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α-Lipoic acid prevents 3,4-methylenedioxy-methamphetamine (MDMA)-induced neurotoxicity

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A single administration of 3,4-methylenedioxymethamphetamine (MDMA, 20 mg/kg, i.p.), induced significant hyperthermia in rats and reduced 5-hydroxytryptamine (5-HT) content and [^{3}H]paroxetine-labeled 5-HT transporter density in the frontal cortex, striatum and hippocampus by 40–60% 1 week later. MDMA treatment also increased glial fibrillary acidic protein (GFAP) immunoreactivity in the hippocampus. Repeated administration of the metabolic antioxidant α-lipoic acid (100 mg/kg, i.p., b.i.d. for 2 consecutive days) 30 min prior to MDMA did not prevent the acute hyperthermia induced by the drug; however, it fully prevented the serotonergic deficits and the changes in the glial response induced by MDMA. These results further support the hypothesis that free radical formation is responsible for MDMA-induced neurotoxicity. *NeuroReport* **10:3675–3680** © **1999 Lippincott Williams & Wilkins.**

Key words: 5-HT (serotonin, 5-hydroxytryptamine); Glial fibrillary acidic protein (GFAP); α-Lipoic acid (thioctic acid); 3,4-Methylenedioxymethamphetamine (MDMA, Ecstasy)

Introduction

It is well established that single or repeated administration of high doses of the ring-substituted amphetamine 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy) produces neurochemical and histological evidence of long-term deficits in serotonergic function in the brain of rodents and primates. Such changes are shown by a decrease in the content of 5-hydroxytryptamine (5-HT) and its major metabolite 5-hydroxyindoleacetic acid (5-HIAA) in several brain regions, a decline in the activity of tryptophan hydroxylase, reduction in the number of [^{3}H]paroxetine-labeled 5-HT transporters and a reduction in the density of serotonergic terminals [1]. Enhanced expression of glial fibrillary acidic protein (GFAP) has also been shown in different strains of mice after repeated MDMA administration [2].

The precise mechanism by which MDMA selectively damages 5-HT axon terminals remains unknown; however, hyperthermia appears to play a key role in the reactions leading to MDMA-induced serotonergic damage, probably by potentiating free radical formation, as there is a substantial body of evidence indicating that increased free radical formation is responsible for MDMA-induced neurotoxicity [3–5].

α-Lipoic acid has generated considerable clinical interest as a thiol-replenishing and redox modulating agent, and it is being used to treat complications associated with diabetes [6]. It is a metabolic antioxidant which is taken up and reduced in cells to dihydrolipoate. Both α-lipoate and its reduced form have been shown to protect against excitotoxicity, ischemia-reperfusion injury, mitochondrial dysfunction and other acute or chronic damage to neural tissue involving free radical formation [7,8].

In the present study we analysed the potential neuroprotective effects of α-lipoic acid against MDMA-induced toxicity. 5-HT content, [^{3}H]-paroxetine binding and GFAP expression were taken as indicators of neurotoxicity. The effects of α-lipoic acid on the body temperature of the rats were also analysed, as many drugs, including free radical scavengers, prevent MDMA-induced neurodegeneration by producing hypothermia [3,9–11].

Materials and Methods

Animals and treatments: Male Wistar rats (220–240 g) were housed in plastic cages in a temperature controlled room (22 ± 1°C) with free access to food and water and maintained on a 12:12 h light:dark cycle (lights on at 07.00 h). Rats received vehicle or α-lipoic acid (100 mg/kg i.p. twice daily for 2 days, in 1 ml of sodium bicarbonate 2.5% w/v). Thirty minutes after the fourth dose of α-lipoic acid or vehicle, rats were injected with either MDMA (20 mg/kg, i.p.) or saline (control group). Seven days after MDMA, rats were killed by decapitation, the

brain was removed rapidly and placed on ice. The hippocampus, striatum and frontal cortex were dissected free, frozen on dry ice and stored at −80°C until chromatographic and binding studies were performed. The animals used for immunohistochemical studies were terminally anaesthetized with an overdose of pentobarbitone and perfused transcardially with phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS, pH 7.4. The brains were removed and immersed in a 30% sucrose solution for 48–72 h at 4°C. Coronal sections (40 μm) were cut in a freezing microtome and stored at 4°C in a cryoprotectant solution (phosphate buffer, glycerin and ethylene glycol, 2:1:1). In all cases, the doses of MDMA refer to the hydrochloride.

All procedures for the treatment of these animals were in compliance with the European Community Council Directive and were approved by the Ethical Committee of the University of Navarra.

Temperature measurements: Rectal temperature of the rats was measured at an ambient temperature of 22 ± 1°C with a lubricated digital thermometer probe (pb 0331, Panlab, Barcelona) inserted 3 cm into the rectum, the rat being lightly restrained by holding in the hand. Temperature was recorded before any drug treatment and thereafter every 30 min for 240 min. Probes were re-inserted from time to time until the temperature stabilized.

Determination of 5-HT: Concentrations of 5-HT in the brain regions of the rats were determined by high performance liquid chromatography with electrochemical detection as described previously [12].

[^{3}H]paroxetine binding: Binding studies were performed according to the procedure described by Marcusson *et al.* [13], with minor modifications. The brain regions studied were homogenized in 15 ml ice-cold buffer (Tris–HCl 50 mM, 120 mM NaCl, 5 mM KCl, pH 7.4) and centrifuged at $48\,000 \times g$ for 10 min at 4°C. The pellet was resuspended in buffer and incubated at 37°C for 10 min. After a second centrifugation in the same conditions the resultant pellet was resuspended in buffer (1.5 mg tissue/400 μl buffer). The incubation mixture contained 400 μl tissue suspension, 200 μl increasing concentrations of [^{3}H]paroxetine (0.02–0.4 nM) and 1.4 ml incubation buffer in the absence and presence of fluoxetine 10 μM. Tubes were incubated for 60 min at 22°C. After rapid filtering through GF/C Whatman filters; the filters were rinsed with 4×5 ml ice cold buffer and placed in vials containing 4 ml liquid scintillation cocktail (Biogreen3, Scharlau). All the determinations were carried out in duplicate.

Immunohistochemistry: Staining was carried out with a polyclonal antibody against GFAP (Sigma). The basic ABC immunohistochemical procedure was as follows: free-floating sections were rinsed in 25 mM Tris-buffered saline (TBS), treated in 0.3% hydrogen peroxide to block endogenous peroxidase followed by 0.3% Triton-X in TBS for 30 min, soaked in carrier medium consisting of 3% goat serum in 0.1% Triton-X–TBS for 1 h. Subsequently sections were incubated in solutions of primary antibody (dilutions of 1:1000, 1:500 and 1:250) in carrier medium overnight. After rinsing, sections were incubated with the secondary antibody, biotinylated goat anti-rabbit (Vector labs) diluted 1:500. After washing with TBS, sections were processed with the avidin–biotin technique (Elite ABC kit, Vector labs) and developed with diaminobenzidine (DAB) and hydrogen peroxide (Vector labs), mounted on gelatine-coated slides, dehydrated through ethanol, cleared in xylene and coverslipped. Negative controls were carried out in sections incubated with no primary antibody. In these sections no noticeable immunostaining was observed.

Drugs: MDMA-HCl was either from Sigma (UK) or was a gift from the Servicio de Restricción de Estupefacientes (Dr L. Domínguez, Madrid); [^{3}H]-paroxetine (22.5 Ci/mmol) was obtained from New England Nuclear (Boston, MA); 5-HT creatinine sulfate and DL-6,8-Thioctic acic (α-lipoic acid) were from Sigma (UK); fluoxetine-HCl was generously donated by Eli-Lilly and Co. (Indianapolis, IN); all other chemicals were from Merck (Darmstadt, Germany).

Statistics: The data were analysed by one-way ANOVA followed by Tukey's *post-hoc* test. For the rectal temperature analysis, two-way ANOVA for repeated measures was used to compare treatment groups. In this case, treatment was used as the between subjects factor and time as the repeated measure. Single time point comparisons between groups were made using Tukey's test. Treatment differences were considered statistically significant at $p < 0.05$.

Results

MDMA-induced serotonergic deficits: As expected, 7 days after a single dose of MDMA (20 mg/kg, i.p.) a significant decrease of 5-HT content (~40–60%; $p \leq 0.05$) was found in the frontal cortex, in the hippocampus and in the striatum of the rat (Fig. 1).

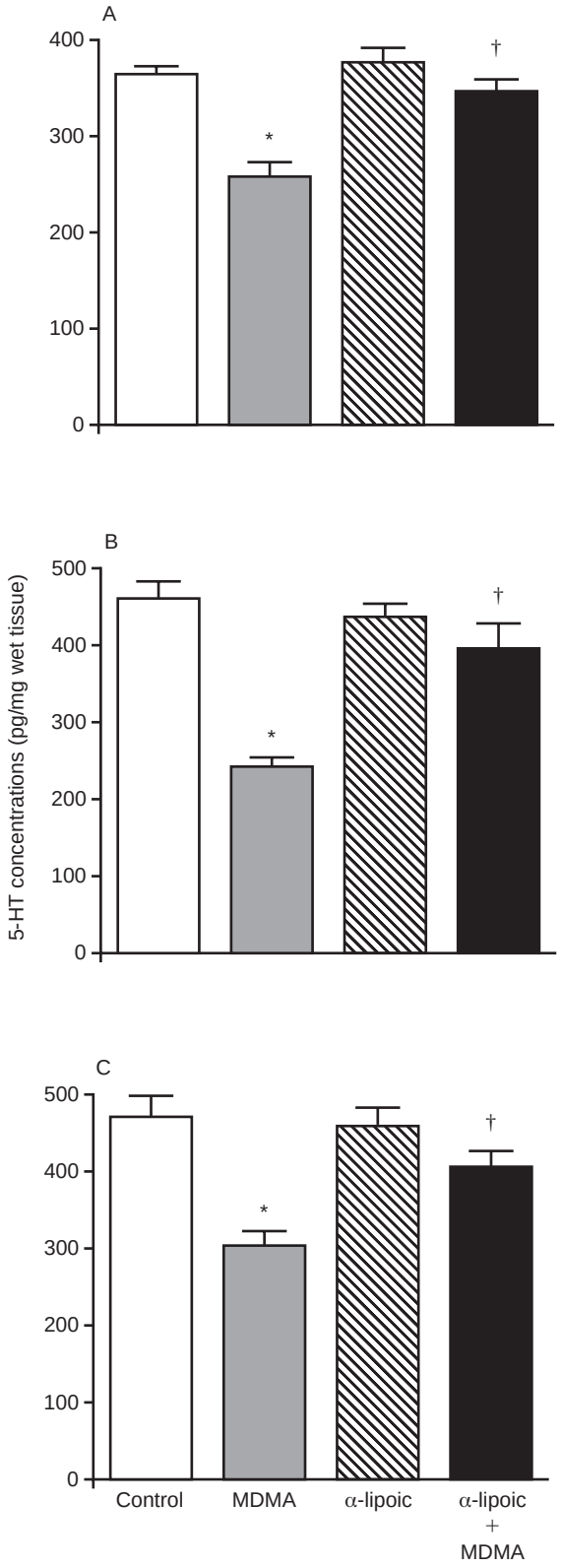

FIG. 1. Effect of MDMA (20 mg/kg, i.p.) on 5-HT content in the frontal cortex (**A**) the hippocampus (**B**) and the striatum (**C**) of the rat. Animals received vehicle or α-lipoic acid 100 mg/kg i.p., b.i.d. for 2 consecutive days. Thirty minutes after the fourth dose of α-lipoic acid or vehicle, rats were injected with either MDMA 20 mg/kg i.p. or saline (control group). Animals were killed 7 days after MDMA administration. Data are means ± s.e.m. pg/mg wet tissue of 8–10 rats. * $p < 0.05$ *vs* control group (vehicle + saline), † $p < 0.05$ *vs* MDMA-treated group (vehicle + MDMA).

Repeated administration of α-lipoic acid (100 mg/kg, i.p.), a dose usually employed in different animal models of neurotoxicity [10], did not produce by itself any change in the concentration of 5-HT; however, it completely prevented the loss of 5-HT induced by MDMA in all the brain regions examined.

Seven days after the administration of MDMA the number of [^{3}H]paroxetine binding sites was significantly decreased ($p < 0.05$), in the frontal cortex and in the striatum (by ~35%) and in the hippocampus (by ~45%). Again, the administration of α-lipoic acid completely prevented MDMA-induced loss of 5-HT transporter density in all the brain regions examined (Fig. 2).

MDMA-induced hyperthermia: MDMA (20 mg/kg, i.p.) caused a significant rise of core temperature which lasted ~4 h. The highest rise in temperature for the MDMA-treated group (~2°C), was obtained during the first 90 min after drug administration. α-Lipoic acid by itself produced a sustained hypothermia (−3.6°C) which lasted for more than 3 h following administration; however, α-lipoic acid pretreatment did not prevent the hyperthermic response to MDMA. The highest rise in core temperature of rats given the combined treatment of α-lipoic acid and MDMA was obtained during the first 90 min (~2.4°C; Fig. 3).

GFAP immunoreactivity: Astrocytes with the typical stellate morphology were observed in control animals, especially in the molecular layer of the dentate gyrus and stratum lacunosum-moleculare of the CA1 region. In sections from MDMA-treated animals an increase in GFAP immunoreactivity in both, the stratum lacunosum-moleculare and the molecular layer of the dentate gyrus was noticeable (Fig. 4). However, the most striking changes could be seen in the pyramidal cell layer of the CA1 region, with marked increase in GFAP-positive cells, which were round and had few or no processes observed at all antibody dilutions.

The treatment of the animals with α-lipoic acid completely prevented the changes in the glial response, and the pattern of GFAP immunoreactivity in these animals matched that of the control animals (Fig. 4). α-Lipoic acid alone did not alter the pattern of GFAP immnoreactivty in the hippocampus.

Discussion

According to previous studies [1], 7 days after a single dose of MDMA (20 mg/kg, i.p.), a marked reduction in 5-HT levels in the frontal cortex, in the striatum and in the hippocampus of the rat was

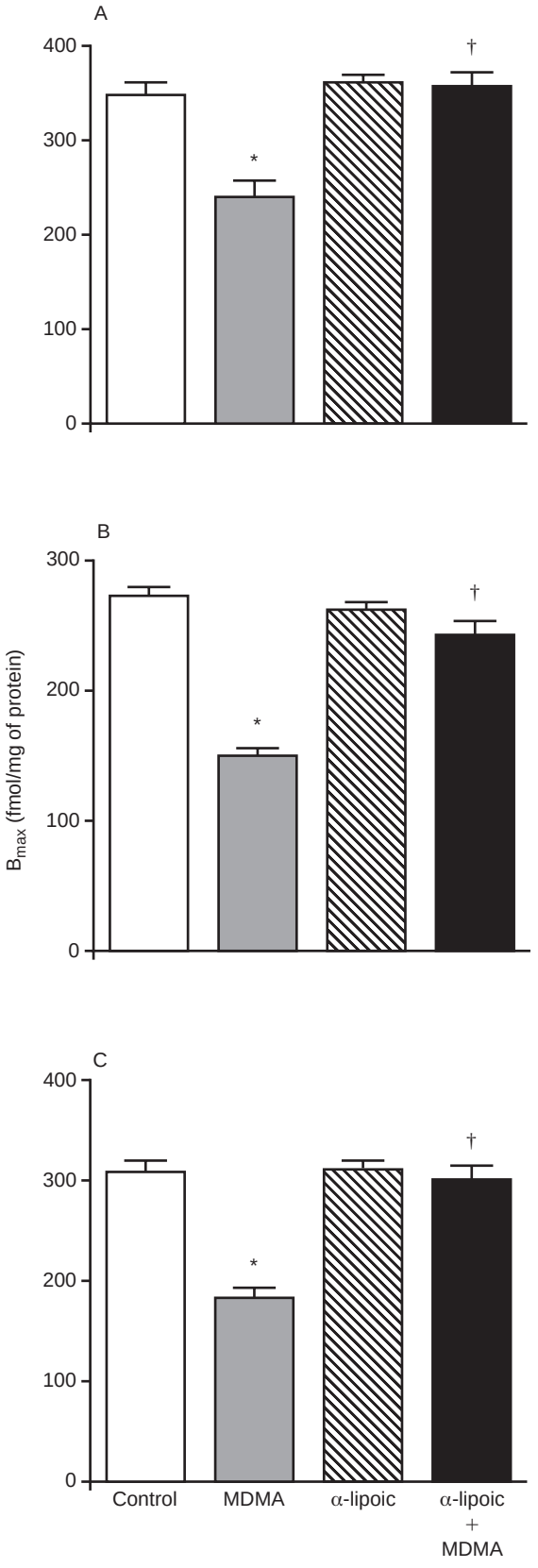

FIG. 2. Effect of MDMA (20 mg/kg, i.p.) on [^{3}H]paroxetine binding in (**A**) the frontal cortex, (**B**) the hippocampus and (**C**) the striatum of the rat. Treatments are described in the legend to Fig. 1. Data are means ± s.e.m. fmol/mg protein of 8–10 rats. * $p < 0.05$ *vs* control group (vehicle + saline), † $p < 0.05$ *vs* MDMA-treated group (vehicle + MDMA).

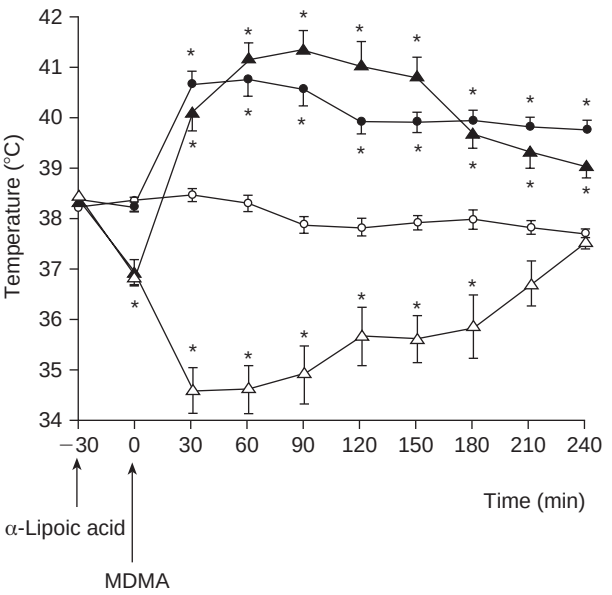

FIG. 3. Rectal temperature of rats after the administration of vehicle followed by saline (**a**, open circles), vehicle followed by a single dose of MDMA 20 mg/kg, i.p. (**b**, closed circles), α-lipoic acid 100 mg/kg i.p., b.i.d. for 2 consecutive days 30 min before saline (**c**, open triangles), or α-lipoic acid 30 min before MDMA (**d**, closed triangles). Values are means ± s.e.m.; $n = 7–10$. * $p < 0.05$ or better *vs* control group (vehicle + saline).

observed. Likewise, [^{3}H]paroxetine binding sites were significantly decreased in the three brain regions suggesting a degeneration of 5-HT nerve terminals [14].

One of the most widely documented reactions to nervous system damage is reactive gliosis [15]. Astrocytic reactivity results in an enhanced expression of GFAP that can be used to localize and quantify chemically induced neurotoxicity [16]. In the MDMA-treated animals, an increase in the hippocampal GFAP immunoreactivity could be seen, specially in the CA1 region, which is considered to be the main termination field of the serotonergic innervation to the rat hippocampus [17]. From a morphological point of view, these astrocytes, although GFAP-immunoreactive, displayed profound morphological differences from the normal; however, this is not the first report that describes this kind of glial response [18].

It has been suggested that MDMA-induced neurotoxicity occurs when endogenous free radical scavenging mechanisms become overwhelmed or exhausted [19]; however, there is some controversy about the mechanisms by which MDMA increases free radical formation. It has been proposed that MDMA generates reactive oxygen species as a result of its metabolism into catechols and reactive quinones [4,20]. Other authors have suggested that the large concentrations of extracellular dopamine that follows MDMA could be the source of reactive

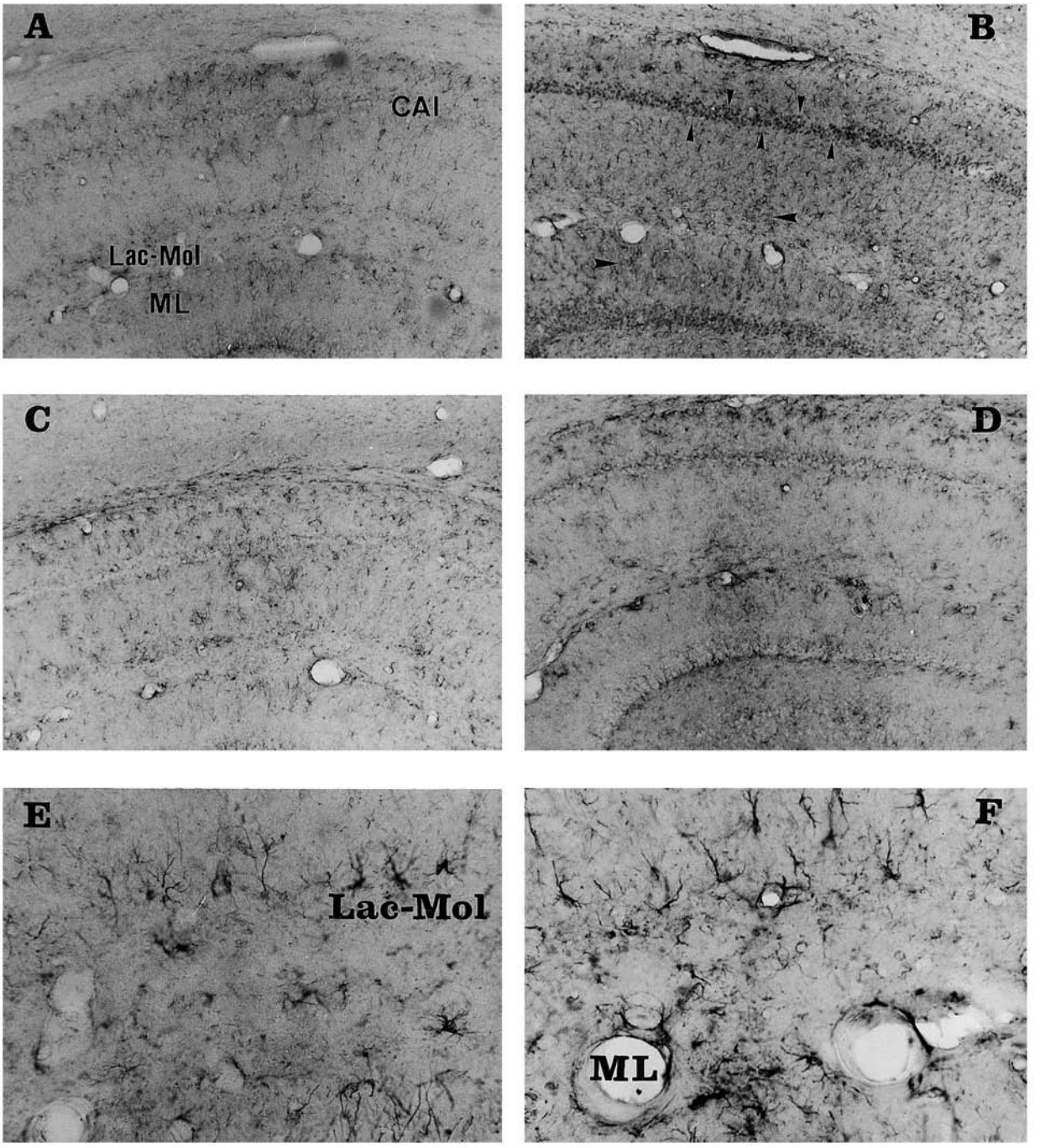

FIG. 4. GFAP immunoexpression at low magnification (×10) in sections of animals treated with (**A**) vehicle, (**B**) MDMA 20 mg/kg i.p., (**C**) α-lipoic acid 100 mg/kg i.p., b.i.d. for 2 consecutive days, (**D**) α-lipoic acid + MDMA. Note that the increase in GFAP immunoreactivity after the administration of MDMA (see in B the CA1 region, molecular layer and stratum lacunosum-moleculare) is prevented by the treatment with α-lipoic acid (D). A higher magnification (×25) showing the increases in GFAP immunostaining induced by MDMA administration (**F**) compared with control animals (**E**). ML molecular layer of the dentate gyrus; Lac-Mol: stratum lacunosum-moleculare.

oxygen species responsible for 5-HT neurotoxicity [5]. Another hypothesis proposed by Poblete and Azmitia [21] suggests that 5-HT released by MDMA increases glycogen phosphorylase activity promoting the breakdown of glycogen and ultimately depriving the neuron of its energy source. Depletion of available energy may then contribute to final terminal degeneration [22].

Independently of the source of free radicals, our results indicate that the metabolic antioxidant α-lipoic acid administered before MDMA completely prevents the loss of 5-HT content and the decrease of [^{3}H]paroxetine-labeled 5-HT transporters in the frontal cortex, hippocampus and in the striatum and also abolishes the increases in the glial response observed in the hippocampus 7 days after MDMA.

Finally, there appears to exist a correlation between hyperthermia and neurotoxicity engendered by MDMA [23]. Furthermore, many drugs that protect against MDMA-induced neurotoxicity lower the core temperature of the rats, and this protection is abolished when temperature of the animals is kept elevated [3,9–11]. If we take into account that formation of hydroxyl radicals in the brain is a temperature-dependent process [24,25], this would explain why drugs that cause hypothermia prevent MDMA-induced toxicity, and also why hyperthermia exacerbates its damage. In contrast, α-lipoic acid afforded complete protection against MDMA-induced damage even though it did not prevent the hyperthermic effect of MDMA.

Conclusion

Whether MDMA-induced free radical formation and further neurotoxicity is due to the depletion of synaptic energy stores; to the metabolism of MDMA or to the oxidation of dopamine inside the serotonergic terminals, still remains unknown, however, the present findings support the hypothesis that MDMA-induced neurotoxicity is related to oxidative damage as it is prevented by the potent metabolic antioxidant α-lipoic acid. It is worth noting that α-lipoic acid afforded complete protection against MDMA-induced serotonergic deficits even though it did not prevent the acute hyperthermia induced by MDMA. This and other aspects of regulation of cell functions by α-lipoic acid may account for its therapeutic effects in pathologies with redox imbalances and perhaps contribute to healthy ageing.

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