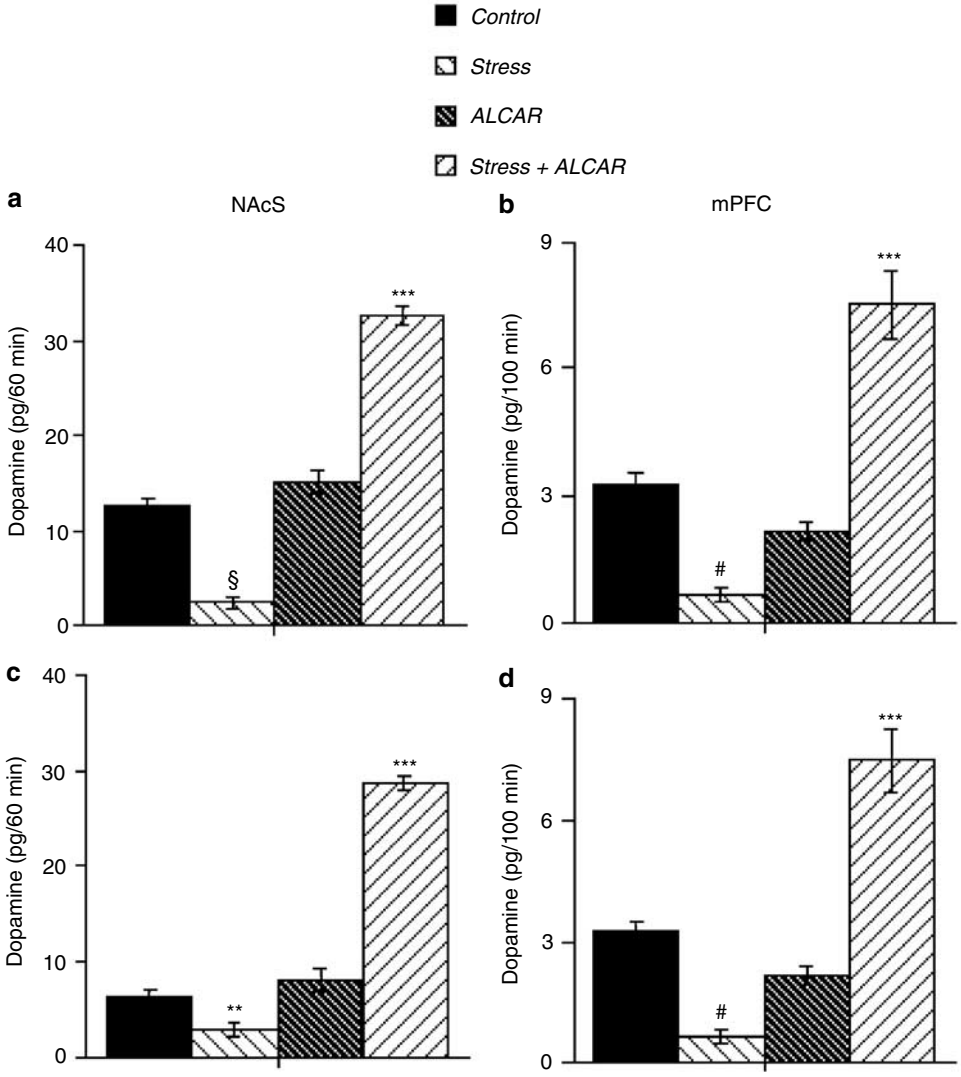

Figure 2 DA output in the NAcS (a, c) and mPFC (b, d) in response to VS pellet consumption. Rats underwent the protocol described in Figure 1. When baseline levels were assessed, rats were presented twice with five VS pellets. Values represent the mean ± SEM of the sums of four samples for each rat collected after the first (a, b) and second VS meal consumption (c, d) minus the mean basal DA level. ***Significantly different from DA output in the *Control*, *ALCAR*, and *Stress* groups ($p < 0.001$). **Significantly different from DA output in the *ALCAR* group ($p < 0.01$). §Significantly different from DA output in the *Control* and *ALCAR* groups ($p < 0.001$). #Significantly different from DA output in the *Control* group ($p < 0.001$).

increases compared to basal values, but as absolute increase (DA concentration in each sample collected following cocaine administration minus the basal value). In the NAcS, cocaine administration produced a significant variation in DA levels in all groups (repeated measures ANOVA, $p<0.001$ in all four groups: *Control*, $F_{15,23}=138.8$; *Stress*, $F_{15,23}=16.28$; *ALCAR*, $F_{27,39}=196.2$; *Stress+ALCAR*, $F_{27,39}=142.0$) (Figure 3a). In particular, Bonferroni's test demonstrated that cocaine administration induced in all groups a significant elevation of extraneuronal DA levels after 15 min ($p<0.001$ for all comparisons). However, when peak increases in DA levels were compared between groups they were significantly different (ANOVA, $F_{28,31}=89.54$, $p<0.001$). The maximum DA increase was lower in the *Stress* group than in the *Control*, *ALCAR*, and *Stress+ALCAR* groups ($p<0.001$ for the three comparisons, Bonferroni's test), and it was higher in the *ALCAR* group than in the *Control* and *Stress+ALCAR* groups ($p<0.001$ for both comparisons, Bonferroni's test) (Figure 3a). In the mPFC, cocaine administration produced a significant variation in DA levels in all groups (repeated measures ANOVA, $p<0.001$ in all four groups: *Control*, $F_{15,23}=47.73$; *Stress*, $F_{15,23}=39.75$; *ALCAR*, $F_{24,35}=101.6$; *Stress+ALCAR*, $F_{27,39}=84.78$) (Figure 3b). In particular, Bonferroni's test demonstrated that cocaine administration induced in all groups a significant elevation of extraneuronal DA levels after 25 min ($p<0.001$ for all comparisons). However, when peak increases in DA levels were compared between groups they were significantly different (ANOVA, $F_{27,30}=20.05$, $p<0.001$). The maximum DA increase was lower in the *Stress* group than in the *Control*, *ALCAR*, and *Stress+ALCAR* groups ($p<0.01$ compared *Control* group, $p<0.001$ compared to *ALCAR*, and *Stress+ALCAR* groups, Bonferroni's test), and it was higher in the *ALCAR* and *Stress+ALCAR* groups than in the *Control* group ($p<0.05$ for both comparisons, Bonferroni's test) (Figure 3b). DA accumulation was also calculated as the sum of the absolute amounts of the monoamine in each of the four samples collected following cocaine administration. In the NAcS, analysis of data by ANOVA indicated a significant difference between

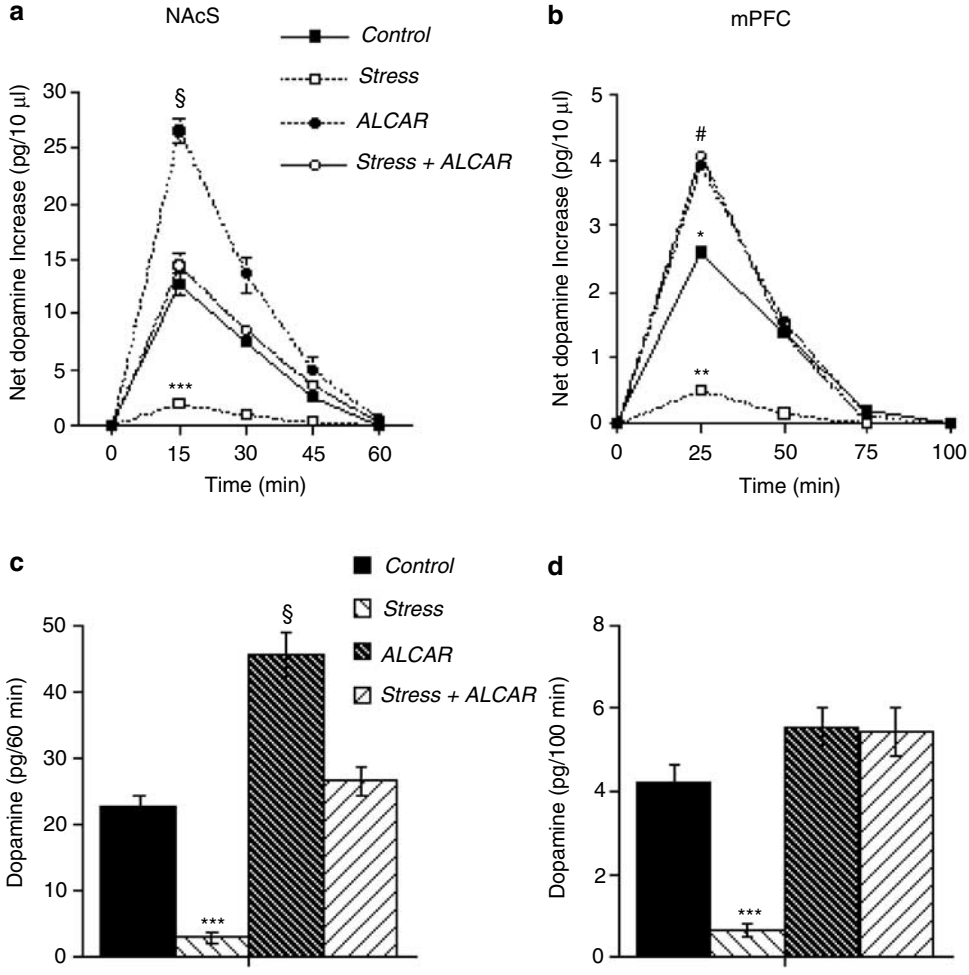

Figure 3 DA output in the NAcS (a, c) and mPFC (b, d) in response to acute cocaine administration. Rats underwent the protocol described in Figure 1. When baseline levels were reached, after VS consumption, rats were injected with cocaine (5 mg/kg, i.p.). (a, b) Values represent the net increase in DA levels (sample concentration minus basal value). (c, d) Values represent the mean ± SEM of the sums of four samples for each rat collected after cocaine administration, minus the mean basal DA level. ***Significantly different from DA output in the *Control*, *ALCAR*, and *Stress+ALCAR* groups ($p<0.001$). **Significantly different from DA output in the *Control* group ($p<0.01$). *Significantly different from DA output in the *ALCAR* and *Stress+ALCAR* groups ($p<0.05$). §Significantly different from DA output in the *Control* and Stress+*ALCAR* groups ($p<0.001$). #Significantly different from DA output in the *Stress* group ($p<0.001$).

groups (ANOVA, $F_{28,31} = 42.86$, $p < 0.001$). Bonferroni's test demonstrated that total DA accumulation was significantly reduced in the *Stress* group compared to the *Control*, *ALCAR*, and *Stress+ALCAR* groups ($p < 0.001$ for all comparisons) (Figure 3c). Moreover, DA accumulation was significantly higher in the *ALCAR* group compared to the *Control* and *Stress+ALCAR* groups ($p < 0.001$ for both comparisons) (Figure 3a). In the mPFC, analysis by ANOVA showed a significant difference between groups (ANOVA, $F_{26,29} = 18.43$, $p < 0.001$). Bonferroni's test demonstrated that DA accumulation was significantly reduced in the *Stress* group compared to the *Control*, *ALCAR*, and *Stress+ALCAR* groups ($p < 0.001$ for all comparisons) (Figure 3d). That is, ALCAR administration prevented the stress-induced decrease in DA output in the NAcS and mPFC.

Experiment 2: Effect of Long-Term ALCAR Treatment on VAB Acquisition and Dopaminergic Output in the NAcS of Rats Exposed to Chronic Stress

A total of 50 rats were divided into five groups of 10 animals each (Table 2). Rats in groups 1 and 2 were injected with saline (1 ml/kg, i.p.) twice a day and handled daily for the duration of the experimental protocol (*Control*), or they were trained in the Y-maze (*VAB*). Group 3 was administered ALCAR (10 mg/kg, i.p.) twice a day for 8 days, and then trained in the Y-maze while continuing ALCAR treatment (*ALCAR+VAB*). Groups 4 and 5 were exposed to the pretest, tested 24 h later for escape, and then exposed to the chronic stress protocol for 7 days while treated with saline (1 ml/kg, i.p., *Stress+VAB*) or ALCAR (10 mg/kg, i.p., *Stress+ALCAR+VAB*).

Effect of Long-Term ALCAR on the Acquisition of VAB in Previously Stressed Rats

After 1 week of treatment plus exposure to stress or handling, rats in the *Stress+VAB*, and *Stress+ALCAR+VAB* groups presented a clearcut escape deficit (Table 3). Analysis of the number of escapes by ANOVA, followed by Bonferroni's test, demonstrated a significantly lower score in the *Stress+VAB*, and *Stress+ALCAR+VAB* groups compared to the *VAB* and *ALCAR+VAB* groups ($F_{28,31} = 286.58$, $p < 0.001$; Bonferroni's test $p < 0.001$ for all four comparisons).

On day 9, 24 h after the escape test, groups 2–5 began training in the Y-maze while continuing their treatment. At the end of the training protocol (day 30), performance in the Y-maze was assessed. Rats exposed to chronic stress (*Stress+VAB*) did not acquire the appetitive behavior (Figure 4). Rats treated with ALCAR during stress exposure and during Y-maze training plus stress exposure acquired appetitive behavior as efficiently as the rats in the *VAB* group (Figure 4). Analysis of data by ANOVA showed a significant difference between group scores ($F_{36,39} = 29.796$, $p < 0.001$). Bonferroni's test demonstrated that the number of correct choices was significantly lower in the *Stress+VAB* group than in the *VAB*, *ALCAR+VAB*, and *Stress+ALCAR+VAB* groups ($p < 0.001$ for all comparisons) (Figure 4).

The day after the Y-maze test (day 31) all groups were again tested for escape. Rats in the *Stress+VAB* group presented a clearcut escape deficit, whereas animals treated with ALCAR showed a complete reversal of the stress-induced avoidance deficit (Table 3). Analysis of data by ANOVA followed by Bonferroni's test demonstrated a significantly lower number of escapes in the *Stress+VAB* group compared to the *VAB*, *ALCAR+VAB*, and *Stress+ALCAR+VAB* groups ($F_{28,31} = 155.50$, $p < 0.001$; Bonferroni's test $p < 0.001$ for all three comparisons).

Extraneuronal DA Output in the NAcS in Stressed Rats Trained in the Y-Maze

At 2 days after the escape test, rats from all groups were implanted with microdialysis probes in the NAcS and were dialyzed 24 h later (day 34). Rats trained in the Y-maze while exposed to stress showed a DA output in the NAcS significantly lower than that of control animals, and ALCAR treatment completely antagonized this effect. Analysis of data by ANOVA showed a significant difference between the basal extraneuronal DA levels in the different experimental groups ($F_{35,39} = 108.42$, $p < 0.001$). Bonferroni's test demonstrated a significant decrease in DA basal levels in the *Stress+VAB* group compared to the *Control*, *VAB*, *ALCAR+VAB*, and *Stress+ALCAR+VAB* group values ($p < 0.001$ for all comparisons). Moreover, rats in the *ALCAR+VAB* group had significantly higher basal levels of extraneuronal DA than those of the *Stress+ALCAR+VAB* group ($p < 0.01$), while they were significantly lower than those of the *VAB* group ($p < 0.001$) (Figure 5a). DA accumulation was calculated as described above as the sum of the absolute amounts of the monoamine in each of the four samples collected following cocaine administration (5 mg/kg, i.p.). Analysis of data by ANOVA indicated a significant difference between groups (ANOVA, $F_{35,39} = 615.84$, $p < 0.001$). Bonferroni test demonstrated that DA accumulation was lower in the *Stress+VAB* group than in the *Control*, *VAB*, *ALCAR+VAB*, and *Stress+ALCAR+VAB* groups ($p < 0.001$ for all comparisons) (Figure 5b). Moreover, DA accumulation was significantly higher in

Table 2 Experimental Design of Experiment 2: Effect of Long-Term ALCAR Treatment on VAB Acquisition and Dopaminergic Output in the NAcS of Rats Exposed to Chronic Stress

Group	Day 2	Day 1	Days 1–7	Day 8	Days 9–29	Day 30	Day 31	Day 34
Control			Saline+handling		Saline+handling			Dialysis
VAB			Saline+handling	E test	Saline+Y-maze training	VAB test	E test	Dialysis
ALCAR+VAB			ALCAR+handling	E test	ALCAR+Y-maze training	VAB test	E test	Dialysis
Stress+VAB	Pretest	E test	stress+saline	E test	Stress+saline+Y-maze training	VAB test	E test	Dialysis
Stress+ALCAR+VAB	Pretest	E test	stress+ALCAR	E test	Stress+ALCAR+Y-maze training	VAB test	E test	Dialysis

E test: escape test.
VAB test: test in the Y-maze to verify appetitive behavior acquisition.

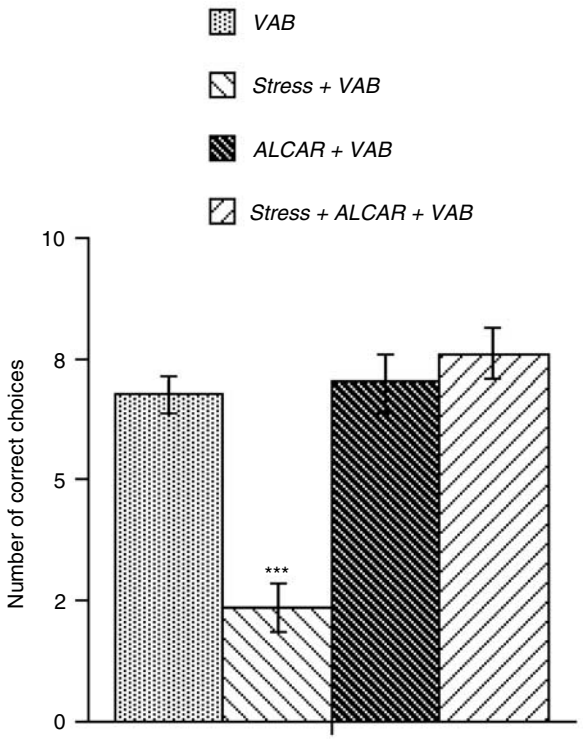

Figure 4 Number of correct choices scored by rats in *VAB*, *Stress+VAB*, *ALCAR+VAB*, and *Stress+ALCAR+VAB* groups at the end of Y-maze training. Rats were exposed to chronic stress procedure while treated with saline (1 ml/kg) or ALCAR (10 mg/kg) i.p. twice a day (*Stress+VAB* and *Stress+ALCAR+VAB* groups). After 1 week of treatment, the two groups of stressed rats and a group of control rats (*VAB*) began training in the Y-maze. After 10 training sessions rats were tested for performance in the Y-maze. Scores are expressed as mean ± SEM of the number of correct choices out of 10 trials. ***Significantly different from the score of the *VAB*, *ALCAR+VAB*, and *Stress+ALCAR+VAB* groups ($p<0.001$).

Table 3 Effects of Long-Term ALCAR Treatment on the Escape Response During Exposure to Chronic Stress and Y-Maze Training

	Number of escapes	
Group	**After 8 days**	**After stress+Y-maze training**
VAB	25.4 ± 0.8***	24.4 ± 1.1
ALCAR+VAB	22.8 ± 1.2***	22.4 ± 0.9
Stress+VAB	1.4 ± 0.5	1.4 ± 0.5**
Stress+ALCAR+VAB	1.0 ± 0.5	22.6 ± 0.8§

After exposure to the sequence of pretest and escape test, rats began treatment (ALCAR, 10 mg/kg or saline, 1 ml/kg, i.p. twice a day) plus exposure to chronic stress. After 8 days they were tested for escape. Then, rats resumed treatment plus exposure to chronic stress and began training in the Y-maze for the acquisition of VAB. After 3 weeks of training, rats were tested again for escape. Values are the mean ± SEM of the number of escapes. ***$p<0.001$ *vs* score of the *Stress+VAB*, and *Stress+ALCAR+VAB* groups after 8 days of treatment (ANOVA with Bonferroni's test). **$p<0.001$ *vs* score of the *VAB*, *ALCAR+VAB*, and *Stress+ALCAR+VAB* groups after stress+Y-maze training (ANOVA with Bonferroni's test). §$p<0.001$ *vs* score of the *Stress+ALCAR+VAB* group after 8 days of treatment (paired *t*-test).

the *VAB* and *ALCAR+VAB* groups than in the *Control* and *Stress+ALCAR+VAB* groups ($p<0.001$ for all comparisons) (Figure 5b).

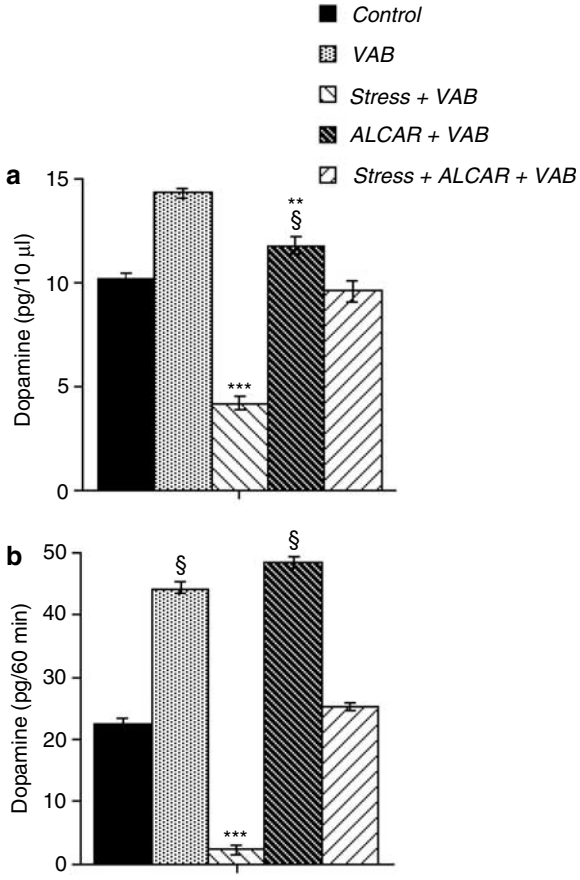

Figure 5 DA output in the NAcS in *Control*, *VAB*, *Stress+VAB*, *ALCAR+VAB*, and *Stress+ALCAR+VAB* groups. Rats underwent the experimental procedures described in Figure 4. Rats in the *Control* group were treated with saline (1 ml/kg, i.p. twice a day) and handled daily. All rats were implanted with a probe in the NAcS and microdialysis experiments were carried out 24 h after surgery. (a) Basal extraneuronal DA levels. Values represent the mean ± SEM of DA levels. (b) DA output in response to acute cocaine administration (5 mg/kg, i.p.). Values represent the mean ± SEM of the sums of four samples for each rat collected after cocaine administration, minus the mean basal DA level. ***Significantly different from values in the *Control*, *VAB*, *ALCAR+VAB*, and *Stress+ALCAR+VAB* groups ($p<0.001$). **Significantly different from values in the *Stress+ALCAR+VAB* group ($p<0.01$). §Significantly different from values in the *VAB* group ($p<0.001$). #Significantly different from values in the *Control* and *Stress+ALCAR+VAB* groups ($p<0.001$).

DISCUSSION

The present study shows that rats stressed for 7 days had only a meager interest in VS pellets and that only six out of 10 of them consumed the VS meal twice. In these rats, the increase in DA output after VS consumption did not reach significance in the NAcS, and it was significantly lower than that of *Control* rats in the mPFC. This condition is reminiscent of that of rats exposed to the chronic mild stress (CMS) procedure, a model of depression induced by chronic sequential exposure to a variety of mild stressors (Willner, 1997). CMS exposure produces decreased drinking of a sweetened solution (Willner, 1997) and reduced DA response to palatable food consumption in mesolimbic areas (Di Chiara and Tanda, 1997). Moreover, both these experimental models reduce the motivation elicited in rats by a palatable food, as chronic stress exposure prevents the

acquisition of VAB (Ghiglieri *et al*, 1997), and CMS inhibits palatable food-induced place preference (Papp *et al*, 1991). Repeated ALCAR administration consistently antagonized the effects of chronic stress exposure on VS consumption, as rats in the *Stress+ALCAR* group showed basal DA levels similar to those of *Control* rats, and, when presented twice with VS meals, they quickly ate all the pellets. VS consumption in these animals was accompanied by an increase in extraneuronal DA in the mPFC and NAcS two to three times higher than that observed in the *Control* and *ALCAR* groups, and this dopaminergic response was associated with a short-lived increase in motility and intense stereotypies. Moreover, no habituation was observed in the DA response to a second VS meal in the NAcS of *Stress+ALCAR* rats, at variance with *Control* and *ALCAR* rats. A lack of habituation in the NAcS dopaminergic response to repeated palatable food consumption was reported in food-deprived rats, as the state of necessity strengthens the dopaminergic response (Bassareo and Di Chiara, 1999b). Thus, the daily administration of ALCAR, initiated after exposure to pretest and escape test, counteracted the stress-induced decrease in DA output and strengthened the dopaminergic response to VS in the *Stress+ALCAR* rats. After cocaine administration, rats in the *Stress+ALCAR* group showed DA accumulation values in the mPFC and NAcS similar to those of *Control* rats, and lower than those of the *ALCAR* group, selectively in the NAcS. The acute inhibition of the monoamine transporter produced by cocaine, which does not interfere with monoamine release (Di Chiara and Imperato, 1988; Hurd and Ungerstedt, 1989), induces an extraneuronal accumulation of monoamines proportional to the amount taken up by nerve terminals, and it can be used as an indicator of monoaminergic neuronal activity (Gambarana *et al*, 1999a, b).

In the second part of the study, we tested whether the increase in DA output in the mPFC and NAcS observed in the *Stress+ALCAR* group in response to VS consumption was predictive of the capability of rats exposed to unavoidable stress and treated with ALCAR to learn VAB. Training in the Y-maze was initiated after 7 days of chronic stress exposure and ALCAR treatment, that is, in a condition of intense dopaminergic responsiveness to VS consumption (see the *Stress+ALCAR* group in experiment 1). At the end of the training procedure, *Stress+ALCAR+VAB* rats had acquired VAB and they performed as efficiently in the Y-maze as control animals (*VAB* group). Moreover, in contrast with *Stress+VAB* rats, when tested for escape they showed a complete reversal of the avoidance deficit. That is, ALCAR treatment enabled stressed rats to learn the appetitive behavior and, in turn, VAB acquisition seemed to strengthen the protective effect of ALCAR in the development of escape deficit. The finding that VAB acquisition coincided with the recovery of avoidance competence was not completely unexpected, as rats chronically treated with lithium show an avoidance deficit similar to that of chronically stressed rats (Gambarana *et al*, 1999b), which disappears as a consequence of VAB acquisition (Masi *et al*, 2000). Thus, repeated exposure to a hedonic stimulus, contingently rewarding a specific behavioral pattern, induces the development of a motivated behavior in rats chronically treated with lithium or exposed to stress while treated with ALCAR. The development of motivated behavior in these animals is accompanied by the reversal of the avoidance deficit sustained either by lithium or by stress exposure. When dialyzed at the end of Y-maze training, *VAB* and *ALCAR* rats showed a DA output twice as high as that of *Control* animals. The *Stress+VAB* group (ie rats that had been exposed to chronic stress for 30 days and had not acquired VAB) showed a DA output in the NAcS as low as that observed after the 7-day stress exposure in experiment 1. Rats in the *Stress+VAB+ALCAR* group had extraneuronal DA values similar to those of *Control* rats. Thus, VAB acquisition during ALCAR treatment not only reverted the chronic stress-sustained escape deficit, but it also prevented the decrease in DA output in the NAcS.

The role of DA in reward-related learning has been studied in different brain areas by using elaborated behavioral models and discrete brain lesions (Beninger, 1983; Di Chiara, 1995; Montague *et al*, 1996; Berridge and Robinson, 1998; Packard and Knowlton, 2002; Cardinal *et al*, 2002). Activation of the mesolimbic DA system, as quantified by electrophysiological, microdialysis, or voltammetric measures, can be triggered in animals by encounters with food, sex, drugs of abuse, and by secondary reinforcers of these incentives (Apicella *et al*, 1991; Blackburn *et al*, 1989; Fiorino *et al*, 1997; Kiyatkin and Gratton, 1994; Kiyatkin and Rebec, 1997; Kiyatkin and Stein, 1996; Mark *et al*, 1994; Mirenowicz and Schultz, 1996; Phillips *et al*, 1993; Schultz *et al*, 1992, 1997). As each of these incentives can induce the acquisition of instrumental behaviors aimed at earning and/or consuming it, an increase in DA release in the NAcS has been associated with reward and reinforcement (Beninger and Miller, 1998; Hernandez and Hoebel, 1988; Kelley and Delfs, 1991; Kiyatkin, 1995; Nader *et al*, 1997; Robbins *et al*, 1989; Robbins and Everitt, 1996; Di Chiara, 1998). DA release in the mesolimbic system was initially correlated with the hedonic properties of reward (Wise, 1982), a hypothesis presently revised to include the incentive-motivational component of reward (Wise, 1982; Berridge and Robinson, 1998; Di Chiara, 1995; Robbins and Everitt, 1992, 1996). In a model of conditioned taste aversion, DA release in the NAcS in response to an unfamiliar taste was correlated to the formation and consolidation of a gustatory short-term memory trace, the duration of which is crucial in associating a taste with a possible incoming postingestive change (Fenu *et al*, 2001). This effect is mediated by DA D_1 receptor stimulation, as the systemic or local administration of selective antagonists prevents the development of conditioned taste aversion when the conditioned taste stimulus is associated with an unconditioned aversive stimulus, such as i.p. lithium administration (Fenu *et al*, 2001). As the systemic administration of DA D_1 receptor antagonists also prevents the acquisition of palatable food-induced conditioned place preference (Acquas and Di Chiara, 1994), it was concluded that DA in the NAcS enables the association between a perceived taste and its biological outcome; thus, it facilitates the learning of either appetitive or aversive behaviors (Fenu *et al*, 2001). In searching for a role of mesolimbic DA in VAB acquisition, we observed that rats that have acquired VAB consistently show a significant increase in both the basal levels and the cocaine-induced accumulation of extraneuronal DA in the NAcS (Masi *et al*, 2001). When

VAB-competent rats are exposed to chronic stress while continuing the training, they maintain a high frequency of correct choices in the Y-maze (Masi *et al*, 2001). Moreover, they show a NAcS DA output similar to that of control animals, and higher than that of chronically stressed rats; yet they rapidly develop escape deficit (Masi *et al*, 2001). Thus, we hypothesized that DA output in the NAcS could play a role in the acquisition and maintenance of an appetitive behavior, while the expression of a deficit in avoidance appears to be independent of it (Masi *et al*, 2001). However, this hypothesis was in contrast with the observation that lithium-treated rats have a markedly reduced DA output in the NAcS, but they rapidly acquire VAB (Masi *et al*, 2000). Rats treated with ALCAR during chronic stress exposure showed a DA output in the mPFC and NAcS and a capacity to learn VAB unmodified compared to control animals, and a DA response to VS consumption significantly higher than control animals. These results underscore the relevance of the phasic dopaminergic response to VS consumption as a predictive element of a rat's competence to learn VAB; accordingly, only rats that showed an increased extraneuronal DA output in the mPFC and NAcS in response to VS consumption acquired VAB. Microdialysis experiments on the DA response to VS ingestion and training in the Y-maze were conducted in rats that had not been previously exposed to VS. We may, therefore, assume that DA output in the mesolimbic areas in response to the first VS meal increased only in the animals (*Control*, *ALCAR*, and *Stress+ALCAR*) competent to learn VAB. Thus, the increased release of mesolimbic DA in response to a novel palatable food seems to be a crucial step in the formation of a gustatory short-term memory trace that can be associated with an antecedent contingent behavior (present study) or with a contingent postingestive modification (Fenu *et al*, 2001). In our experimental conditions, the initially fortuitous daily repetition of a DA-dependent association between ingestion of palatable food and entering one of the Y-maze arms, slowly motivated rats to express an appetitive behavior aimed at earning VS. That is, DA response to VS consumption seems necessary to trigger a cascade of molecular mechanisms underpinning the associative process between a perceived taste and a closely antecedent behavioral experience, such as entering the baited arm. In conclusion, the present study shows that ALCAR, administered to stressed rats, preserved the DA response to palatable food consumption; thus, allowing stressed rats to learn VAB. Moreover, it supports the hypothesis that mesolimbic DA plays a central role in associative learning.

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