ACETYL-L-CARNITINE PROVIDES EFFECTIVE IN VIVO NEUROPROTECTION OVER 3,4-METHYLENEDIOXIMETHAMPHETAMINE-INDUCED MITOCHONDRIAL NEUROTOXICITY IN THE ADOLESCENT RAT BRAIN


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Abbreviations: ALC, acetyl-L-carnitine; ANOVA, analysis of variance; bp, base pairs; BSA, bovine serum albumin; CoA, acetyl-coenzyme A; COXI, cytochrome c oxidase; DNPH, 2,4-dinitrophenylhydrazine; EGTA, ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid; FFA, free fatty acid; GSH, reduced glutathione; HPLC/EC, high performance liquid chromatography with electrochemical detection; HSD, honest significant difference; LC, L-carnitine; MAO-B, monoamine oxidase B; MDA, malondialdehyde; MPT, membrane permeability transition pore; mtDNA, mitochondrial DNA; NDII, NADH dehydrogenase; PCR, polymerase chain reaction; PND, postnatal day; ROS, reactive oxygen species; SERT, 5-HT transporter; TBA, thiorbarbituric acid; TCA, trichloroacetic acid; UCP3, uncoupling protein 3; VTA/SN, ventral tegmental area and substantia nigra; 5-HIAA, 5-hydroxyindoleacetic acid.

Abstract—3,4-Methylenedioxymethamphetamine (MDMA, ecstasy) is a worldwide abused stimulant drug, with persistent neurotoxic effects and high prevalence among adolescents. The massive release of 5-HT from pre-synaptic storage vesicles induced by MDMA followed by monoamine oxidase B (MAO-B) metabolism, significantly increases oxidative stress at the mitochondrial level. L-Carnitine and its ester, acetyl-L-carnitine (ALC), facilitate the transport of long chain free fatty acids across the mitochondrial membrane enhancing neuronal anti-oxidative defense. Here, we show the potential of ALC against the neurotoxic effects of MDMA exposure. Adolescent male Wistar rats were assigned to four groups: control saline solution, isovolumetric to the MDMA solution, administered i.p.; MDMA (4 × 10 mg/kg MDMA, i.p.); ALC/MDMA (100 mg/kg 30 min of ALC prior to MDMA, i.p.) and ALC (100 mg/kg, i.p.). Rats were killed 2 weeks after exposure and brains were analyzed for lipid peroxidation, carbonyl forma-

The amphetamine derivative 3,4-methylenedioxymethamphetamine (MDMA or ecstasy), is presently one of the most abused recreational drugs among adolescents and young adults. Despite the increasing number of publications reporting the toxic effects of MDMA, long term consequences of this drug are still mostly unknown among consumers. Acute doses of MDMA markedly affect the levels of 5-HT and associated behaviors, leading to hyperactivity and increased social interaction, but decreasing alertness and blurring perception (Morgan, 2000; Baylen and Rosenberg, 2006).

Long-term exposure to MDMA has been implicated in the etiology of several neuropsychological disorders such as depression, increased and phobic anxiety, obsessive traits, paranoid thoughts, sleep disorders, memory deficits, impulsiveness and addiction (for review see Britt and McCance-Katz, 2005). After the initial marked release of 5-HT and associated increase of dopamine (DA) transmis-

tion, mitochondrial DNA (mtDNA) deletion and altered expression of the DNA-encoded subunits of the mitochondrial complexes I (NADH dehydrogenase, NDII) and IV (cytochrome c oxidase, COXI) from the respiratory chain. Levels of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) were also assessed. The present work is the first to successfully demonstrate that pretreatment with ALC exerts effective neuroprotection against the MDMA-induced neurotoxicity at the mitochondrial level, reducing carbonyl formation, decreasing mtDNA deletion, improving the expression of the respiratory chain components and preventing the decrease of 5-HT levels in several regions of the rat brain. These results indicate potential benefits of ALC application in the prevention and treatment of neurodegenerative disorders. © 2009 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: ecstasy, 5-HT, oxidative stress, neurodegeneration, mitochondrial DNA.
reactive oxygen species (ROS) (Jayanthi et al., 1999; Zhou et al., 2003; Hrometz et al., 2004).

Recently, we have shown that exposure of adolescent rats to a neurotoxic dose of MDMA results in mitochondrial oxidative damage to several brain regions. Increased lipid and protein peroxidation, mitochondrial DNA (mtDNA) deletion and subsequent impaired expression of subunits of the mitochondrial complexes I (NADH dehydrogenase, NDII) and IV (cytochrome c oxidase, COXII) were concomitantly observed (Alves et al., 2007). Moreover, we demonstrated that selegiline, a selective inhibitor of monoamine oxidase B (MAO-B), was able to robustly prevent all these events, evidencing the role of MAO-B in the observed oxidative stress (Alves et al., 2007). MDMA-induced release of monoamine neurotransmitters (mainly 5-HT) from storage vesicles was shown to increase MAO-B catalyzed oxidative deamination that is followed by the production of hydrogen peroxide and highly reactive hydroxyl radicals (HO·) that mediate oxidative stress damage (Alves et al., 2007). MAO’s location in the outer membrane of the mitochondria (Zhuang et al., 1988, 1992) facilitates oxidative damage of mitochondrial macromolecules through diffusion of peroxides into the mitochondrial matrix.

Additionally, it has been shown that exposure to MDMA reduces concentrations of neuronal antioxidant elements, such as ascorbic acid (Shankaran et al., 2001), glutathione (Capela et al., 2007a), and vitamin E (Johnson et al., 2002), and increases the concentration of ROS, evidencing the neuroprotective role of antioxidants against MDMA neurotoxicity. Increasing the levels of ascorbic acid was also shown to prevent the neurochemical and behavioral responses to MDMA administration (Shankaran et al., 2001). Likewise, N-acetylcyesteine, a glutathione precursor, also reduced MDMA-induced neurotoxicity (Capela et al., 2006, 2007a,b).

Neuroprotective effects of carnitines in various conditions of metabolic stress have been reported (Binienda and Virmani, 2003; Virmani and Binienda, 2004). Absorbed from diet or biosynthesized in the liver and kidneys, carnitine is incorporated into the total body carnitine pool that comprises L-carnitine (LC) and short chain esters such as acetyl-L-carnitine (ALC). Both LC and ALC, as a “shuttle,” facilitate the transport of long chain free fatty acids (FFAs) across the mitochondrial membrane for β-oxidation. While LC deficiency may impair the FFAs’ oxidation and the utilization of carbohydrates (Schulz, 1994), LC has been shown to prevent oxidative stress–related damage induced by methamphetamine (Binienda and Virmani, 2003). It has been suggested that LC administration enhanced glutathione activity preventin lipid peroxidation in aging rats (Rani and Panneerselvam, 2001).

Studies have shown that ALC is more easily transported across the blood–brain barrier than LC when administered therapeutically, supporting ALC application in acute and chronic neurological disorders (Kido et al., 2001).

Based on the characteristics of ALC, the aim of present study was to evaluate the ALC neuroprotective potential against the MDMA-induced oxidative stress in the brain mitochondria. A model of MDMA “binge” administration in adolescent rats that was previously demonstrated to induce significant neurotoxicity was used (Alves et al., 2007).

**EXPERIMENTAL PROCEDURES**

**Animal model**

Wistar rats born from nulliparous females purchased from Charles River Laboratories España S.A. (Barcelona, Spain) were used. Animals were maintained under a 12-h light/dark cycle, in a temperature- and humidity-controlled room and given *ad libitum* access to food and water. Institutional guidelines regarding animal experimentation were followed. Rats were handled daily and regularly weighed. Cylindrical plastic tubes and soft paper for nest construction were made available to reduce stress. All procedures used were approved by the Portuguese Agency for Animal Welfare (general board of Veterinary Medicine in compliance with the Institutional Guidelines and the European Convention). The number of animals used was reduced to the minimum necessary to obtain valid results. Human end-points were clearly defined to avoid unnecessary pain and distress. On postnatal day 40 (PND 40), animals were randomly assigned to the different experimental groups and caged in pairs. Two days later, an s.c. probe (Implantable Programmable Temperature Transponder, IPTTTM-200, BMDS, Seafood, DE) (Kort et al., 1998) was inserted in the dorsal region, to allow the measurement of body temperature throughout the experiment. On PND 45, the animals assigned to the MDMA group received a freshly prepared solution of MDMA at 10 mg/kg of body weight, i.p. every 2 h in a total of four injections. Rats in the ALC/MDMA group received the same MDMA dose preceded in 30 min by a single i.p. injection of 100 mg/kg ALC. Control animals received equal i.p. doses of saline vehicle (0.9% W/V) in the same protocol and the ALC group rats were administrated i.p. a single dose of ALC (100 mg/kg). Dose was selected as the lowest dose used in works that report effective neuroprotection after ALC supplementation.

MDMA (HCl salt) was extracted and purified from high purity MDMA tablets that were provided by the Portuguese Criminal Police Department. The obtained salt was pure and fully characterized by NMR and mass spectrometry methodologies. ALC was kindly provided by Sigma-tau Health Science S.p.A., Pomezia, Italy.

**Mitochondria isolation for determination of lipid peroxides and protein carbonyls**

Two weeks after exposure, animals were killed by decapitation and brains were rapidly removed on ice. Mitochondria were isolated from whole brain by slight modification of a previously described method (Rosenthal and Bodley, 1987). Briefly, the whole encephalon was washed, minced and homogenized at 4 °C in isolation medium (250 mM sucrose, 5 mM Hepes, 1 mM EGTA, 1 mg/mL bovine serum albumin (BSA), pH 7.4) containing 5 mg of bacterial protease (P-5380, Sigma, Steinheim, Germany). Single brain homogenates were brought to 30 mL with isolation medium and centrifuged at 1260 × g (SOKRAL RC 5B Plus, Kendra Laboratory Products, USA) for 5 min. The supernatant was centrifuged at 20,000 × g for 10 min. The pellet including the fluffy synaptosomal layer was resuspended in 10 mL of isolation medium containing 0.02% digitonin (to release mitochondria from the synaptosomal fraction) and centrifuged at 20,000 × g for 10 min. The brown mitochondrial pellet (without the synaptosomal layer) was resuspended in 10 mL of washing medium (225 mM sucrose, 5 mM Hepes, pH 7.4) and centrifuged at 20,000 × g for 10 min. The main portion of the mitochondrial pellet was resuspended in 300 μL of washing medium. Mitochondrial protein were determined by the biuret method, calibrated with BSA (Gornall et al., 1949).

**Quantification of lipid peroxidation**

Lipid peroxidation was determined by measuring malondialdehyde (MDA) equivalents, using the thiobarbituric acid (TBA) as-
say, according to a modified procedure (Rohn et al., 1993). Mitochondrial protein (3 mg) was incubated for 30 min at 25 °C in 3 mL of medium (potassium chloride 175 mM, Tris 10 mM, pH 7.4 and rotenone 3 μM). Aliquots of 0.3 mL were then incubated with 2.7 mL of TBA reactive substances (TBARS) reagent (TBA 9%, HCl 0.6 N and butylated hydroxytoluene (BHT), 0.0056%). The mixtures were warmed to 80–90 °C, for 15 min, and cooled by immersion in ice during 10 min before centrifugation at 15000×g for 5 min. Lipid peroxidation, reflected by the production of MDA equivalents, was estimated by spectrophotometry determination, at 535 nm, of the MDA equivalents produced. The amount of MDA equivalents formed was calculated using a molar extinction coefficient of 1.56×10^5 mol^-1 cm^-1 and expressed as nmol MDA equivalents/mg protein (Buege and Aust, 1978).

**Quantification of protein carbonyls**

Protein carbonyls were quantified through the spectrophotometric carbonyl assay (Reznick and Packer, 1994), using 2,4-dinitrophenylhydrazine (DNPH). Two samples of 1 mL of each mitochondrial extract 1 mg/mL were placed in glass tubes. To one tube 4 mL of 10 mM DNPH in 2.5 M HCl solution was added, and to the other tube of the same sample, only 4 mL of 2.5 M HCl (blank tube). Tubes were left for 1 h at room temperature in the dark and vortexed every 15 min. At this point, 5 mL of 20% trichloroacetic acid (TCA) (w/v) solution was added to both DNPH and HCI samples to a final concentration of 10% (w/v) TCA. The tubes were left on ice for 10 min and then centrifuged for 5 min. The resultant supernatant was discarded. Another wash was subsequently performed with 4 mL of 10% TCA and the protein pellets were broken mechanically. The protein pellets were washed three times with ethanol-ethyl acetate (1:1) (v/v). The final pellet was dissolved in 6 M guanidin hydrochloride solution and left for 10 min at 37 °C under agitation in a waterbath. All samples were centrifuged to remove any insoluble material remaining in suspension. The concentration of DNPH was determined at 360 nm, and the molar absorption coefficient of 22×10^5 M^-1 cm^-1 was used to quantify the levels of protein carbonyls. Protein concentration in the samples was calculated by determining the absorbance at 280 nm. Protein carbonyl content was expressed as nmol protein carbonyl formed/mg mitochondrial protein (Reznick and Packer, 1994).

**DNA isolation for polymerase chain reaction (PCR)**

Two weeks after exposure, animals were killed by decapitation. Brains were rapidly removed and dissected on ice, following orientation marks provided by Paxinos and Watson (2005), into: the prefrontal cortex, striatum, hippocampus, amygdala, ventral mesencephalon (comprising the ventral tegmental area and substantia nigra, VTA/SN) and raphe nuclei. DNA from the different brain regions was extracted with GenomicPrep Cells and Tissue DNA Isolation Kit (Amersham Biosciences, Buckinghamshire, UK), according to instructions of manufacturer. Extracted DNA (5 μL) was applied on a 1% agarose gel to quantify the amount of DNA used on the subsequent PCR protocols.

Photographs were taken under UV transillumination (Typhoon 8600, Molecular Dynamics, Amersham Pharmacia Biotech, Buckinghamshire, UK) and the semiquantitative analysis of amplified DNA was made with the software Image Quant 5.1 (Molecular Dynamics®). Negative controls were included containing all the abovementioned PCR components except template DNA. Ten-microliter aliquots of the PCR products were separated by electrophoresis through a 1–1.5% agarose gel in Tris-acetate (TAE) containing ethidium bromide at 45 V/cm. Photographs were taken under UV transillumination (Typhoon, Molecular Dynamics, Amersham Pharmacia Biotech) and the semiquantitative analysis of amplified DNA was made with the software Image Quant 5.1 (Molecular Dynamics®).

**Western blot analysis of isolated whole brain mitochondria**

Isolated whole brain mitochondria were resuspended in extraction buffer (20 mM Tris–HCl, pH 7.6, 250 mM sucrose, 40 mM potassium chloride, 2 mM EDTA). The homogenate was centrifuged at 600×g for 10 min at 4 °C and the supernatant was taken for mitochondrial Western blot analysis, 15 μg of protein was loaded per lane and separated on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels. The gels were transferred to a polyvinylidene fluoride (PVDF) membrane for protein blotting (0.2 μm, Bio-Rad) membranes by electroblotting 1 h at 150 mA. The filters were blocked in 5% non fat dry milk and 0.1% Tween 20 overnight at 4 °C. Blots were then incubated with mouse monoclonal antibody against complex IV (COXII) subunit I (COXII) (Invitrogen, Eugene, OR, USA, 2 μg/mL) or complex I (NDII) subunit 2 (NDII) (Invitrogen, 0.5 μg/mL) diluted in Tris buffered saline tween 20 (TBST) 0.1% (TBS; 20 mM Tris, 137 mM NaCl, pH 7.6) for 1 h at room temperature. Membranes were washed three times for 10 min in the same buffer and incubated for 1 h with horseradish peroxidase (HRP)–conjugated goat anti-mouse IgG (Immun-Star™: 1:20,000, Bio-Rad). Immunoreactive proteins were revealed using enhanced chemiluminescence method (Immun-Star™ HRP Chemiluminescent Kit, Bio-Rad). Blots were analyzed with Quantity One Software (Bio-Rad) version 4.5.

**Neurochemical determinations**

Levels of 5-HT, 5-hydroxyindoleacetic acid (5-HIAA) were assayed by high performance liquid chromatography, combined with electrochemical detection (HPLC/EC) using a Gilson instrument (Gilson, Inc., Middleton, WI, USA), fitted with an analytical column (Supelco Supelcosil LC-18 3 μM; 7.5 cm×4.6 mm; flow rate: 0.8–1.0 mL/min; Supelco, Bellefonte, PA, USA). Two weeks after exposure, animals were killed by decapitation. Brains were rapidly removed and the same brain regions used for DNA isolation and PCR were dissected on ice following orientation marks provided by Paxinos and Watson (2005): prefrontal cortex, striatum, hippocampus, amygdala, ventral mesencephalon (comprising the VTA/SN) and raphe nuclei. Tissue samples were frozen by immersion in 4-methylbutane cooled over dry ice and stored at −70 °C until used for neurochemical determinations by HPLC-EC analysis. Tissues were homogenized in 0.2 N perchloric acid, disrupted by ultrasonication and centrifuged (15,000×g; 5 min),
supernatant was collected and filtered through a 0.2 µm nylon filter (microcentrifuge filter from Costar, Corning, NY, USA). Aliquots of 50 µl were injected into the HPLC system, using a mobile phase of 0.7 M aqueous potassium phosphate (monobasic) (pH 3.0) in 10% methanol, 1-heptanesulfonic acid (222 mg/L) and Na-EDTA (40 mg/L) (Ali et al., 1993).

Concentrations of neurotransmitters were calculated using standard curves. Standards were purchased from Sigma (St. Louis, MO, USA). Final results were expressed in terms of monamine content per amount of protein. Mitochondrial protein was determined by the biuret method, calibrated with BSA (Gornall et al., 1949).

**Statistical analyses**

Weight evolution was analyzed using SPSS for Windows (SPSS Statistical Software Programs version 15.0; SPSS, Inc., Chicago, IL, USA). A one-way analysis of variance (ANOVA, treatment) was used, since different animal were used for each analyzed PND. Significant differences were further tested using the post hoc Tukey’s HSD for unequal n. Significant differences are marked as follows: * P<0.05 and *** P<0.001, for MDMA when compared with control; † P<0.05, ‡‡‡ P<0.01, for ALC/MDMA when compared with control saline.

**RESULTS**

**MDMA-induced decreased weight gain**

Daily changes in animal body weight gain were monitored throughout the experiment and until kill. On the day of exposure (PND 45) no differences between animals from the four experimental groups were observed. On PND 49, the weight gain for animals exposed to MDMA was already reduced when comparing to control rats (P<0.05). Four days later, on PND 53, the group exposed to MDMA was still presenting a reduced gain of weight (P<0.05) and the ALC/MDMA group also showed a reduced weight gain (P<0.01). On PND 57 these differences were still present, and on the day of kill (PND 59) were even more evident (P<0.001 and P<0.01 for MDMA and ALC/MDMA respectively, when comparing to the saline group). However, the weight gain in the ALC group was not different from the control group (Fig. 1).

**MDMA-induced hyperthermia**

MDMA administration resulted in hyperthermia. Analysis of body temperature data, throughout the day of exposure indicated that ALC was unable to modify the hyperthermic MDMA effect when administered 30 min prior to MDMA (Fig. 2). Rats treated either with MDMA or ALC/MDMA had significantly higher body temperature (P<0.001) 30 min after the first injection and until the end of the measuring period, as compared with both saline and ALC group for most time points.

**ALC administration did not attenuate the MDMA-induced increase in lipid peroxidation**

Lipid peroxidation was assessed by means of MDA equivalents formation in isolated whole brain mitochondria homogenates of adolescent male Wistar rats 14 days post-exposure. Animals treated with MDMA presented significantly higher levels of MDA equivalents (P<0.01) as compared with the saline control. No differences between MDMA and ALC/MDMA groups were observed at the
tested level (Fig. 3). However, the levels of MDA equivalents formed under exposure to ALC/MDMA or ALC were also increased ($P<0.01$ and $P<0.05$) showing a clear change in the processing of lipids in the presence of ALC. ALC administration prevented the MDMA-induced increased formation of protein carbonyls

The levels of protein carbonyls were evaluated in the mitochondrial fraction of whole brain homogenates of adolescent male rats 14 days after exposure. The administration of a neurotoxic dose of MDMA produced a significant increase in protein carbonyls of whole brain mitochondria when compared with the control group ($P<0.001$). In animals treated with ALC prior to MDMA, carbonyl levels were significantly lower ($P<0.05$) than those of the MDMA group and simultaneously very similar to the levels of carbonyls formed in the ALC group, which were also higher than those of the control ($P<0.05$) (Fig. 4). Therefore, the administration of ALC was clearly efficient in protecting the cells from the action of MDMA, leading to a significant reduction of protein carbonyls in the mitochondria.

ALC administration prevented the MDMA-induced deletion of mtDNA

MtDNA from the prefrontal cortex, the striatum, the amygdala, the ventral mesencephalon (dissected as the VTA/SN), the hippocampus and the raphe nuclei, were analyzed by PCR using the Taq DNA polymerase to verify the presence of a deletion corresponding to the genes NDI and NDII of the mitochondrial complex I and COXI of complex IV. Considering the set of primers used, a band corresponding to NDI, NDII and COXI deletion was expected to be observed. Fig. 5A shows a representative image of the obtained PCR results, the selected image corresponds to a frontal cortex mitochondrial extract. The deletion was sub-

![Graph](image-url)
stantially more evident in animals exposed to MDMA \((P<0.001)\) in the striatum, VTA/SN, hippocampus and raphe nuclei, \(P<0.01\) in the frontal cortex and \(P<0.05\) in amygdala as compared with the other experimental groups) (Fig. 5B). A previous administration of ALC produced a significant attenuation on the level of mtDNA deletion \((P<0.001)\) in the striatum, VTA/SN, hippocampus and raphe nuclei, \(P<0.01\) in the frontal cortex and \(P<0.05\) in amygdala as compared with the MDMA group) as evidenced in Fig. 5B.

**ALC administration prevented the MDMA-induced decrease in the expression of the mitochondrial subunit NDII and COXI**

Expression of the mitochondrial respiratory chain components NDII and COXI was evaluated by Western blot, 2 weeks after exposure to MDMA and ALC. Expression of NDII was significantly decreased in MDMA rats compared with control \((P<0.01)\), ALC and ALC/MDMA \((P<0.001)\) in both cases) (Fig. 6A). Likewise, expression of COXI was also considerably decreased \((P<0.001)\) in the MDMA group compared with the other experimental groups. Animals treated with ALC prior to MDMA presented values of expression that were not significantly different from controls (Fig. 6B). These results evidence that pre-administration of ALC successfully prevented the MDMA-induced decreased expression of NDII and COXI.

**ALC administration prevented the MDMA-induced decrease in 5-HT content**

Analysis of the neurochemical data revealed a highly significant influence of treatment in the levels of 5-HT and 5-HIAA \((P<0.001)\). In all assessed brain regions, exposure to MDMA induced a reduction of the 5-HT levels 2 weeks after exposure (Fig. 7). This decrease in the 5-HT content was accompanied by a decrease in the levels of 5-HIAA, although it only achieved significance in the hippocampus and raphe nuclei (Fig. 7). In animals treated with ALC prior to MDMA, the levels of 5-HT were clearly higher than those observed for MDMA-exposed animals, which evidences that pre-administration of ALC was also effective in preventing the MDMA-induced loss of 5-HT.

**DISCUSSION**

The present study confirms our previous report of MDMA-induced oxidative stress in the brain mitochondria (Alves et al., 2007). The protection exerted by ALC against MDMA exposure, reinforces the notion that the neurotoxicity induced by MDMA is linked with oxidative stress observed primarily in the mitochondria. To our knowledge, this is the first study that reports the neuroprotective effects of ALC against the MDMA-evoked neurotoxicity.

Exposure to MDMA significantly attenuated body weight gain throughout the 2 weeks that followed exposure. This observation is consistent with previous studies where an anorectic effect was noticed after the fourth day of dosing and was still evident almost 2 weeks after the last MDMA administration (Piper et al., 2005). A trend toward a further decrease in body weight gain observed after ALC/MDMA exposure indicates that, besides anorexia (Conductier et al., 2005), other factors may also be involved. The half-life of ALC is close to 12 h. Therefore, albeit its role in fatty acid utilization (Penn et al., 1997; Mollica et al., 2001; Iossa et al., 2002), a single ALC administration would not account for a decrease in body weight over a 2-week period. However, i.p. injection of ALC itself may lead to some stress reaction underlying this effect. It was observed that LC i.p. administration was associated with a localized inflammatory macrophages activation in the peri-
toneal cavity (Dionyssopoulou et al., 2005). Nevertheless, our results also show that ALC alone did not affect the normal weight gain.

Hyperthermia is a common feature of exposure to MDMA involving a complex interaction between the hypothalamic–pituitary–thyroid axis and the sympathetic nervous system (Sprague et al., 2003). Despite former reports where no hyperthermic response was observed in adolescent models of MDMA-exposure (Jean et al., 2007), we have previously shown that, under the present protocol, adolescent rats display a robust increase in body temperature as monitored through s.c. inserted probes (Alves et al., 2007). Thermogenesis is a multifaceted process that involves the action of the CNS and the peripheral nervous system, as well as cell signaling (see Mills et al., 2004 for review). The activation of mitochondrial uncoupling protein 3 (UCP3) was pointed out as a key factor in the abnormal thermoregulation induced by MDMA (Sprague et al., 2007). Increased noradrenaline levels were also shown to be involved in the development of hyperthermia, both by direct activation of UCP3 through the α1 and β3 adrenergic receptors, and by vasoconstrictive prevention of heat dissipation (Sprague et al., 2007). Likewise, increased levels of plasma FFAs were implicated in the activation of UCP3 in skeletal muscle mitochondria (Sprague et al., 2007). Carnitine was previously shown to increase levels of FFAs in the rat frontal cortex that in turn, may contribute to an increased activation of UCP3 (Binienda et al., 1999). In that sense, administration of ALC before MDMA was not expected to affect the MDMA-induced hyperthermic response. Increased levels of FFAs may result from an ALC-induced improved ratio of free-to-esterified acetyl-coenzyme A (CoA), with a consequent increase in oxidative phosphorylation of lipids. In agreement with this hypothesis, we observed a significant increase in the formation of lipid peroxides in groups that were administered with ALC.

The neuroprotective effects of ALC may be exerted through attenuation of mitochondrial membrane permeability transition pore (MPT) opening. Mitochondria control apoptosis via release of cytochrome c into the cytosol through the MPT. Consequently, the administration of ALC may reduce the activation of the caspase cascades, leading to restrained apoptosis (Wieckowski et al., 2000). The overall benefit of ALC administration against MDMA neurotoxicity is probably related with an increased protection of the mitochondrial membrane integrity (Kashiwagi et al., 2001). The β-oxidation of FFA involves the formation of long-chain fatty acid esters of CoA and their transport into the mitochondria. Previous studies have hypothesized that the protective actions of LC could be conveyed by restoring mitochondrial production of energy via changes in cell membrane viscosity (Binienda et al., 1999). We have recently reported that a neurotoxic dose of MDMA in adolescent rats results in a strong mitochondrial oxidative damage increasing mitochondrial peroxidation, mtDNA deletion and impaired expression of NDII and COXI (Alves et al., 2007) with a consequent impairment of energy production (reduced levels of ATP, data not shown). Here, we demonstrate that a previous administration of ALC was able to significantly prevent the deletion of the mtDNA portion that encodes the expression of NDII and COXI. In accordance, there is a recovery in the expression of the NDII and COXI proteins. Preventing the diminished expression of NDII and COXI enhances the functionality of the respiratory chain and favors the production of energy, highlighting the protective role of ALC in the mitochondria.

Despite the already discussed increase in lipid peroxidation, in the present work we show a significant reduction...
in the formation of protein carbonyls in the mitochondria, demonstrating the protective role of ALC at this level. Proteins are major targets for ROS, leading to the formation of oxidized forms of proteins that are easily recognized by proteases, increasing degradation levels and resulting in loss of enzymatic activity (Grune et al., 2001). Of notice, the enzymes involved in the oxidative-damage defense, such as glutathione peroxidase, reduced glutathione (GSH), oxidized glutathione or superoxide dismutase, are among the proteins targeted by ROS, which reinforces the relevance of protecting the mitochondria against protein carbonylation. Increased levels of protein carbonyls are a characteristic feature of aging, which may be explained by increased mitochondrial oxidant production and a progressive decline in proteasome activity (Davies et al., 2001; Grune et al., 2001). Confirming the role of ALC in preventing the increase in protein carbonyls, there are several studies that report the use of ALC to delay age-related processes (Abdul et al., 2006; Calabrese et al., 2006; Sethumadhavan and Chinnakannu, 2006; Savitha et al., 2007; Tamilselvan et al., 2007).

In the present study, lipid peroxidation produced by MDMA, despite the protective actions of ALC on oxidation of proteins, indicates the importance of assessing the overall neuroprotection exerted by ALC with regard to brain function. A combined treatment regimen of ALC and the mitochondrial coenzyme, lipoic acid (Hagen et al., 2002), may be effective in attenuating side effects of ALC-enhanced β-oxidation of FFAs, i.e. lipid peroxidation. Such treatment might be the right direction in formulating a therapeutic strategy for ALC as a neuroprotectant against the MDMA-induced neurotoxicity.

Acute effects of MDMA on the efflux of 5-HT from serotonergic neurons through SERT and consequent long-term depletion of 5-HT that correlates with damage of serotonergic nerve terminals have been widely described and revised (see Ricaurte and McCann, 2001; Green et al., 2003; Gudelsky and Yamamoto, 2008; Schaefer et al., 2008; Skelton et al., 2008). Although MDMA is generally viewed to be selectively neurotoxic to 5-HT terminals, studies reporting the role of oxidative and bioenergetic stress in the mechanisms underlying MDMA induced 5-HT neurotoxicity (Gudelsky and Yamamoto, 2003; Darvesh and Gudelsky, 2005; Quinton and Yamamoto, 2006; Alves et al., 2007), demonstrate the relevance of free radical formation in the neurotoxicity of MDMA and may substantiate the neurotoxic effects of MDMA also at the VTA/SN and raphe nuclei, regions of origin of the dopaminergic and serotonergic neurons. In the present work, we have observed a clear reduction in the levels of 5-HT 2 weeks after exposure to MDMA. This loss of 5-HT content was evident in all assessed brain regions, although not so pronounced in areas that are mainly dopaminergic, such as the VTA/SN and striatum. In the hippocampus and raphe nuclei, 5-HT was markedly decreased and was accompanied by a decrease in the levels of 5-HIAA. Interestingly, in animals previously treated with ALC, exposure to MDMA no longer resulted in a loss of 5-HT. In all assessed brain regions the levels of 5-HT and 5-HIAA were similar in both ALC/MDMA and control groups, showing the ALC protective effect also at this endpoint. In accordance, ALC was reported to increase the levels of both 5-HT and dopamine and act positively on pathologies such as increased impulsivity in adolescent rats (Adriani et al., 2004), attention deficit/hyperactivity disorder in children (Tomioi et al., 2008) and fatigue syndromes (Carroll et al., 2007). Of note, ALC treatment seems not to affect healthy subjects, where neurotransmitter levels are kept at the normal ranges (Adriani et al., 2004), just as observed in the present work for the ALC control group.

Another interesting aspect is the increased 5-HT turnover observed in the prefrontal cortex, striatum and amygdala of ALC control animals. This has been consistently described in the previous studies (Adriani et al., 2004) and may be attributed to an increased mitochondrial membrane integrity (Kashiwagi et al., 2001), which may improve MAO function.

ALC has been considered a therapeutic compound of great interest in various neurological problems, especially chronic neurodegenerative disorders. The substantia nigra (SN) is a brain region with an increased vulnerability to oxidative damage, because of its high content of oxidizable components, high metabolic rate and relatively low antioxidant complement. Therefore, treatment with carnitine may slow progress of Parkinson’s disease (Kidd, 2000; Beal, 2003). Likewise, ALC may be useful as a possible therapeutic agent in Alzheimer’s disease. The presence of ALC in primary cortical neuronal cultures, significantly attenuated amyloid-beta peptide-induced cytotoxicity, decreasing protein oxidation, lipid peroxidation and apoptosis in a dose-dependent manner (Abdul et al., 2006). Moreover, ALC was also shown to elevate cellular GSH and heat shock proteins levels (Abdul et al., 2006) which reinforces its neuroprotective potential against neurodegeneration associated with mitochondrial oxidative damage.

The present work successfully demonstrates that pretreatment with ALC confers effective neuroprotection against the MDMA-induced neurotoxicity in the rat brain. We report a significant prevention of mitochondrial oxidative damage, with reduced carbonyl formation, decreased mtDNA deletion, and improved expression of respiratory chain components. Moreover, we demonstrated a significant ALC-exerted prevention of the 5-HT loss typically observed in MDMA-induced neurotoxicity. These results reinforce the beneficial potential of ALC as a neuroprotectant in therapy of neurodegenerative disorders.

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