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Effects of Long-term Acetyl-L-carnitine Administration in Rats: I. Increased Dopamine Output in Mesocorticolimbic Areas and Protection toward Acute Stress Exposure

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Acetyl-L-carnitine (ALCAR) is the acetyl ester of carnitine that has been reported to be beneficial in depressive disorders and Alzheimer's disease. A 7-day administration of ALCAR in rats increased dopamine and serotonin output in the nucleus accumbens shell and it prevented the development of escape deficit produced by acute exposure to unavoidable stress. No tolerance developed to this protective effect, which appeared to be mediated by (1) the activation of 5-HT$_{1A}$ receptors, as it was antagonized by the administration of WAY100635 30 min before stress exposure; and (2) a process of neuronal plasticity dependent on NMDA receptor activity, as subcutaneous dizocilpine infusion during ALCAR treatment prevented the development of the protective effect on stress. Chronic stress exposure maintains an escape deficit condition that is reverted by a long-term treatment with antidepressants, but the same condition was not modified by long-term ALCAR administration. Thus, ALCAR cannot be defined as an antidepressant.

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KEY WORDS: *Acetyl-L-carnitine; Dopamine; Escape deficit; Nucleus accumbens shell; Serotonin; Stress*

Acetyl-L-carnitine (ALCAR) is the acetyl ester of carnitine and both carnitine and ALCAR play an essential regulatory role in fatty acid oxidation (Fritz 1963; Bieber 1988). ALCAR has a modulating influence on the structure, function and metabolism of cell membranes (Villa et al. 1988; Aureli et al. 1990; Arduini et al. 1993; Butterfield and Rangachari 1993), and on energy metabolism (Aureli et al. 1990; Rosenthal et al. 1992; Blokland et al. 1993; Liu et al. 1993; Prickaerts et al. 1995; Rao et al. 1997). There is increasing evidence that abnormalities in fatty acid and membrane phospholipid metabolism play a part in a wide range of neurodevelopmental and psychiatric disorders (Richardson and Ross 2000; Bell et al. 2000; Richardson and Puri 2000). ALCAR also inhibits apoptosis (Galli and Fratelli 1993), activates protein kinase C (Pascale et al. 1994), exerts anti-amnesic activity (Pascale et al. 1994), and improves performance in spatial learning tasks in aged rats (Ghirardi et al. 1988). In controlled studies, ALCAR has been reported to have beneficial effects in major depressive disorders and Alzheimer's disease, both of which are highly prevalent in the geriatric population (Bella et al. 1990; Garzya et al. 1990; Pettegrew et al. 1995, 2000). How-

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ever, the reported antidepressant activity of ALCAR in the elderly might not necessarily be explained in terms of altered fatty acid membrane composition. The data regarding endogenous ALCAR distribution in rat brain, as well as those on the entry of ALCAR through the blood brain barrier, offer an unsatisfactory rationale for the reported pharmacological effects. Total carnitine in different brain areas appears to be homogeneously distributed, and its concentration is about 2.5 times higher in the hypothalamus than in the other areas. ALCAR is present at 25–35%, long chain acyl-carnitine at 10–15%, and free carnitine at 45–60% (Bresolin et al. 1982). Only a very small amount of [^{3}H]-acetyl-carnitine was found in the brain after a pulse i.v. injection with a tracer dose (Farrel et al. 1986). Furthermore, a PET study in primates and humans on the brain localization of ALCAR, labeled with [^{11}C] in different positions and pulse injected i.v., came to the likely conclusion that only the acetic acid moiety enters the brain (Kuratsune et al. 1997). A study of Alzheimer patients, treated for 10 days with ALCAR by i.v. infusion and for a further 50 days by oral administration (2000 mg/day divided into three daily doses), showed a significant increase in the CSF concentration of acetyl-L-carnitine at days 10 and 60 of treatment compared with basal values (Parnetti et al. 1997).

Data presented in the literature suggest that ALCAR may prevent some of the neurochemical sequelae of stress exposure. In fact, repeated ALCAR treatments prevent a stress induced decrease in nerve growth factor binding in rat brain (Foreman et al. 1995), and ALCAR counteracts the increase in β-endorphine induced by repeated exposure to stress (Bidzinska et al. 1993). Moreover, ALCAR seems to improve the feedback control of hypothalamus-pituitary-adrenal axis response to stress, at least in aged rats (Angelucci and Ramacci 1989; Patacchioli et al. 1989). Thus, in the present study we investigated the effect of ALCAR in a series of behavioral paradigms induced by exposure to unavoidable stress that consistently respond to classical antidepressants (Gambarana et al. 1995a, 2001; Ghiglieri et al. 1997). The first model (*acute escape deficit*) consists of a decreased response to aversive stimuli and is a modified version of the *learned helplessness* paradigm. It allows us to evaluate the activity of a treatment in preventing the development of behavioral sequelae following unavoidable stress exposure. The second paradigm is a condition of *chronic escape deficit*, which begins as acute escape deficit and then can be indefinitely sustained by the repeated administration of mild stressors. This model allows us to evaluate the capacity of a treatment to revert the condition of escape deficit. Moreover, we have measured the effect of repeated ALCAR administration on the extraneuronal concentration of dopamine (DA) and serotonin (5-HT), both in basal conditions and after inhibition of the monoamine transporters with cocaine, in the nucleus accumbens shell (NAcS) and medial prefrontal cortex (mPFC). The aims of our study were to assess a possible antidepressant activity of ALCAR in animal models, and to investigate whether such activity may be related to a change in the transmission of one or more monoamines in the frontomesolimbic system.

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 5 per cage (59 × 38.5 × 20 cm) 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 (lights off at 7:00 A.M.) was used. Experiments were carried out from 9:00 A.M. to 5:00 P.M. 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 one week of habituation to the animal colony; 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 n. 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 Ethical Committee.

Antinociceptive Test

Pain threshold was assessed by the radiant heat tail-flick method according to D'Amour and Smith (1941). The animals were taken into the test room, placed on the tail-flick apparatus (Ugo Basile, Italy), and the radiant heat intensity was adjusted to obtain average latency values of 5 s in control animals. A 10 s cut-off point was employed to prevent tissue damage to the tail.

Locomotor Activity

Rats were individually placed in locomotor activity cages (M/P40 Fc Electronic Motility Meter, Motor Products, Stockholm). Each cage had 40 photo-conductive sensors in the floor area (21 × 32 cm) at a fixed distance of 4 cm. These sensors and the arena were uniformly illuminated by an incandescent lamp mounted 60 cm above the floor. Motor activity was defined as the number of interruptions of a beam during a 30-min observa-

tion period. Five days before the test, animals were placed in the motility cage for 15 min/day. On test day, rats were observed for 30 min after a 10-min habituation period.

Acute Escape Deficit

Apparatus. A dark Plexiglas cage (30 × 60 × 30 cm) 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 × 10 cm 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 in the exposure to unavoidable stress (pre-test) 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.

Chronic Escape Deficit

For the chronic stress procedure, rats were initially exposed to the sequence of pre-test and escape test trials described above. Then, rats selected on the basis of their failure to escape (0–3 escapes/30 trials), were subjected to the following procedure, 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).

Microdialysis Procedure

Anesthetized 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 ± 1.2 mm, V − 8.0 mm) and the mPFC (AP + 3.7 mm, L ± 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 × 30 × 30 cm) 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, a solution of artificial cerebrospinal fluid (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). At least 4–5 samples were obtained for the estimation of basal levels. Then, the animals received a cocaine injection (5 mg/kg i.p.) and four samples were collected.

Dialysate samples were immediately analyzed by reverse-phase High Performance Liquid Chromatography (HPLC) with electrochemical detection. Monoamines were eluted on a C-18 reverse phase column (Supelco LC18 DB). The detector was an ESA Coulochem II with a 5014 A microdialysis 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, U.S.A.) 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 the positioning of the probes. Microdialysis data was utilized only when the correct positioning of the probes had been microscopically confirmed on cresyl violet-stained brain sections.

Drugs

ALCAR, SCH 23390, d,l-propranolol, WAY100135 and cocaine were dissolved in 0.9% saline and injected in a volume of 1 ml/kg rat body weight. Dizocilpine was dissolved in 0.9% saline and released by s. c. implanted osmotic minipumps (ALZET® pumps, Palo Alto, CA). Pentobarbital was dissolved in a mixture of 12% ethanol, 38% propylene glycol, 50% deionized/distilled water (vol/vol) and was 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 ± S.E.M. Comparisons were made by *t*-test when two experimental groups were compared. Otherwise, comparisons were made by 1-way analysis of

variance (ANOVA), followed by post-hoc Bonferroni's or Dunnett's test, when applicable ($p < .05$). When the criteria for parametric statistics were not met by the data, non-parametric analysis of variance (Kruskal-Wallis test) was used; when differences between groups were significant ($p < .05$), the data were subjected to post-hoc analysis using Dunn's test, a variation of Bonferroni's test for non-parametric ANOVA.

RESULTS

Effect of a Single Administration of ALCAR on Acute Escape Deficit

The effect of a single treatment with different doses of ALCAR on the development of acute escape deficit by rats was initially examined. Fifty rats were divided into five groups of 10 animals each that received 3, 6, 10, or 20 mg/kg of ALCAR or saline (1 ml/kg) i.p. 60 min before the inescapable shock session, respectively, and that were tested for escape 24 h later. A sixth group of 10 saline treated rats was only tested for escape (*Naive)*. ALCAR treated rats performed in the same way as control rats (Table 1). Analysis of the data by ANOVA test demonstrated a significant difference between groups ($F_{54,59} = 232.20$, $p < .001$); post-hoc Bonferroni's test showed that only the score of *Naive* rats was significantly different from the score of control and ALCAR treated rats ($p < .001$, for all comparisons).

Effect of a 7-day ALCAR Treatment on Acute Escape Deficit

Rats received 3, 6, or 10 mg/kg of ALCAR or saline (1 ml/kg) twice a day, i.p. for seven days (n = 10 for each group); 18–20 h after the last treatment they were exposed to the pre-test and 24 h later to the escape test. A group of saline treated rats (n = 10) was only tested for escape (*Naive)*. Analysis of the data by ANOVA showed a significant difference between groups ($F_{25,29} = 103.61$, $p < .001$). Bonferroni's test demonstrated that rats treated for seven days with ALCAR 10 mg/kg twice a day had a significantly higher number of escapes than rats in the control group and rats treated with ALCAR 3 or 6 mg/kg twice a day ($p < .001$, for all three comparisons) (Table 1).

Table 1. Effect of a Single or Repeated Treatment with ALCAR on the Development of Acute ED

	Number of escapes	
Group	Single administration	7-day administration
Naive	23.9 ± 0.9	23.0 ± 1.0
Ctr	1.4 ± 0.5***	1.0 ± 0.5**
ALCAR 3 mg/kg	1.5 ± 0.5***	3.2 ± 1.0**
ALCAR 6 mg/kg	2.1 ± 0.5***	2.7 ± 0.8**
ALCAR 10 mg/kg	2.2 ± 0.6***	20.3 ± 1.6
ALCAR 20 mg/kg	1.9 ± 0.5***	—

ALCAR was administered i.p. 60 min before the exposure to unavoidable stress (single administration) or it was administered twice a day for 7 days before the sequence of pre-test and escape test. *Naive* and *Ctr* groups received saline acutely or for 7 days. Data are expressed as mean ± S.E.M. of the number of escapes out of 30 trials.

*** $p < .001$ vs. the score of *Naive* group (ANOVA with Dunnett multiple comparisons test).

** $p < .01$ vs. the score of the *Naive* and *ALCAR 10 mg/kg* groups (ANOVA with Dunnett multiple comparisons test).

Effect of a 7-day ALCAR Treatment on Monoaminergic Transmission

Rats received saline (1 ml/kg, n = 10, *Control*) or ALCAR (10 mg/kg, n = 10, *ALCAR*) i.p. twice a day for seven days; on day 8 they were implanted with microdialysis probes in the NAcS and mPFC, and on day 9 they underwent microdialysis. In the NAcS, basal levels of extraneuronal DA and 5-HT were higher in the *ALCAR* group than in the *Ctr* group ($p < .01$ and $p < .05$, respectively, unpaired *t*-test) (Figure 1, panels A and B). Then, when baseline levels had been determined, the animals received an injection of cocaine (5 mg/kg i.p.) and four samples were collected. Because of the significant difference in the basal levels of extraneuronal monoamines between the groups, variations in levels after acute uptake inhibition were not calculated as percentage increases compared with basal values. DA and 5-HT accumulation 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 cocaine administration. The output of DA and 5-HT in the *ALCAR* group was significantly higher than in the *Ctr* group ($p < .001$ and $p < .01$, respectively, unpaired *t*-test) (Figure 1, panels C and D).

In the mPFC, no significant differences were observed between groups in the basal values and in the accumulation after uptake inhibition of DA and 5-HT (data not shown).

Lack of Tolerance to the Protective Effect of ALCAR

In order to establish whether tolerance might develop to the protective effect of ALCAR on acute stress, rats were exposed to the sequence of pre-test and test after 5, 10, 20, or 40 days of ALCAR treatment (10 mg/kg, i.p. twice a day) (n = 10 for each group). Analysis of the data by ANOVA showed a significant difference between group performances ($F_{54,59} = 132.36$, $p < .001$). Dunnett's test indicated that no tolerance developed to the effect of ALCAR after a long-term treatment, as there was a significant difference between the number of escapes of the *Ctr* group and the *Naive* and *ALCAR* groups (Table 2).

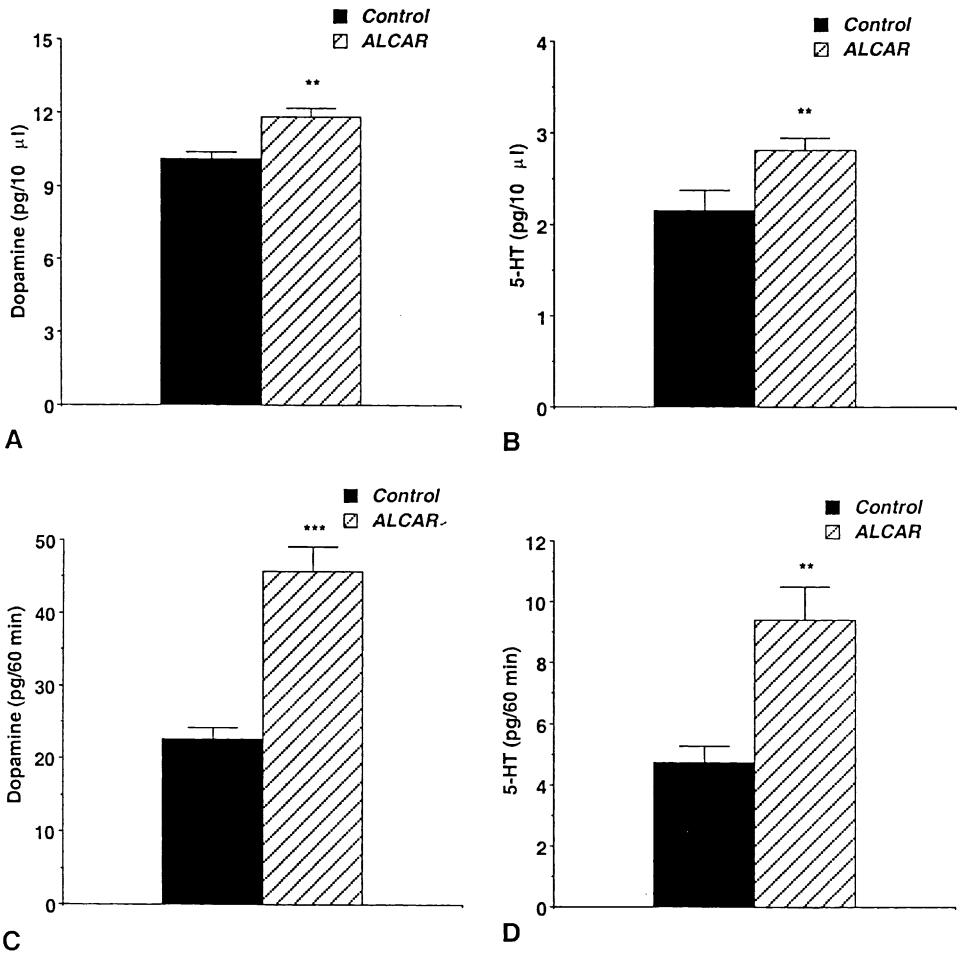

Figure 1. Dopamine and serotonin basal levels (A and B) and accumulation (C and D) in the NAcS after a 7-day ALCAR treatment. Rats received saline (Control) or ALCAR (ALCAR) for seven days, they underwent surgery on day 8, and then microdialysis experiments were performed 48 h after the last treatment. (*Control*, n = 10; *ALCAR*, n = 10). A, B. Dopamine and serotonin basal levels. Values represent the mean of four samples for each rat ± S.E.M.

** $p < .01$ compared with the extraneuronal levels in the *Control* group.

C, D. Dopamine and serotonin accumulation after cocaine administration (5 mg/kg, i.p.). Values represent the mean ± S.E.M. of the sums of four samples collected from each rat after cocaine administration, minus the mean basal level.

*** $p < .001$, compared with the output in the *Control* group.

** $p < .01$, compared with the output in the *Control* group.

Effect of Long-term ALCAR on Pain Threshold and Motor Activity

To exclude a non-specific analgesic effect of repeated ALCAR treatment, which would constitute a major bias in evaluating a reduction of behavioral sequelae to a nociceptive stimulus, two groups of 10 rats each were tested with the radiant heat tail-flick method after seven days of treatment with ALCAR (10 mg/kg) or saline (1 ml/kg) i.p., twice a day. In the tail-flick test the ALCAR treated rats did not show any modification in pain threshold compared with the saline treated rats (mean latency value ± S.E.M. for the *Ctr* group: 4.6 ± 0.1 s, for the *ALCAR* group: 4.9 ± 0.2 s).

In order to verify whether repeated ALCAR treatment had a stimulant activity, which would constitute a bias in evaluating avoidance (Gambarana et al. 1995b), the two groups of rats were tested for spontaneous locomotor activity after 15 days of treatment, 2 h after the last drug administration. No differences were observed in spontaneous locomotor activity between the *ALCAR* and the *Ctr* groups (mean motility counts in 30 min ± S.E.M. for the Ctr group: 1786 ± 132, for the ALCAR group: 1823 ± 205).

Pharmacological Characterization of ALCAR Protective Effect on Stress

Experiment 1. To verify whether the observed protective effect was related to a prominent function of a monoaminergic receptor system, 50 rats were treated with ALCAR (10 mg/kg, i.p. twice a day) for seven days. Then, 30 min before the exposure to the pre-test:

Table 2. No Tolerance Developed to the Protective Effect of a Long-term Treatment with ALCAR

Group	Number of escapes
Naive	25.8 ± 1.0
Ctr	1.1 ± 0.7**
ALCAR 5 days	25.1 ± 0.9
ALCAR 14 days	24.1 ± 0.9
ALCAR 30 days	25.6 ± 0.7
ALCAR 40 days	23.9 ± 1.0

ALCAR was administered at the dose of 10 mg/kg i.p. twice a day for the time indicated. Data are expressed as mean ± S.E.M. of the number of escapes out of 30 trials.

** $p < .01$ vs. the score of the *Naive* group (ANOVA with Dunnett multiple comparisons test).

10 rats received a D_1 dopaminergic antagonist, SCH 23390 (0.03 mg/kg, i.p.); 10 rats received a 5-HT_{1A} serotonergic antagonist, WAY-100635 (0.2 mg/kg, s. c.); 10 rats received a β-adrenergic antagonist, propranolol (5 mg/kg, i.p.); and 10 rats received a M_1-M_2 muscarinic receptor antagonist, scopolamine (0.1 mg/kg, s. c.); 10 received saline (1 ml/kg). Ten rats (*Controls*) were treated with saline (1 ml/kg i.p. twice a day) for seven days and on day 8 they were exposed to the pre-test session. Twenty-four hours after the pre-test all rats were tested for escape. Table 3 shows that only the pretreatment with the selective 5-HT_{1A} serotonergic antagonist WAY 100635 significantly inhibited the protective effect of a 7-day ALCAR treatment (Kruskal-Wallis non-parametric ANOVA, KW = 51.165, $p < .001$; Dunn's test: $p < .001$).

Experiment 2. In order to establish whether the effect of a repeated ALCAR treatment on the development of the escape deficit was dependent on the activity of the NMDA receptor system, the non-competitive NMDA receptor antagonist dizocilpine was utilized. Thirty rats were implanted with osmotic minipumps, which delivered dizocilpine (0.1 mg/kg/day s. c.) for seven days; during the infusion period 10 rats were injected with ALCAR (10 mg/kg i.p. twice a day) and were then exposed to the sequence of pre-test and escape test (*Dizocilpine + ALCAR + Stress*). The other 20 animals received saline i.p. twice a day; 10 of them were exposed to the sequence of pre-test and escape test (*Dizocilpine + Stress*), and 10 to the escape test only (*Dizocilpine*). Twenty rats received saline (1 ml/kg i.p. twice a day) for seven days; 10 of them were exposed to the sequence of pre-test and escape test (*Stress*), and 10 to the escape test only (*Naive*). Analysis of the number of escapes by ANOVA showed a significant difference between groups ($F_{54,59} = 255.61$, $p < .001$). Dunnett's test demonstrated a significant lower number of escapes in the *Stress*, *Dizocilpine + Stress*, and *Dizocilpine + ALCAR + Stress* groups compared with the scores of the *Naive*, *Dizocilpine*, and *Saline + ALCAR + Stress* groups ($p < .01$, for the four comparisons) (Table 4).

Table 3. Selective Antagonism of ALCAR Effect on Acute ED by WAY100635 Administration

Treatment	Number of escapes
Naive	26.0 ± 0.8
Ctr	3.0 ± 1.7***
ALCAR + saline	25.0 ± 1.8
ALCAR + SCH 23390	22.4 ± 1.4
ALCAR + WAY100635	4.3 ± 1.0***
ALCAR + propranolol	22.2 ± 2.0
ALCAR + scopolamine	18.6 ± 3.2

Antagonists were administered 30 min before the pre-test as follows: SCH-23390 = 0.03 mg/kg i.p.; WAY100635 = 0.2 mg/kg s. c.; propranolol = 5 mg/kg i.p.; scopolamine = 0.1 mg/kg s. c. Data are expressed as mean ± S.E.M. of the number of escapes out of 30 trials.

*** $p < .001$ vs. the score of the *Naive* and *ALCAR + saline* groups (Kruskal-Wallis with Dunn multiple comparisons test).

Effect of Long-term ALCAR treatment on the Development of Chronic Escape Deficit

Experiment 1. Rats were exposed to the chronic stress procedure for three weeks while receiving saline (1 ml/kg, *Stress*, n = 10) or ALCAR (10 mg/kg, *Stress + ALCAR*, n = 10) twice a day i.p. Ten rats were injected with saline (1 ml/kg) i.p. twice a day for three weeks (*Naive*). The day after the end of the protocol all rats were tested for escape. Analysis of the data by ANOVA showed a significant difference between groups ($F_{27,29} = 135.54$, $p < .001$); Dunnett's test demonstrated that ALCAR treatment did not reinstate a normal avoidance response in rats exposed to stress ($p < .01$ compared with the *Naive* group) (Table 5).

Experiment 2. The persistence of the protective effect of a 7-day ALCAR treatment during long-term treatment and concomitant stress exposure was tested. Ten rats treated i.p. twice a day for two weeks with saline (1 ml/kg) and 10 treated with ALCAR (10 mg/kg) underwent the sequence of pre-test and escape test on days 15 and 16. They were then exposed to the chronic stress procedure for three weeks, while continuing the treatment (*Chronic Stress* and *ALCAR + Chronic Stress*). Ten more rats were injected i.p. twice a day for five weeks with saline (1 ml/kg) (*Naive*), and 10 with ALCAR (10 mg/kg) (*ALCAR*). The day after the end of the protocol all rats were tested for escape. Analysis of the data by ANOVA showed a significant difference between the number of escapes of experimental groups ($F_{26,29} = 98.347$, $p < .001$); post-hoc analysis by Dunnett's test demonstrated scores significantly lower in

Table 4. Continuous Infusion with Dizocilpine Antagonized the Effect of Long-term ALCAR Treatment on Acute ED Development

Group	Number of escapes
Naive	24.8 ± 0.9
Stress	1.1 ± 0.4***
Dizocilpine	23.5 ± 0.8
Dizocilpine + Stress	1.3 ± 0.5**
Saline + ALCAR + Stress	22.7 ± 1.0
Dizocilpine + ALCAR + Stress	6.7 ± 0.6*

Dizocilpine was administered s. c. by osmotic mini-pump at the dose of 0.1 mg/kg/day for seven days. ALCAR was administered at the dose of 10 mg/kg i.p. twice a day for seven days. Data are expressed as mean ± S.E.M. of the number of escapes out of 30 trials.

*** $p < .001$ vs. the score of the *Naive* group (ANOVA with Bonferroni multiple comparisons test).

** $p < .001$ vs. the score of the *Dizocilpine* group (ANOVA with Bonferroni multiple comparisons test).

* $p < .001$ vs. the score of the *Saline + ALCAR + Stress* group (ANOVA with Bonferroni multiple comparisons test).

Table 5. ALCAR Treatment and Chronic Stress Exposure

Group	Number of escapes
Naive	24.0 ± 0.9
Stress	1.8 ± 0.6**
Stress + ALCAR	3.5 ± 1.5**

Rats were exposed to the sequence of pre-test and escape test, then they began saline (*Stress*) or ALCAR treatment (10 mg/kg b. i. d., i.p.) (*Stress + ALCAR*) and the exposure to the chronic stress protocol. Data are expressed as mean ± S.E.M. of the number of escapes out of 30 trials.

** $p < .01$ vs. the *Naive* group (ANOVA with Dunnett multiple comparisons test).

the *Stress* and *ALCAR + Stress* groups compared with the scores of the *Naive* and *ALCAR* groups (Table 6).

DISCUSSION

ALCAR administered at the dose of 10 mg/kg twice a day for seven days protected rats from the behavioral sequelae of an unavoidable stress and no tolerance developed to this effect after 40 days of continuous treatment. A higher dose of ALCAR administered acutely before the pre-test did not prevent escape deficit development. Animals treated for seven days with ALCAR had levels of extraneuronal DA in the NAcS significantly higher than the control group, and the accumulation of the monoamine after uptake inhibition in this area exceeded that of control rats. Moreover, they presented a significant increase in both basal and cocaine-induced accumulation of extraneuronal 5-HT 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 the 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, 1999b). No modification in either DA or 5-HT extraneuronal concentrations were observed in the mPFC.

Table 6. ALCAR Treatment and Chronic Stress Exposure

Group	Number of escapes
Naive	26.0 ± 1.0
Stress	1.6 ± 0.6***
ALCAR	23.3 ± 1.3
ALCAR + Stress	4.0 ± 1.1**

Rats were pretreated for 14 days (10 mg/kg b. i. d., i.p.), exposed to the sequence of pre-test and escape test, and then they resumed treatment plus exposure to the chronic stress protocol (*ALCAR + Stress*) or handling (*ALCAR*). Data are expressed as mean ± S.E.M. of the number of escapes out of 30 trials.

***$p < .001$ vs. the *Naive* group (ANOVA with Bonferroni multiple comparisons test).

**$p < .001$ vs. the *ALCAR* group (ANOVA with Bonferroni multiple comparisons test).

Rats receiving ALCAR treatment under a continuous dizocilpine infusion did not show protection and scored similarly to the *Stress* group when tested for escape 24 h after the pre-test. The dose of dizocilpine used (0.1 mg/kg/24 h) does not produce apparent behavioral alterations, and it prevents the development of the protective effect of imipramine (Meloni et al. 1993) and fluoxetine (Gambarana, unpublished results) on the behavioral and neurochemical sequelae of inescapable stress. Thus, the protective effect of ALCAR on stress appeared to be mediated by a process of neuronal plasticity dependent on NMDA receptor activity. Rats repeatedly treated with ALCAR had a pain threshold, measured with the tail-flick test, similar to that of control animals. Moreover, their spontaneous motor activity did not differ from that of control animals.

The long-term administration of classical antidepressants, such as imipramine and fluoxetine, produces a protective activity on the development of the behavioral sequelae of unavoidable stress similar to that observed with ALCAR (Gambarana et al. 1995a). This protective effect appears to be related to the functional integrity of a specific type of monoamine receptor. Thus, the acute administration of the selective D_1 dopaminergic antagonist SCH 23390 before the pre-test selectively antagonizes the protective effect of imipramine, while pindolol antagonizes that of fluoxetine (Gambarana et al. 1995a). In order to assess whether the protective effect of ALCAR was sustained by a specific receptor system, rats treated with ALCAR for seven days were acutely administered different selective monoamine receptor antagonists before the pre-test. The doses of each antagonist used were chosen on the basis of previously published results (Gambarana et al. 1995a, 1999c) or of preliminary experiments (scopolamine). Preliminary behavioral data indicated that scopolamine (0.05–0.2 mg/kg) did not induce overt behavioral modifications (Masi, unpublished results), while some cognitive impairment has been described in rats after scopolamine administration at the same doses (Kirkby et al. 1996). The protective effect of repeated ALCAR administrations was specifically antagonized by WAY100635, a selective silent antagonist of 5-HT_{1A} receptors (Forster et al. 1995), given 30 min before the pre-test. This data strongly suggests that 5-HT_{1A} receptor activation plays a relevant role in the mechanism of action of ALCAR.

The involvement of brain monoamines in response to stress has been widely investigated (Bliss et al. 1968; Konstandi et al. 2000) and both 5-HT (Courzon 1971; Petty et al. 1992; Chaouloff 2000) and DA (Pani et al. 2000) are considered to play a crucial role in coping with stressful situations. Dopaminergic neurons in the

ventral tegmental area are rapidly activated by sudden environmental events, including stressful circumstances (Kaneyuki et al. 1991), and acute exposure to different forms of stress has been reported to increase DA release in the PFC (Sorg and Kalivas 1993; Finlay et al. 1995; Yoshioka et al. 1996) as well as in the NAc (Rouge-Pont et al. 1993; Kalivas and Duffy 1995; Tidey and Miczek 1996). It appears that the PFC exerts an inhibitory influence on subcortical dopaminergic transmission. Accordingly, the NAc dopaminergic response to stress, measured by voltammetry, is dampened by the concurrent activation of dopaminergic terminals in the meso-prefrontal cortex, and this action is mediated, at least in part, by D_1 dopaminergic receptor stimulation (Doherty and Gratton 1996). Long-term exposure to different stress procedures impairs an animal's responsiveness to both aversive and pleasurable stimuli (Overmier and Seligman 1967; Papp et al. 1991; Moreau et al. 1992; Gambarana et al. 1995a), and it decreases DA output in mesolimbic areas (Di Chiara et al. 1999; Gambarana et al. 1999a; Mangiavacchi et al. 2001). Moreover, 5-HT output in the mPFC increases in response to acute exposure to unavoidable stress (Petty et al. 1992), although no difference has been observed between helpless and non-helpless control rats in the spontaneous firing of serotonergic neurons in the dorsal raphe (Maudhuit et al. 1997). After chronic exposure to unavoidable stress 5-HT output decreases in the mPFC and NAcS (Mangiavacchi et al. 2001). Some of these stress procedures have been validated as models of depression, as the behavioral and neurochemical deficit that they induce reproduces depressive symptoms (*face validity*), and is reverted by the administration of antidepressant compounds (*predictive validity*) (Willner 1997; Gambarana et al. 2001). Thus, rats subjected to the chronic mild stress (CMS) model, a chronic sequential exposure to a variety of mild stressors, show decreased drinking of a sweetened solution, a condition equated to anhedonia (Willner 1997). These rats also show a decrease of D_2 DA receptor binding and mRNA levels in mesolimbic areas (Papp et al. 1994; Dziedzicka-Wasylewska et al. 1997), and a blunted phasic DA response to a feeding of palatable food in the NAcS and in the mPFC (Di Chiara and Tanda 1997). Both the behavioral and neurochemical changes induced by unavoidable stress exposure in the learned helplessness or CMS paradigms could be reversed by the chronic administration of classical antidepressant drugs (Muscat et al. 1990, 1992; Petty et al. 1992, 1994; Papp et al. 1994, 1996; Di Chiara and Tanda 1997; Dziedzicka-Wasylewska et al. 1997). Antidepressant compounds also revert the chronic escape deficit, and the drugs that we have tested in this paradigm, such as imipramine, fluoxetine, clomipramine, phenelzine, reboxetine, and a total extract of *Hypericum perforatum*, reinstated an avoidance response within three weeks of continuous treatment (Gambarana et al. 1995a, 1999c, 2001; Gambarana, unpublished results). Consequently, we consider that the reversal of a behavioral deficit sustained by chronic stress is an effect that is crucial to the definition of antidepressant activity. Thus, as ALCAR administered for three weeks to chronically stressed rats did not revert the escape deficit condition, it cannot be defined an antidepressant. Indeed, in rats previously treated for 14 days with ALCAR, and that scored like naive animals when tested for escape 24 h after the pre-test, the exposure to chronic stress neutralized the protective effect of ALCAR. Thus, a repeated treatment with ALCAR appeared to share only some effects with imipramine, as it was able to prevent the development of the sequelae of a single, but not of a repeated, unavoidable stress. Moreover, it produced a significant increase in dopaminergic and serotonergic transmission in the NAcS, an effect that did not appear sufficient to protect rats from the disrupting sequelae of chronic stress exposure.

ALCAR has been reported to affect the cholinergic, serotonergic, dopaminergic, glutamatergic, and GABAergic systems, yet no data indicate whether these are direct or indirect effects of ALCAR (Fariello et al. 1988; Harsing et al. 1992; Tempesta et al. 1985). Moreover, the existing data on brain ALCAR distribution and on ALCAR pharmacokinetics do not add any sufficient information that could be used to clarify our results.

In conclusion, although repeated ALCAR administration consistently produced an increase in DA and 5-HT output in the NAcS associated with a protective effect on acute stress exposure, on the basis of the present results, ALCAR does not seem to conform to the criteria that we use to define a compound as an antidepressant (Gambarana et al. 2001).

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