# DNA fragmentation and oxidative stress in the hippocampal formation: a bridge between 3,4-methylenedioxymethamphetamine (ecstasy) intake and long-lasting behavioral alterations

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**Intake of 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) in humans leads to marked behavioral alterations. In a recent paper, we demonstrated that chronic MDMA intake produces a latent hippocampal hyperexcitability that parallels a reduced threshold for limbic seizures and a slowing of electroencephalographic activity. These phenomena suggest an alteration in hippocampal function. So far, only a few studies have focused on the hippocampal formation as a potential target for the effects induced by MDMA. In this study we sought to evaluate whether the intrinsic cells of the hippocampus might be modified chronically by ecstasy intake. In particular, we examined whether administration of MDMA, at doses producing hippocampal hyperexcitability also produces rearrangements of DNA strands measured by the comet assay. We found that MDMA, at very low doses, comparable with those self-administered by humans, produces acute oxidative stress and DNA single and double-strand breaks, which persist together with long-lasting metabolic changes in the hippocampal formation. These persisting effects are accompanied by behavioral sensitization, reduced seizure threshold and long-lasting slowing of electroencephalographic activity, and hyperexcitability of the hippocampus, without affecting the basal ganglia. The present data indicate that the intake of very low doses of MDMA, comparable to those consumed by humans, produces selective hippocampal alterations which may underlie cognitive impairment and seizure susceptibility. *Behavioural Pharmacology* 18:471–481 © 2007 Lippincott Williams & Wilkins.**

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## Introduction

The amphetamine derivative 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) is widely abused by humans and leads to acute and chronic behavioral alterations (Parrott *et al.*, 2001; Landry, 2002). These include changes in neuronal excitability, which are responsible for convulsive seizures occurring immediately following MDMA intake (Zagnoni and Albano, 2002; Ben-Abraham *et al.*, 2003), and long-lasting alterations in electroencephalographic (EEG) activity in chronic ecstasy abusers. These latter effects are associated with persistent behavioral changes (Dafters *et al.*, 1999; Gamma *et al.*, 2000). Consistent with these findings, recent data demonstrated that, in mice, repetitive doses of MDMA produce persistent changes in EEG activity (Giorgi *et al.*, 2005). These effects are associated with a reduced seizure threshold following injection of kainic acid.

Oxidative stress plays a major role in MDMA-induced neurotoxicity and various experimental studies have shown that systemic MDMA administration increases free radicals and oxidative species (Cadet *et al.*, 2001; Shankaran *et al.*, 2001; Camarero *et al.*, 2002). In contrast, overexpression of superoxide dismutase (SOD) type 1 reduces MDMA toxicity, both *in vitro* (Cerruti *et al.*, 1995) and *in vivo* (Jayanthi *et al.*, 1999). Therefore, in this study we used different techniques to study whether acute oxidative stress may lead to long-lasting hippocampal alterations and behavioral effects induced by MDMA. In particular, we determined, first, whether small doses of MDMA produce oxidative stress in the hippocampus compared with striatum. For this purpose, we measured the activity of SOD type 1, glutathione (GSH) levels and lipid peroxidation following MDMA. As we reported previously that MDMA produces long-lasting behavioral changes suggesting neuronal plasticity in the hippocampus, we evaluated whether oxidative stress may also lead to rearrangement in DNA structure in hippocampal cells. To do this we employed the comet assay as a sensitive

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technique to measure DNA strand breaks following oxidative stress in the hippocampus and striatum.

We further investigated whether oxidation-induced DNA rearrangements were accompanied by persistent hippocampal hyperexcitability and behavioral sensitization to locomotor activity and limbic seizures, and whether these effects were related to persistent alterations in the hippocampus but not in the striatum. Although species differences play a significant role in the effects induced by MDMA (de la Torre and Farre, 2004), the occurrence of these effects at very low doses makes it more likely that such a specific phenomenon extends to ecstasy abusers.

Thus, we measured acute oxidative stress occurring immediately after MDMA administration in the hippocampus and striatum and evaluated the oxidative stress on the DNA of intrinsic hippocampal cells by using the comet assay at early and delayed time intervals following MDMA. Similarly, we measured the early and delayed metabolic activation measured by the uptake of [$^{14}$C]2-deoxyglucose ([$^{14}$C]2-DG).

To relate early MDMA-induced hippocampal alterations with the delayed behavioral consequences described in abusers, we evaluated the susceptibility to kainate-induced limbic seizures at prolonged time intervals. At this time interval we also evaluated the persistence of hippocampal hyperexcitability; this was tested by measuring brain metabolism in the hippocampus and striatum after a low dose of kainic acid during MDMA withdrawal. At the same time interval we measured the long-lasting effects on the DNA integrity at the striatal and hippocampal levels.

## Methods

### Subjects and experimental design

Male C57 black mice (C57BL/6J), 9–10 weeks old, were obtained from Harlan (San Pietro al Natisone, Italy). Animals were housed in the animal facility, with free access to food and kept under closely controlled environmental conditions (12 h light/dark cycle, lights on between 07.00 and 19.00 h). As the toxicity of amphetamine derivatives is highly variable, critically depending on room temperature and number of animals per cage, the temperature was kept constant at 21°C. Twenty-four hours before treatment, mice were housed one per cage (size of the cage: 11 × 10 cm wide and 15 cm high). Mice were treated in accordance with the Guidelines for Animals Care and Use of the National Institute of Health. All possible efforts were made to reduce animal suffering and to reduce the number of animals used, and all procedures were approved by the local Animal Care Committee.

On the day of the experiment, mice received intraperitoneal (i.p.) injections of MDMA hydrochloride (2.5 mg/kg daily, for 5 days) or saline (controls). Some mice in each group were killed immediately after the last MDMA injection to measure the occurrence of DNA breaks, SOD activity, lipid peroxidation and GSH levels, or 5 days later to measure further the DNA breaks. To measure brain metabolism, we used the [$^{14}$C]2-DG assay (Sokoloff *et al.*, 1977); some mice were injected with the radioactive glucose analogue [$^{14}$C]2-DG 1 h before the killing either on day of last MDMA injection or 5 days later.

Finally, a further subgroup of MDMA-treated or saline-treated mice was killed at 14 days after the last MDMA injection, after being EEG recorded, and evaluated for seizure threshold.

All mice were killed by decapitation and brain samples were used to measure the DNA damage, SOD type 1 activity, lipid peroxidation, GSH levels, and catecholamine content (see later).

For measuring markers of oxidative stress, on the day of the analysis brain samples were sonicated (VibraCell, Sonics & Materials, Inc., Newtown, Connecticut, USA) in phosphate-buffered saline, pH 7.4 (Sigma, St Louis, Missouri, USA). Homogenates were centrifuged at 18 000 *g* for 8 min, at 4°C, and supernatants were used for the biochemical assays.

For each procedure, groups were composed of 5–7 mice.

### Locomotor activity during 3,4-methylenedioxymethamphetamine treatment

During MDMA administration mice were monitored to record the MDMA-induced increase of the locomotor activity. After each injection and 5 days after the last MDMA dose mice were put into a box (80 × 80 × 20 cm) with transparent walls and a floor divided into 64 squares of 100 cm$^2$ each. For each mouse, locomotor activity was measured by counting open field activity and rearing episodes. These were measured, daily, by two independent, blind observers. In particular, open field activity was evaluated by the number of squares crossed by the mice with all four legs during constant time intervals, each lasting 3 min. Rearing behavior was evaluated using the same box with transparent walls, with a normal, white floor. Rearing episodes were counted during periods lasting 3 min and each episode was defined by the rearing of the mouse standing on the hind limbs.

### Electroencephalographic evaluation

Five mice per group were implanted with epidural electrodes 5 days after MDMA/saline administration. Briefly, they were anaesthetized with chloral hydrate

(400 mg/kg, i.p.) and placed in a stereotaxic apparatus. Each mouse was implanted with one electrode (active recording electrode) above the left frontal cortex, and one (reference electrode) below the occipital bone. Electrodes consisted of 1.4-mm-diameter stainless-steel microscrews (Centrostyle, Vedano Olona, Varese, Italy), connected to a female pin, and kept in place by dental acrylic cement; for each mouse a reference electrode (above cerebellum) and one recording electrode (above left-frontal cortex) were used.

Starting at 7 days after surgery (i.e. 12 days after the last dose of MDMA), two EEG recording sessions, 48 h apart, were performed for each animal, and mice were killed after the second recording (i.e. 14 days after the last dose of MDMA).

Each EEG session used freely moving mice in a Faraday cage. During the first recording session only baseline EEG was collected whereas during the second recording session (i.e. at 14 days after MDMA) EEG was collected for up to 1 h after i.p. injection with kainic acid (10 mg/kg, see below). On the day of recording the female electrode microconnectors were connected via shielded cables to a dedicated digital EEG apparatus (BE-Light, EBNeuro, Florence, Italy). Before each recording session electrode impedance was measured and only signals recorded from electrodes with impedance $< 3\,k\Omega$ were used for analysis. EEG signals were notch-filtered, low-pass and high-pass filtered (64 and 0.5 Hz, respectively), and stored with a 128 Hz sampling rate on a PC equipped with the software Galileo-NT (EBNeuro), for off-line analysis.

The power spectral analysis of the EEG was performed by computing the fast Fourier transformation (Cooley and Tukey, 1965), via the spectral analysis software of GAL-NT (EBNeuro). In particular, the mean EEG power spectrum for the range of frequencies between 0.1 and 64 Hz was calculated from 64-s long segments, by averaging the power spectrum of its 8-s long consecutive epochs.

### Seizure evaluation

At 2 weeks after the last dose of either MDMA or saline, each mouse was challenged with a single injection of kainic acid (Sigma Chemical Co.) at the dose of 10 mg/kg i.p., during EEG recording, to evaluate the potential effects of MDMA on limbic seizure threshold. Behavioral seizures were rated by an observer blind to the treatment and scored according to a modified Racine's scale for limbic seizures (Racine, 1972; Giorgi *et al.*, 2005), as follows: score 1 = repetitive intense jaw clonus; score 2 = forelimb clonus; score 3 = brief (< 5 s) rearing with forelimb clonus; score 4 = prolonged (> 10 s) bilateral forelimb clonus; score 5 = clonus of the four limbs with rearing and loss of balance.

### Biochemical procedures: HPLC and antioxidant activity analysis

Monoamines were measured by HPLC with a reverse-phase column ($250 \times 4.5$ mm, $C_{18}$, SGE) and two coulometric electrochemical detectors as described previously (Fornai *et al.*, 1999). The mobile phase consisted of a citrate-phosphate buffer (0.04 mol/l citric acid, 0.06 mol/l $Na_2HPO_4.2H_2O$) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.6 mM 1-heptanesulphonic acid sodium salt, and 10% methanol.

The standard curve for each compound was calculated using regression analysis of the ratios of the peak areas for various concentrations of each compound recorded at the reducing electrode.

The determination of antioxidant activity was carried out by using specific colorimetric kits for Cu/ZnSOD type 1 activity (Dojindo Molecular Technologies Inc., Gaithersburg, Maryland, USA), GSH content (Chemicon International Inc., Temecula, California, USA) and lipid peroxidation (malondialdehyde/4-hydroxyalkenal concentrations; Calbiochem, San Diego, California, USA). Readings were performed on a colorimetric microplate reader (Model 550, Bio-Rad, California, USA).

Protein content of brain samples was estimated using a commercially available kit (Bio-Rad), after dissolving the precipitates in 0.5 N NaOH.

### Evaluation of DNA damage through the comet assay

DNA integrity was evaluated by the use of alkaline single cell gel electrophoresis or comet assay, according to Singh *et al.* (1988) with minor modifications.

Briefly, isolated cells were embedded in agarose on a microscope slide, lysed with detergents, and treated with high salts. Any breaks present in the DNA cause the supercoiling to relax locally and negatively charged loops of DNA are then free to extend and migrate in the electric field toward the anode as a 'comet tail'.

After killing, separate specimens from the hippocampal area were washed in cold PBS and then placed in 1 ml of chilled mincing solution (HBSS $Ca^{2+}$, $Mg^{2+}$ free, 20 mM $Na_2EDTA$, 10% dimethylsulfoxide, pH 7.5). The tissue was cut in to small pieces by scissors. After 15 min, the supernatant was centrifuged for 10 min at 1000 rpm. Assessment of cell viability in individual cells was not possible under these experimental conditions because during the mincing process the cell membrane was disrupted (Singh *et al.*, 1995). For this reason, to check for potential occurrence of brain cell death, a small portion of each area was fixed in 10% formaldehyde, dehydrated, and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin to detect the occurrence of cell death (Sasaki *et al.*, 1999; Tsuda *et al.*, 2000).

The pellet obtained was mixed with 75 µl agarose (0.5% low-melting point agarose, prepared in $Ca^{2+}$-$Mg^{2+}$-free PBS) and layered on conventional slides, pre-dipped in 1% normal melting point agarose (NMPA) (Klaude *et al.*, 1996). Then, a third layer of 85 µl low-melting point agarose was added. Slides were immersed in ice-cold freshly prepared lysis solution (2.5 mol/l NaCl, 100 mM $Na_2EDTA$, 10 mM Tris–HCl, 1% Triton X-100 and dimethylsulfoxide 10%, pH 10) to lyse the cells and allow DNA unfolding. After 1 h at 4°C in the dark, slides were covered with an alkaline solution (1 mM $Na_2EDTA$, 300 mM NaOH, pH > 13) in horizontal electrophoresis units for 20 min to allow DNA unwinding and expression of alkali-labile sites. Successively, slides underwent electrophoresis (25 V; 300 mA, 20 min) in an ice-cold bath. Next, slides were washed gently with a neutralization buffer (0.4 mol/l Tris–HCl, pH 7.5) to remove alkali and detergents, dipped in 100% cold methanol and dried. After drying, slides were stained with 100 µl ethidium bromide (2 µl/ml). All the steps described above were conducted under yellow light or in the dark, to prevent nonspecific DNA damage. DNA migration is proportional to the level of DNA damage.

### The comet assay: background, principles, and applications

Cells embedded in agarose on a microscope slide were lysed with detergent and high salt to form nucleoids containing supercoiled loops of DNA linked to the nuclear matrix. Electrophoresis at high pH results in structures resembling comets, observed by fluorescence microscopy; the intensity of the comet tail relative to the head reflects the number of DNA breaks. The rationale for this is that loops containing a break lose their supercoiling and become free to extend toward the anode. DNA breaks may represent the direct effect of some damaging agent, or they may be intermediates in cellular repair. DNA strand breaks may also come from the action of free radicals indirectly generated by oxidative stress processes (Tice *et al.*, 2000; Collins, 2004). The comet assay has applications in testing novel chemicals for genotoxicity, monitoring environmental contamination with genotoxins, and fundamental research in DNA rearrangements.

Owing to the versatility of the technique, the comet assay has been used successfully during the last few years (for a brief review see Frenzilli *et al.*, 2006). As oxidative stress is known to play a key role in a number of neurodegenerative disorders like Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, recent studies have applied the comet assay to evaluate the possible loss of DNA integrity in such patients (see, e.g. Migliore *et al.*, 2002).

Increasing attention has been paid to the effects of drugs of abuse in target areas. A significant increase in single-strand breaks in rat brain cells was found after ethanol administration (Singh *et al.*, 1995). Furthermore, Shafer *et al.* (1994) demonstrated by comet assay that if morphine exposures persist through one or more cell cycles, then DNA rearrangements occur.

The alkaline version of the comet assay has been used to evaluate the potential DNA alterations caused by MDMA in mouse brain cells (Fornai *et al.*, 2004). The data obtained in our laboratory indicate that high doses of MDMA induce DNA fragmentation and oxidized bases in monoaminergic as well as nonmonoaminergic neurons in the nigrostriatal pathway.

Such findings might help to explain long-term changes in chronic MDMA abusers. Thus, the use of the comet assay under the present experimental conditions, whereas the effects on DNA were studied following very low doses of MDMA comparable with those abused by humans, is particularly relevant.

### Measure of local cerebral glucose utilization

Besides MDMA-induced changes of brain electrical activity, we wanted to evaluate whether such functional alteration was also accompanied by brain metabolism modification, both in acute and chronic conditions. For this purpose, we used the autoradiographic [$^{14}$C]2-DG method. In particular, brain [$^{14}$C]2-DG uptake was measured either immediately after the last MDMA injection, or 5 days later. In addition, [$^{14}$C]2-DG uptake was measured in mice administered kainic acid at a subthreshold dose of 10 mg/kg 1 h or 5 days after the last MDMA injection.

These studies were carried out according to the procedure originally described by Sokoloff *et al.* (1977), with some modifications as reported by Giorgi *et al.* (2005). Briefly, 1 h after administration of [$^{14}$C]2-DG (125 µCi/kg, specific activity: 60 mCi/mM; Amersham, UK), mice were killed by decapitation and brains were removed rapidly, frozen in isopentane at −40°C and then stored at −80°C. Coronal brain sections (20 µm), cut in a cryostat at −21°C, were dried and then exposed for 10 days and autoradiographed on Kodak Min-R radiograph films (Kodak, Rochester, NY, USA), along with a set of calibrated $^{14}$C plastic standards (Amersham, Milan, Italy). Autoradiograms were analyzed by quantitative densitometry using a computerized image-processing system (NIH Imaging, Bethesda, Maryland, USA). In particular, optical density measurements were made in at least eight sections per mouse. Corpus callosum was used as a control area, as it does not incorporate the tracer.

### Statistical analysis

For locomotor activity (open field activity and rearing) each mouse was tested twice daily, 2 min apart. Results obtained by each observer for each mouse were considered for each count. The cumulative data are shown as the mean ± SEM from groups of 5–7 mice.

For the biochemical assay, results from control and treated mice are expressed as the mean ± SEM of values obtained from the same groups of mice.

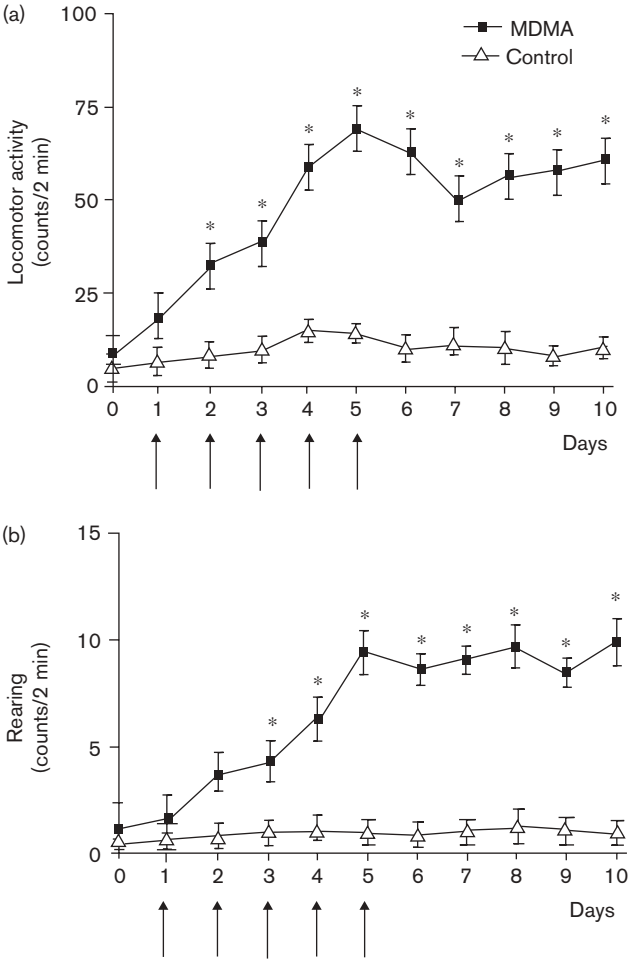
The effects of MDMA on behavior, monoamines and [$^{14}$C]2-DG uptake were compared using analysis of variance (ANOVA) with Sheffè's post-hoc analysis. The null hypothesis was rejected when $P < 0.05$.

For the comet assay, nuclei were observed individually under a fluorescence microscope (×200) and an image analyzer (Kinetic Imaging Ltd, Komet, version 4, Liverpool, UK) was used to evaluate the percentage of DNA migrated in the tail of at least 25 cells per slide. Slides were coded and scored blindly to avoid risk of bias. Four parallel tests per experimental point were performed for a total of at least 100 cells, and the mean calculated. The data were analyzed using multifactor ANOVA (MANOVA).

For the EEG data, 64-s-long epochs were selected randomly from EEG segments recorded during waking immobility to avoid potential movement artifacts. Each power spectrum obtained from the 64-s-long segments was subdivided into eight groups of frequencies ranging 1.5 Hz, starting from 0 Hz (0–1.5, 1.5–3, 3–4.5 Hz, and so on, up to 12 Hz) and the absolute and relative power was calculated for each range of frequencies and expressed as spectral mean percentage relative power ± SEM. For each frequency range, the relative powers from control animals and MDMA-pretreated mice were compared with ANOVA.

Analysis of seizures was carried out for each group by measuring behavioral seizure incidence and maximal seizure score. Difference in maximal seizure score was compared with $\chi^2$ (data are shown in Fig. 1 as mean ± SEM, for clarity).

**Fig. 1**




Locomotor activity and rearing induced by MDMA. Repeated injections of MDMA, as indicated by arrows, induced a marked increase in the locomotor activity (a) and rearing (b), which remained elevated for 5 days after the last MDMA administration. *$P$<0.05 compared with controls. MDMA, 3,4-methylenedioxymethamphetamine.

## Results

### Locomotor activity

MDMA treatment induced an increase in the locomotor activity and rearing, as shown in Fig. 1. In particular, locomotion was increased significantly compared with saline-injected mice starting from the second MDMA dose, and progressively increased up to the fifth day, corresponding to the last MDMA dose. A similar trend was observed for rearing behavior that was statistically significant compared with controls starting from the third MDMA administration (Fig. 1).

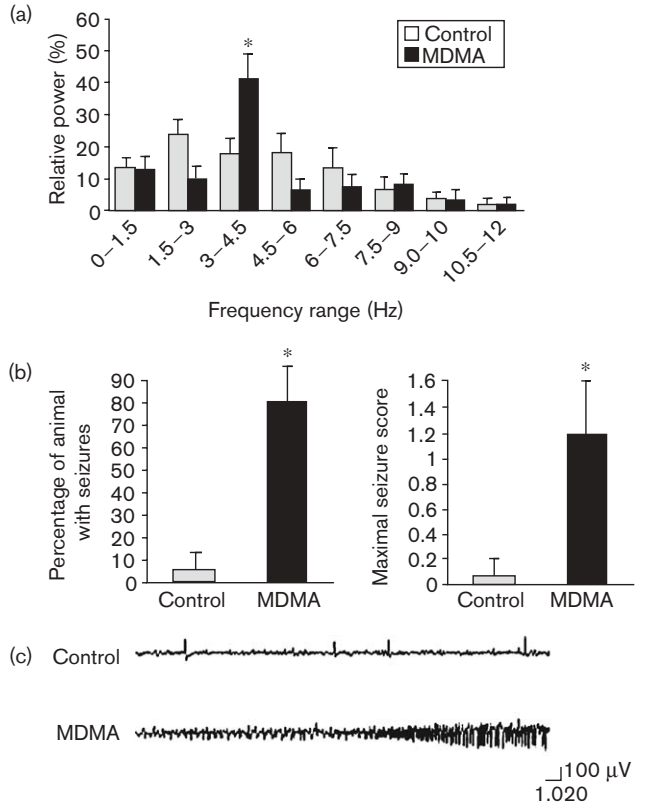
### Electroencephalographic recordings

EEG recordings at 12 days after the last MDMA/saline administration showed significant differences between the two groups. In particular, in MDMA-pretreated mice we observed a peak in the relative EEG power in the range of frequencies between 3 and 4.5 Hz, which was significantly different from the relative power, for the same range of frequencies, in EEG of controls (Fig. 2a). Furthermore, in control mice relative power was higher than in MDMA-pretreated mice for 1.5–3, 4.5–6, and 6–7.5 Hz intervals, even though these differences were not statistically significant.

### Limbic seizures

Behavioral seizures were observed in four out of five of the MDMA-treated mice, which had been injected with a subthreshold dose (10 mg/kg) of kainic acid (Fig. 2b, left graph); two mice showed a maximal seizure score of 2 and the remaining animals showed only strong jaw clonus (i.e. stage 1; Fig. 2b, right graph). In all mice, seizures were accompanied by recruiting polyspike/spike and wave activity in the EEG (Fig. 2c). In contrast, none of the saline-pretreated mice showed behavioral seizures, even though EEG sometimes showed interictal spikes (see Fig. 2c).

**Fig. 2**




Seizure and EEG in MDMA pretreated mice. (a) The relative power spectrum of the EEG collected at 12 days after MDMA (2.5 mg/kg/daily for 5 consecutive days) or saline administration. In MDMA pretreated mice there was a peak in the relative EEG power in the range of frequencies between 3 and 4.5 Hz, significantly different from the relative EEG power, for the same range of frequencies, of controls. (b) The effect of kainic acid (KA, 10 mg/kg i.p.) in mice receiving the first MDMA or saline 14 days before. A significant difference was observed in seizure occurrence (left graph) and maximal seizure score (right graph) in the two groups. (c) Two representative epidural EEG traces (recorded from left-frontal cortex versus reference), collected at 10 min after injection of kainic acid (10 mg/kg i.p.) in mice that had been treated 14 days before with saline (upper trace) or MDMA (lower trace). Although in the EEG from a saline-pretreated mouse there are only sporadic epileptiform spikes, the EEG of a MDMA-preinjected mouse shows seizures featured by recurrent recruiting spike and polyspike activity, one of which is shown in the figure. $^{*}P<0.05$ compared with controls. EEG, electroencephalogram; MDMA, 3,4-methylenedioxymethamphetamine.

### Monoamine levels

Systemic administration of MDMA in mice (2.5 mg/kg, i.p., daily for 5 consecutive days) did not induce any significant decrease in hippocampal or striatal monoamine levels at 5 days after the last MDMA injection, as assessed by measurements of serotonin (5-HT), dopamine, and their metabolites levels (Fig. 3).

### Comet assay

Despite the lack of biochemical and histological damage, EEG changes were accompanied by a significant increase in DNA fragmentation in the hippocampus. Interestingly, when we evaluated the effects of MDMA on DNA integrity in limbic neurons (measured as DNA migration under electrophoresis, % DNA in the comet tail), we found that the hippocampus exhibited a statistically significant increase of DNA migration immediately and 5 days after the last MDMA dose (Fig. 4). The occurrence of a pronounced effect within the hippocampus was confirmed by DNA fragmentation data obtained immediately after the end of MDMA administration, as shown in Fig. 5, when this brain region showed a significant effect compared with controls. In particular, when animals were killed soon after the end of the treatment, a 50% increase of DNA migration ($P < 0.001$) was detected in the hippocampus, in the absence of any effect on DNA integrity within the striatum (Fig. 4). The increase of DNA damage acutely observed in the hippocampus reached a 100% increase at 5 days after the last MDMA injection (Fig. 4). These findings indicate a higher susceptibility of the hippocampus to DNA single and double-strand breaks produced by MDMA, which occurred in the absence of cell death.

### Oxidative stress

When we examined the levels of GSH immediately after the last MDMA injection, a pronounced decrease was found within the hippocampus (−38%) (Fig. 6a). Consistent with this finding, we observed a significant loss of Cu/Zn SOD activity in the hippocampus of MDMA treated mice (−57%) (Fig. 6b). A similar trend was observed for lipid peroxidation levels within the cerebral areas considered, although this effect was not statistically significant (Fig. 6c).

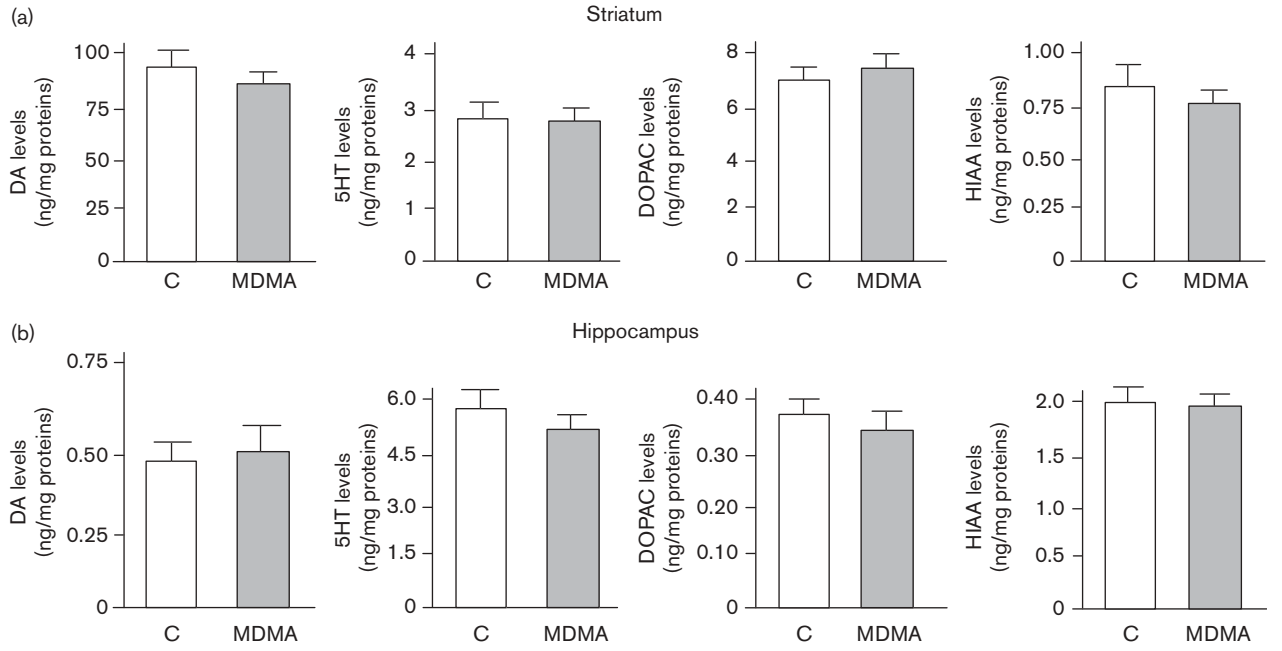
### Local cerebral metabolic glucose utilization

Increased [$^{14}$C]2-DG uptake was observed in the basal ganglia but it was most prominent within limbic brain regions (acute metabolic changes) in mice killed at 1 h after the last MDMA dose (Fig. 7). Levels of [$^{14}$C]2-DG uptake went back to control values in mice killed at 5 days after MDMA treatment (Fig. 7). Injection of kainic acid (10 mg/kg) in naive mice produced a marked activation of the hippocampus (Fig. 7a and b) and a slight activation in the striatum (Fig. 7c and d). This was further enhanced in the hippocampus of those mice that had been treated previously with MDMA, whereas no such enhancement could be detected in the striatum of these mice (Fig. 7).

## Discussion

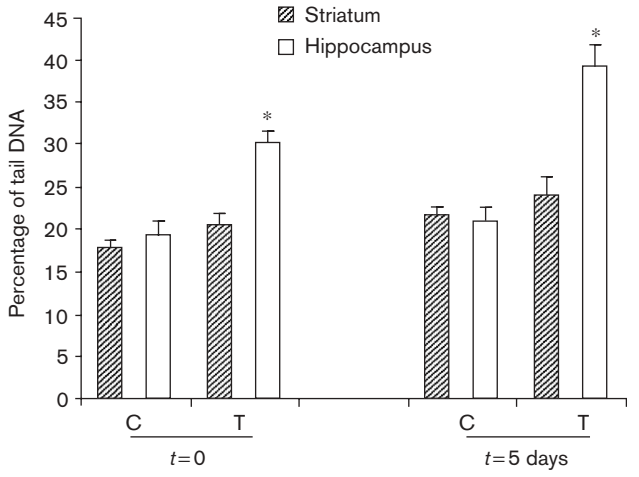
This study demonstrates that administration to mice of very low doses of MDMA (2.5 mg/kg) for 5 days produces oxidative stress specifically in the hippocampus but not in the basal ganglia. Oxidative stress produces an oxidation of DNA and a loss of DNA integrity, specifically in the hippocampal formation but not in the striatum. The oxidative effects induced by MDMA were not sufficient

**Fig. 3**




Striatal and hippocampal monoamine content following repeated MDMA administrations. Repeated injections of MDMA (2.5 mg/kg daily, for 5 days) did not affect levels of 5-HT, DA and their metabolites in the striatum and hippocampus of MDMA-treated mice compared with controls. DA, diamine; MDMA, 3,4-methylenedioxymethamphetamine.

**Fig. 4**

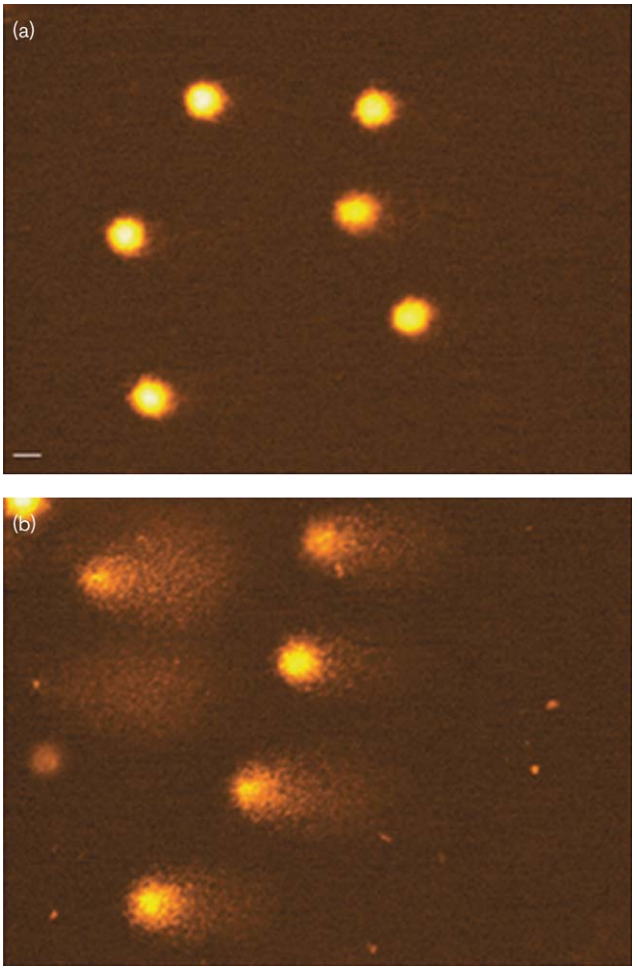



Effects of repeated MDMA treatment on DNA integrity. MDMA administration selectively produced DNA damage in the hippocampus soon after the last injection ($t=0$), in the absence of striatal DNA damage. Such a loss of hippocampal DNA integrity persisted 5 days after the last treatment in mice treated from 10 to 5 days before ($t=5$ days). *$P<0.01$ compared with controls. MDMA, 3,4-methylenedioxymethamphetamine.

to affect the hippocampal and striatal monoamine systems. These low doses, however, produced DNA oxidation and hippocampal DNA plasticity in combination with increased glucose uptake immediately after MDMA administration. Following these acute effects, long-term changes occurred including the onset of slowed baseline EEG activity and increased susceptibility to limbic convulsive seizures and behavioral motor sensitization. The onset of long-lasting behavioral and EEG alterations was sustained by the persistence of hippocampal (but not striatal) DNA alterations and development of hippocampal (but not striatal) metabolic hyperexcitability as revealed by [$^{14}$C]2-DG autoradiography. No striatal or hippocampal loss of dopamine and serotonin was observed at later time intervals following these doses of MDMA.

The increase of DNA alterations in the hippocampal cells found at the end of MDMA treatment was about 50%; and it was further increased at 5 days after treatment, specifically within the hippocampus. In contrast, we found a generalized activation of glucose metabolism immediately after MDMA administration, whereas during MDMA withdrawal low doses of kainic acid produced an exaggerated metabolic activation only in mice treated previously with MDMA. Even in this case, the phenomenon only involved the hippocampal formation. The electrophoretic migration of DNA may be due to oxidative cell damage, as we found previously an increase in caspase III activity in the hippocampus but not in the striatum following high MDMA doses (Tamburini *et al.*,

**Fig. 5**




Hippocampal nuclei. Electrophoresis migration of ethidium bromide-stained nuclei of hippocampal cells in control animals (a) and in mice treated with MDMA (2.5 mg/kg daily, for 5 days) (b). Scale bar = 20 μm. MDMA, 3,4-methylenedioxymethamphetamine.

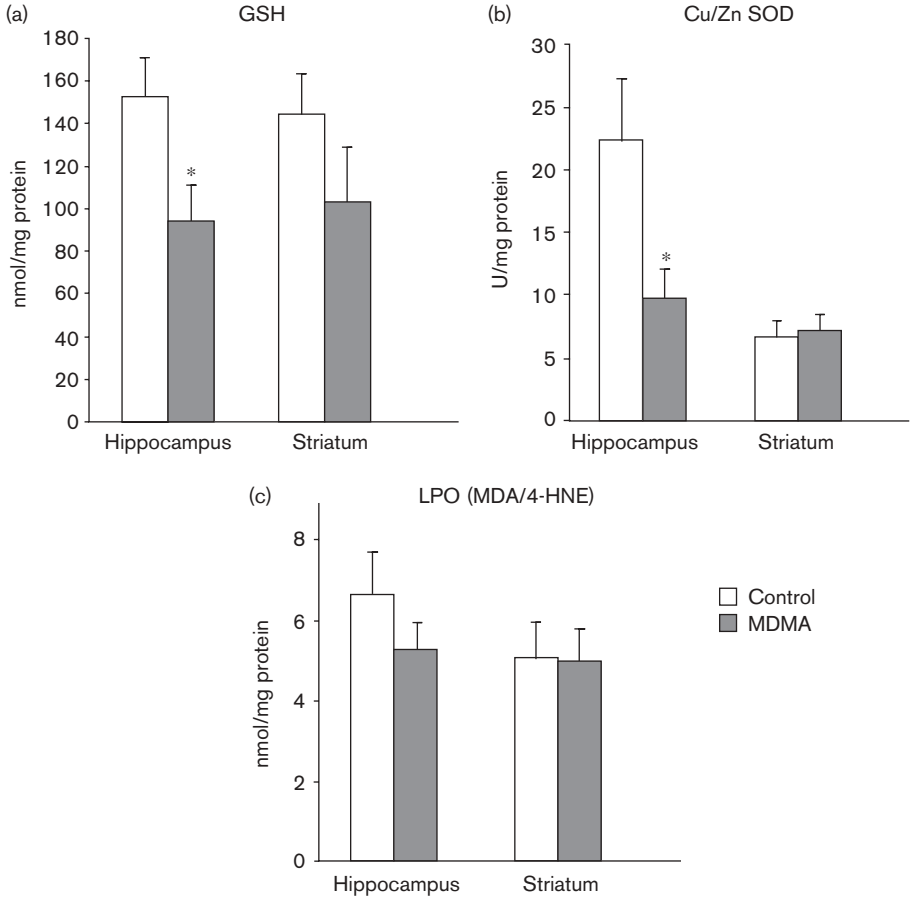
2006). For these low doses, however, it is more likely that they reflect adaptive changes of DNA within hippocampal cells, persisting chronically, following MDMA administration. Further studies are needed to establish potential morphological correlates.

The action of MDMA has been associated with intracellular generation of free radicals and reactive oxygen species, such as hydrogen peroxide, superoxide anion (Jayanthi *et al.*, 1999), and hydroxyl radicals (Coyle and Puttfarcken, 1993). In this study we observed oxidative stress, as shown by the loss of GSH and SOD type 1 in the hippocampus but not in the striatum.

One crucial effect of reactive oxygen species is the oxidative damage of nucleic acid substantiated by single-strand breaks and inter/intrastrand crosslinks (Sarker *et al.*, 1995; Caraceni *et al.*, 1997). In particular, the hydroxyl radical is known to be able to interact with DNA basis (Frenzilli *et al.*, 2001; Regoli *et al.*, 2002). Thus, oxidative stress, which we found to be selective for the hippocampus following low doses of MDMA, represents a trigger for DNA alterations, which then persist in the hippocampal formation during MDMA withdrawal. It is likely that such a delayed DNA rearrangement, which increases compared with early time intervals following MDMA, sustains the plastic changes in the hippocampal formation we found at later time intervals. In particular, following low doses of MDMA a latent hippocampal hyperexcitability to excitatory stimuli develops, which is likely to sustain the decreased threshold to excitotoxins like kainic acid. Similarly, this may sustain the persistent alteration in EEG frequency and the long-lasting behavioral effects we observed as enhanced susceptibility to convulsive seizures and increased motor activity. Although this latter observation may appear out of place, recent findings demonstrate that locomotor activity induced by MDMA is enhanced in the presence of hippocampal alterations (Won *et al.*, 2003). Intriguingly, most of the literature has described the striatum as the main target of MDMA neurotoxicity in mice but other brain regions, including the hippocampus, were suggested to be additional targets of drug (Jayanthi *et al.*, 1999). These authors used higher doses of MDMA in mice (30 mg/kg × 4, 2 h apart, Jayanthi *et al.*, 1999) and found a reduced activity of Cu/Zn SOD, catalase, and an increase in lipid peroxidation, which extended to the hippocampal formation. The increase in oxidative stress and lipid peroxidation is known to represent a powerful trigger for the generation of DNA alterations (Emerit, 1994). In this study, carried out using very low doses of MDMA, oxidative stress involves exclusively the hippocampal formation and the same was true for DNA alterations. Thus, hippocampal selectivity for these MDMA-dependent effects emerges only when using low doses of MDMA, far below those used in previous studies, and comparable with those abused by humans.

The DNA alterations we observed in the hippocampal region immediately after MDMA doubled at 5 days after treatment. Such persistency is noteworthy, as it is known that the repair kinetics of DNA strand breaks usually takes 15 min for single-strand breaks, and 120 min for double-strand breaks to occur (Vijayalaxmi *et al.*, 1993; Plappert *et al.*, 1997). Thus, the persistence and the increase in DNA strand breaks in the hippocampus we found here, at 5 days following MDMA withdrawal, suggests the maintenance of a self-sustaining mechanism, which triggers a high DNA turnover. This event must be placed downstream to the early effects produced by MDMA administration. This might involve the recruitment of an abnormal transduction pathway, which is possibly activated by excitatory amino acids. In fact, at 5 days following MDMA, we were no longer able to detect oxidative stress (data not shown), whereas the

**Fig. 6**

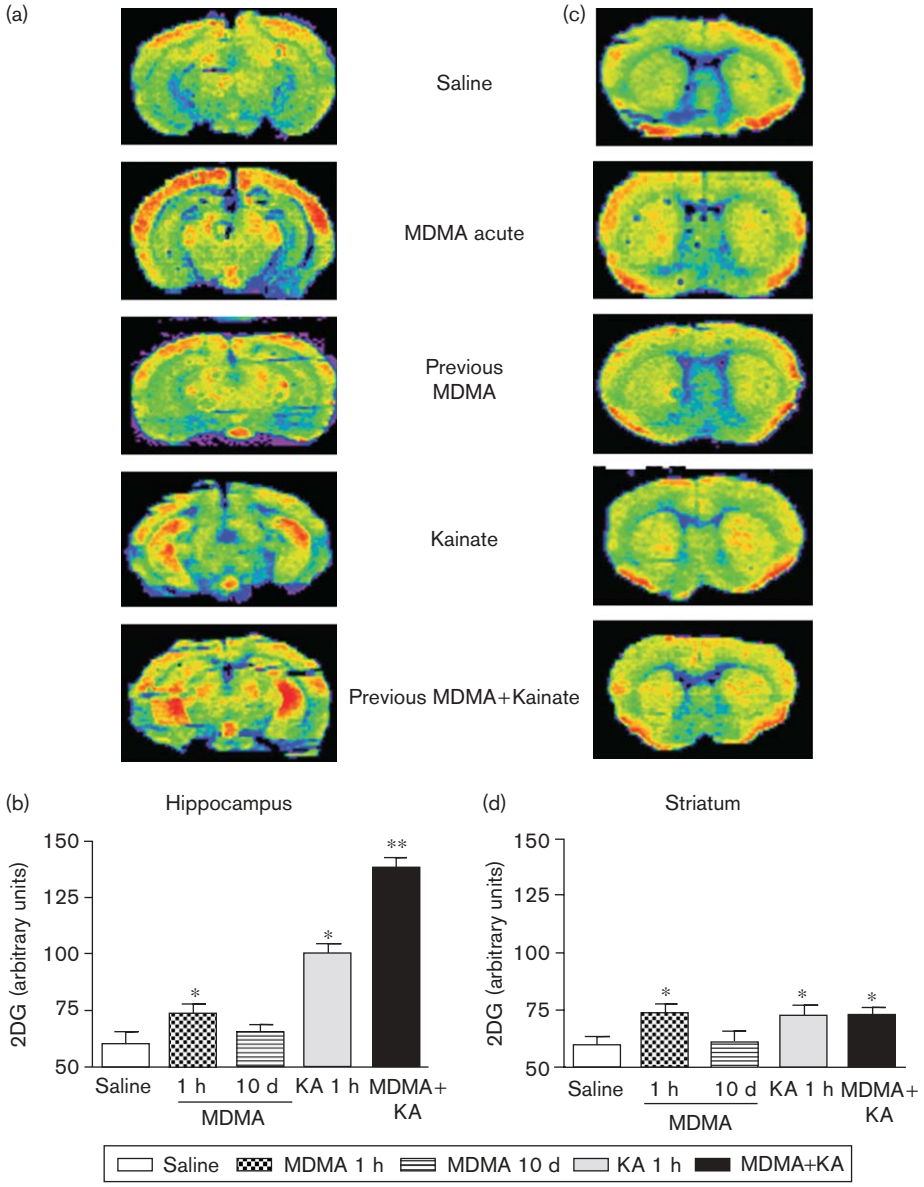



MDMA-induced oxidative stress: acute effects. Histograms show the loss of GSH content (a) and SOD activity (b) immediately after MDMA treatment, which selectively affected the hippocampus. Lipid peroxidation, revealed by the ratio MDA/4-HNE, is also reported (c). *$P<0.05$ compared with controls. MDMA, 3,4-methylenedioxymethamphetamine; SOD, superoxide dismutase.

MDMA-exposed hippocampus exhibited an enhanced response to low doses of the glutamate agonist kainic acid. In addition, the generation of alkali-labile abasic sites might partly account for the persistency of DNA alterations observed at 5 days. In fact, alkali labile sites are also detected by our alkaline comet assay and persist longer than strand breaks. It is difficult to determine the consequence of increased DNA single-strand and double-strand breaks. This phenomenon (which occurs in the hippocampus but not in the striatum, following MDMA at low doses) might not necessarily generate neurotoxicity, but it rather represents the molecular basis for MDMA-induced synaptic plasticity within the hippocampal formation. Such an effect may thus underlie the hippocampal hyperexcitability revealed as an abnormal metabolic activation upon a challenge with a low dose of kainic acid (which does not occur in the striatum). Similarly, the persistence of altered EEG activity, as well as the susceptibility to limbic seizures, may be linked to such MDMA-induced hippocampal DNA rearrangement, which develops concomitantly with the onset of behavioral sensitization.

In summary, our data show that systemic administration of low doses of MDMA to mice causes oxidative stress and the loss of antioxidant systems selectively in the hippocampus. This occurs concomitantly with DNA oxidation; however, whereas the oxidative stress vanishes after a few days, changes in DNA integrity persist and accompany the onset of behavioral sensitization, slowed EEG activity and persistent reduced threshold to convulsive limbic seizures. These effects suggest the presence of plastic changes, which produce a long-lasting altered responsivity of the hippocampal circuitry. This is substantiated by the persistence of a latent metabolic hyperactivity, which can be unraveled following low doses of systemic kainate. This study indicates that the hippocampus is a primary target of MDMA in mice. In contrast, the plastic effects induced by MDMA may contribute to the behavioral alterations including

**Fig. 7**




[$^{14}$C]2-DG uptake after MDMA and kainic acid. (a) Representative sections at the hippocampal level from mice killed at 1 h after [$^{14}$C]2-DG injection. In the autoradiographs the level of radioactivity is shown in a color scale ranging from blue (no uptake) to red (maximal uptake). (b) Semiquantitative densitometry shows that immediately after the MDMA treatment (MDMA 1 h) high levels of [$^{14}$C]2-DG uptake are detected in the hippocampus. This effect vanishes in mice treated from 10 to 5 days before with MDMA (previous MDMA). At this time interval, however, administration of a subthreshold dose of kainic acid produces an increase of [$^{14}$C]2-DG uptake in the hippocampal formation. This effect is significantly enhanced in mice pretreated with MDMA (previous MDMA + kainate). (c) Representative sections at striatal level from mice killed at 1 h after [$^{14}$C]2-DG injection. (d) Semiquantitative densitometry shows that immediately after MDMA administration (MDMA 1 h) a slight increase in the striatal levels of [$^{14}$C]2-DG uptake occurs but there is no enhancement of striatal [$^{14}$C]2-DG uptake in mice pretreated with MDMA (previous MDMA + kainate). *$P$<0.05 compared with controls; **$P$<0.05 compared with MDMA 1 h. KA, Kainate; MDMA, 3,4-methylenedioxymethamphetamine.

cognitive changes reported in MDMA chronically treated rodents (Piper and Meyer, 2004) and chronic MDMA abusers (Dafters *et al.*, 1999; Gamma *et al.*, 2000). Thus, the significance of the present findings, which were obtained with doses comparable with those abused by humans, is particularly relevant for higher species. In fact, the deficits of cognitive functions in which the hippocampal formation is involved, represent the major concern following chronic MDMA abuse. Similarly, alterations of EEG frequency, as well as the occurrence of convulsive seizures, represent common neurological consequences in chronic MDMA abusers.

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