RAT BRAIN RESPONSES TO ECSTASY

Enzymatic–Nonenzymatic Cellular Antioxidant Defense Systems Response and Immuno histochemical Detection of MDMA, VMAT2, HSP70, and Apoptosis as Biomarkers for MDMA (Ecstasy)

Neurotoxicity

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I'm fine with my help.
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Enzymatic–Nonenzymatic Cellular Antioxidant Defense Systems Response and Immunohistochemical Detection of MDMA, VMAT2, HSP70, and Apoptosis as Biomarkers for MDMA (Ecstasy) Neurotoxicity

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3,4-Methylenedioxymethamphetamine (MDMA)-induced neurotoxicity leads to the formation of quinone metabolites and hydroxyl radicals and then to the production of reactive oxygen species (ROS). We evaluated the effect of a single dose of MDMA (20 mg/kg, i.p.) on the enzymatic and nonenzymatic cellular antioxidant defense system in different areas of rat brain in the early hours (< 6 hr) of the administration itself, and we identified the morphological expressions of neurotoxicity induced by MDMA on the vulnerable brain areas in the first 24 hr. The acute administration of MDMA produces a decrease of reduced and oxidized glutathione ratio, and antioxidant enzyme activities were significantly reduced after 3 hr and after 6 hr in frontal cortex. Ascorbic acid levels strongly increased in striatum, hippocampus, and frontal cortex after 3 and 6 hr. High levels of malonaldehyde with respect to control were measured in striatum after 3 and 6 hr and in hippocampus and frontal cortex after 6 hr. An immunohistochemical investigation on the frontal, thalamic, hypothalamic, and striatal areas was performed. A strong positive reaction to the antivesicular monoamine transporter 2 was observed in the frontal section, in the basal ganglia and thalamus. Cortical positivity, located in the most superficial layer was revealed only for heat shock protein 70 after 24 hr.

Key words: MDMA; oxidative stress; HSP70; VMAT2; apoptosis

Previous experimental studies have concluded that 3,4-methylenedioxymethamphetamine (MDMA)-induced neurotoxicity is characterized by the decrease of serotonin and its metabolites, alterations most evident in neocortex of rats, striatum and hippocampus, because deficits in activities of tryptophan hydroxylase (TrpH), the rate-limiting enzyme in the synthesis of the neurotransmitter serotonin, occurred in the rat neostriatum, hippocampus, hypothalamus, and cortex (Schmidt and Taylor, 1987; Schmidt, 1987; Lyles and Cadet, 2003; Peng and Simantov, 2003). Decrease in activity of dopamine transporters and vesicular monoamine transporter 2 (VMAT-2) was associated with increased axon caliber, large swollen varicosities, and dilated proximal axon stumps (Molliver et al., 1990). Evidence documents that microglia activation is closely associated with neurotoxicity and not with other prominent pharmacological effects of methamphetamine such as inhibition of DA or 5HT transporters, stimulation of DA receptor, or hyperthermia (Thomas et al., 2004). Microglia produces a variety of proinflammatory cytokines, prostaglandins, and reactive oxygen species in response to brain injury and disease, which explains how they may contribute to or worsen neuronal damage. Again, studies in rats showed that long-lasting MDMA induced a significant neurotoxicity, accompanied by an extensive fraction of shrunken cells with cytoplasm vacuolization and condensed nuclei indicating apoptotic rather than necrotic cell death (Atlante et al., 1999, 2001). The release of apoptogenic factors into cytoplasm following neurotoxic insult may destabilize the internal cytoskeleton, which may be a major factor in the activation of cell apoptosis (Capela et al., 2007a,b).
The molecular mechanisms involved in the genesis of these toxic effects are not yet fully clarified, but the oxidative stress, exitotoxicity, and mitochondrial dysfunction appear to be causal events that converge to mediate MDMA-induced neurotoxicity, as measured by loss of various markers of dopaminergic and serotonergic terminals. The increase in extracellular dopamine after MDMA leads to the formation of quinone metabolites; the reactive intermediates produced during the oxidation of dopamine into reactive ortho-quinones and/or aminochromes can be conjugated with intracellular glutathione (GSH) to form the corresponding glutathionyl adducts. Quinone thiocarbonyls have ability to interfere with redox cycle, to produce ROS, and to bind covalently to DNA (Miyazaki et al., 2006). In addition, the rise in extracellular glutamate produce by the substituted amphetamines leads to an increase in intracellular calcium concentrations, which leads to the activation of nitric oxide synthase (NOS) and the consequent generation of reactive nitrogen species (RNS). The ROS and RNS can alter proteins, lipids, and DNA as well as inhibit mitochondrial function to produce energy deficits in the nerve terminals. Recent studies support the idea that hyperthermia is an important factor in MDMA-induced neuronal death (Capela et al., 2007a,b). Rats have been reported in many studies to have an acute dose-dependent hyperthermic response following administration of MDMA (Green et al., 2003). It has been established that cytokines such as interleukin-1β, interleukin-6, and tumor necrosis factor-α increase body temperature by direct or indirect mechanisms in the brain, and, in particular, interleukin-1β is involved in the development of the hyperthermic response (Green et al., 2004).

The aim of our experimental study was to evaluate the effect of a single dose of MDMA (20 mg/kg, i.p.) on enzymatic and nonenzymatic cellular antioxidant defense systems in different areas of rat brain, in the early hours (<6 hr) of the administration itself, and to identify the morphological changes in expression of neurotoxicity induced by MDMA and its metabolites on the vulnerable brain areas in the first 24 hr. An immunohistochemical investigation of the frontal, thalamic, hypothalamic and striatal areas was performed with antibodies anti-MDMA, -glial fibrillary acidic protein (GFAP), -HSP 27, -HSP 70, -HSP 90 (heat shock proteins), -VMAT-2, -β-APP (β amyloid precursor protein), -TrypH, and -growth-associated phosphoprotein 43 (GAP-43) and TUNEL assay for apoptosis to evaluate the specific morphological alterations.

MATERIALS AND METHODS

The experimental procedures followed the NIH Principles of laboratory animal care (NIH publication no. 85-23 revised 1996) and were approved by the University of Siena Committee for Animal Experiments.

Animal Model and Experimental Protocol

For the evaluation of oxidative stress, 21 male albino rats (Wistar; Charles River) weighing 200–250 g were used to analyze the effect of MDMA administration (20 mg/kg, i.p.) on rat brains. After treatment, animals were killed by decapitation at the following times: group 1 (9 rats) after 3 hr; group 2 (9 rats) after 6 hr, and group 3 (3 rats) control group.

Both the histopathological examination of the brain and the plasma concentration of MDMA and the metabolite methylenedioxymethamphetamine (MDAA) were carried out on 25 rats, each weighing 200–250 g, divided into three groups of seven animals each treated with MDMA 20 mg/kg, i.p.: group 1 killed 6 hr after treatment; group 2 killed 16 hr after treatment; and group 3 killed 24 hr after treatment of which 2 died spontaneously within 4 hr after administration of MDMA.

One control group of four animals was treated with saline i.p. and killed: 1 rat 6 hr after treatment, 1 rat 16 hr after treatment, and 2 rats 24 hr after treatment were used for histological examination and toxicological analysis. Plasma samples obtained after the treatments were stored at –80°C until MDMA/MDAA gas chromatography/mass spectroscopy (GC-MS) analysis.

Biochemical Analysis: Oxidative Stress Evaluation

The hippocampus, striatum, and frontal cortex of the treated and control animals were immediately resected and frozen at –80°C up to the time of the determination of reduced and oxidized glutathione (GSH, GSSG), malondialdehyde (MDA), and ascorbic acid (AA) levels and of superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GR) enzymatic activities.

GSH/GSSG and protein determination. Brain tissues were homogenized in EDTA K+- phosphate buffer, pH 7.4 (1:3, w/v), at 0°C. Total GSH was analyzed as described by Tietze (1969) and GSSG was determined according to Griffith’s (1980) method. In the remaining aliquot, proteins were assayed according to the method of Lowry (1951).

SOD, GPx, and GR assessment. To measure cytosolic enzyme activity, the brain samples were homogenized according to Whanger and Butler (1988). GPx activity was measured according to Paglia and Valentine (1967). GR activity was analyzed as described by Goldberg and Spooner (1983). Total superoxide dismutase (Cu/Zn superoxide dismutase and Mn-superoxide dismutase) was assayed by spectrophotometric method based on the inhibition of a superoxide-induced NADH oxidation according to Paoletti et al. (1986). The cytosolic protein concentration was determined by using the Lowry (1951) method with BSA as standard.

MDA assessment. The extent of lipid peroxidation in the rat brain was estimated by calculation of MDA levels with an HPLC method and UV detection as described by Shara et al. (1992).

AA assay. Brain tissues were homogenized in EDTA-K+- phosphate buffer, pH 7.4 (1:4, w/v), at 0°C and analyzed as described by Ross (1994).

Morphological Examination

The brains of treated and control animals were fixed in 10% buffered formalin for 48 hr. Paraffin-embedded tissue specimens of brain were sectioned at 4 μm and stained with
hematoxylin and eosin. In addition, immunohistochemical investigation of the frontal section (A) and section C with the structures of thalamus, hypothalamus, and striatum was performed with antibodies anti-MDMA, -GFAP, -HSP27, -HSP70, -HSP90, -VMAT2, -APP, -TrypH, and -GAP-43 and TUNEL assay. We used 4-μm-thick paraffin sections mounted on slides covered with 3-amino-5-propyl-triethoxysilane (Fluka, Buchs, Switzerland). A pretreatment was necessary to facilitate antigen retrieval and to increase membrane permeability to antibodies: for antibody anti-β-APP (Novocastra, Newcastle upon Tyne, United Kingdom), -TrypH (Novocastra), and -GAP-43 (Santa Cruz, CA) boiling in 0.1 M citric acid buffer; for antibody anti-HSP27, -HSP70, and -HSP90, (Novocastra) boiling in 0.25 M EDTA buffer; for antibody anti-VMAT2 (Chemicon, Temecula, CA) 5 min proteolytic enzyme at 20°C (Dako, Copenhagen, Denmark); no pretreatment was necessary for antibody anti-GFAP (Dako, Copenhagen, Denmark). For TdT enzyme the sections were immersed in protease K (20 μg/ml of TRIS) for 15 min at 20°C. The primary antibody was applied in a 1:20 ratio for HSP27; in a 1:50 ratio for VMAT2, HSP90, and TrypH; in a 1:100 ratio for HSP70; in a 1:300 ratio for GFAP and β-APP; and in a 1:2,000 ratio for GAP43. The incubation of primary antibody was for 120 min at 20°C for anti-VMAT2, -GFAP, -HSP27, -HSP90, and -GAP 43 and overnight for anti-β-APP, -HSP70, and -TrypH. For TUNEL assay (Chemicon, Temecula, CA), the sections were covered with the TdT enzyme, diluted in a ratio of 30% in reaction buffer (Aprotag Peroxidase In Situ Apoptosis Detection Kit; Chemicon) and incubated for 60 min at 38°C. Monoclonal antibodies that specifically recognize MDMA and MDAA were kindly supplied by Microgenics GmbH Products Europe (Passau, Germany); they were purified from mouse ascites and available in two clones (clones 1A9 and 5C2; De Letter et al., 2003). Therefore, primary antibody anti-MDMA was tested on brain rat samples treated with MDMA, without pretreatment and with various pretreatments (boiling in 0.25 mM EDTA buffer; boiling in 0.1 M citric acid buffer; Proteolytic Enzyme T 20°C for 5 min; proteinase K for 15 min at 20°C) and at various concentrations (ratio 1:20, 1:50, 1:100, 1:500, 1:1,000, 1:2,000). The samples tested were examined under a light microscope, which detected the best reaction. We used the primary antibody anti-MDMA before a pretreatment with boiling in 0.25 mM EDTA buffer, in a ratio 1:100, with an incubation for 120 min at 20°C. The detection system utilized was the LSAB + Kit (Dako, Carpinteria, CA), a refined avidin–biotin technique in which a biotinylated secondary antibody reacts with several peroxidase-conjugated streptavidin molecules. The positive reaction was visualized by 3,3-diaminobenzidine (DAB) peroxidation, according to standard methods. Then, the sections were counterstained with Mayer’s hematoxylin, dehydrated, coveslipped, and observed under a Leica DM4000B optical microscope (Leica, Cambridge, United Kingdom). The samples were also examined under a confocal microscope, and a three-dimensional reconstruction was performed (True Confocal Scanner; Leica TCS SPE).

For semiquantitative analysis, slides were scored in a blinded manner by two observers (M.N., I.R.). Staining pattern within each sample was categorized as absent (−), weak (+), moderate (++), or intense (+++).

GC-MS Analysis of MDMA and MDAA

MDMA and MDAA were analyzed according a GC-MS method described by Peters et al. (2003). The analytes were analyzed by gas chromatography/mass spectrometry in the selected-ion monitoring mode after mixed-mode solid-phase extraction (HCX) and derivatization with heptafluorobutyric anhydride. The method was fully validated according to international guidelines (De Letter et al., 2002a,b). It was linear from 5 to 1,000 μg/l for all analytes. The limit of quantification was 5 μg/l for all analytes.

Statistical Analysis

Values are presented as means ± SD. The unpaired two-way Student’s t-test was used to compare the results obtained for treated rats with the control group. P < 0.05 was accepted as indicative of significant difference among groups.

RESULTS

Enzymatic and Nonenzymatic Antioxidant Cellular Defense System Evaluation

The acute administration of MDMA produces a decrease of GSH/GSSG ratio and oxidative stress in all brain areas examined. SOD activity was significantly reduced after 3 hr in hippocampus (–60.7%) and after 6 hr in striatum, hippocampus, and frontal cortex (–43.3%, –86.1%, and –23.4%, respectively). GR and GPx activities were reduced after 3 hr (–22%) and after 6 hr (–33.3%) in frontal cortex (Fig. 1). AA levels strongly increased in striatum, hippocampus, and frontal cortex (–33.3%, –84%, and 17.6%) and 6 hr (+162%, +154%, and +23.4%, respectively). High levels of MDA with respect to control were measured in striatum after 3 hr (+276%) and 6 hr (+162%) and in hippocampus (+71.8%) and frontal cortex (+18.22%) after 6 hr (Fig. 2). The numerical values are summarized in Table I.

Morphological Findings

The microscopic evaluation of the sections stained with H&E revealed the following: group III (24 hr): red neurons, nuclear shrinkage, and in the two dead rats (4 hr) perivascular hemorrhages; group I (16 hr): cytoplasmic hypereosinophilia; group I (6 hr): moderate perineuronal edema and initial neuronal cytoplasmic hypereosinophilia. The immunohistochemical study of the samples, for each antibody revealed (Table II) the following.

Anti-MDMA. A markedly positive reaction was noted at the basal ganglia and thalamus in rats sacrificed at 6 hr (group I) after treatment. A positive reaction, gradually weaker, but more uniform, both in the frontal cortex and at the level of the striatum, hippocampus, and thalamus was reported in rats sacrificed after 16 hr (group II) and 24 hr (group III; Fig. 3).
TrypH. TrypH did not provide significant results, except for a weak positive reaction in the external cortical layers of rats sacrificed after 24 hr (group III); the other samples showed a constant negativity.

VMAT2. We found a strong positive reaction to the VMAT2. In detail, we reveal that 1) group I (6 hr) had a positive reaction, particularly in thalamus areas; 2) group II (16 hr) had a weak and uniform positive reaction in the frontal section, in the basal ganglia, and in thalamic areas; c) group III (24 hr) had a marked positive reaction in the deep layers (Fig. 4).

HSP 27, HSP 70, HSP 90. A cortical positivity located in the most superficial layer was revealed only for HSP70 in rats of group I (6 hr). Positivity, gradually increasing, was observed, in both sections, in the group II (16 hr) and rats sacrificed after 24 hr (group III); the reaction was localized exclusively in the cortex but with a more extended involvement of the cortical layers (Fig. 5). A constant negativity was noted in response to HSP90 and -27.

Apoptosis (TUNEL). A weak positive reaction was shown in the deep layers of rats sacrificed at 6 hr (group I) and 16 hr (group II) after treatment. A marked positivity was revealed in the superficial layers (or cortical) of rats sacrificed after 24 hr (group III; Fig. 6). All groups showed a negative reaction to GAP43, β-APP, and GFAP.

MDMA plasma levels. The MDMA plasma levels decreased dramatically with respect to the values detected during the first 6 hr after i.p. administration; in rats that died after 4 hr, concentrations were similar to those observed at hour 6 (Table III).

DISCUSSION

This study demonstrates that the administration of a single dose of MDMA (20 mg/kg ip) was able to alter
significantly the antioxidant defense system, producing
an oxidative stress that may result in lipid peroxidation,
with consequent deterioration of the homeostasis of
Ca\(^{2+}\) and neuronal damage. Numerous mechanisms
have been suggested to be responsible for MDMA toxicity,
such as oxidative stress, metabolic compromise, and
inflammation (Cerretani et al., 2008). The role of oxida-
tive stress in mediating MDMA toxicity is further illus-
trated by a decrease in the activity of the endogenous
antioxidants glutathione peroxidase, catalase, and super-
oxide dismutase observed after MDMA administration
(Yamamoto and Raudensky, 2008). Although decreases
in endogenous antioxidant enzyme may mediate
increased free radical production, acute increases in DA
have been shown to play a predominant role in the
increases in oxidative stress associated with MDMA
administration. The generation of free radical species
subsequent to increases in DA is derived mainly from
autooxidation of DA or enzymatic oxidation by mono-
amine oxidase (MAO) to result in the production of
superoxide and hydrogen peroxide (Yamamoto and
Raudensky, 2008). Furthermore, MDMA depleted intra-
cellular GSH levels in a concentration-dependent man-
ner, an effect that was attenuated by N-acetylcysteine,
an antioxidant and GSH precursor that also reduced
MDMA-induced toxicity in neuronal cultures from rat
cortex (Capela et al., 2007b). It has been described
(Capela et al., 2007a) that thioether MDMA metabolites
time dependently increased the production of reactive
species, concentration dependently depleted intracellular
GSH and increased protein bound quinones, and finally
induced oxidative stress and neuronal death. It is postu-
lated that hepatic metabolism is a key factor in the pro-
duction of MDMA-induced neurotoxicity to 5-HT-
containing neurons. The mechanisms by which MDMA
causes its deleterious effects have yet to be completely

Fig. 2. AA levels increased in striatum, hippocampus, and frontal cortex after 3 hr (+159%, +84%, and
+17.6%) and 6 hr (+162%, +154%, and +23.4%, respectively). High levels of MDA were measured
in striatum after 3 hr (+276%) and 6 hr (+162%); in hippocampus (+71.8%) and in frontal cortex
(+18.22%) after 6 hr. Acute administration of MDMA produces a decrease of GSH/GSSG ratio and
oxidative stress in all brain areas examined.

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clarified. However, there is good evidence to suggest that the mechanism of neurotoxicity involves the formation of MDMA metabolic products such as N-methyl-a-methyl dopamine (MeDA), orthoquinones, and quinone thioethers (Thiriet et al., 2002); MeDA is unstable and is metabolized in the presence of NADPH into a quinone that forms an adduct with GSH. The cathecol thioether metabolites are capable of redox cycling and generating ROS, which is a key feature of MDMA-induced neurotoxicity (de la Torre and Farre, 2004). GSH and related enzymes participate in the protection of neurons from a variety of stresses, and changes in GSH metabolism have been associated with neurodegenerative processes of the brain (Erives et al., 2008).

In our experiment, after a single MDMA injection in rats, we observed presence of oxidative stress in striatum, hippocampus, and frontal cortex, with alteration of antioxidant enzymes, decrease of GSH/GSSG ratio, and increase in MDA levels. The alteration of the antioxidant defense system starts from the early hours (acute phase or reversible) with a subsequent gradual increase, thus giving relief not only to the formation of free radicals but also with regard to the MDMA metabolites in determining neuronal toxicity. Although there is ample scientific evidence confirming MDMA-induced serotonergic depletion, few studies have so far examined the effects of MDMA on markers of neurotoxic damage or on the possible morphological markers indicative of MDMA-induced encephalic damage and their expression history.

The experimental use of anti-MDMA has allowed us to reveal the encephalic distribution of the substance. In this regard, there has been a unique study on encephalic human tissue, taken from two MDMA-related

### TABLE I. Enzymatic and Nonenzymatic Antioxidant Cellular Defence System Evaluation Expresses in Numeric Values

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>3 Hours</th>
<th>6 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MDA frontal cortex</strong></td>
<td>13.23 ± 2.151</td>
<td>13.31 ± 2.709</td>
<td>15.64 ± 0.384</td>
</tr>
<tr>
<td><strong>MDA hippocampus</strong></td>
<td>9.18 ± 2.227</td>
<td>12.05 ± 4.341</td>
<td>15.77 ± 5.015</td>
</tr>
<tr>
<td><strong>MDA striatum</strong></td>
<td>7.06 ± 1.420</td>
<td>20.60 ± 8.069</td>
<td>18.53 ± 7.428</td>
</tr>
<tr>
<td><strong>AA frontal cortex</strong></td>
<td>443.37 ± 48.305</td>
<td>521.46 ± 25.803</td>
<td>547.06 ± 47.456</td>
</tr>
<tr>
<td><strong>AA hippocampus</strong></td>
<td>233.00 ± 124.112</td>
<td>427.91 ± 61.748</td>
<td>592.98 ± 87.833</td>
</tr>
<tr>
<td><strong>AA striatum</strong></td>
<td>143.58 ± 14.055</td>
<td>353.125 ± 66.282</td>
<td>420.130 ± 92.395</td>
</tr>
<tr>
<td><strong>GSH/GSSG ratio</strong></td>
<td>17.58 ± 7.421</td>
<td>6.80 ± 2.084</td>
<td>5.86 ± 0.928</td>
</tr>
<tr>
<td><strong>GSH/GSSG ratio</strong></td>
<td>10.74 ± 4.665</td>
<td>4.13 ± 1.280</td>
<td>4.67 ± 4.782</td>
</tr>
<tr>
<td><strong>GSH/GSSG ratio</strong></td>
<td>7.39 ± 2.261</td>
<td>4.03 ± 0.669</td>
<td>4.24 ± 0.771</td>
</tr>
<tr>
<td><strong>GPx frontal cortex</strong></td>
<td>17.35 ± 2.075</td>
<td>15.07 ± 3.979</td>
<td>11.74 ± 2.503</td>
</tr>
<tr>
<td><strong>GPx hippocampus</strong></td>
<td>17.60 ± 3.864</td>
<td>19.40 ± 2.029</td>
<td>18.90 ± 7.304</td>
</tr>
<tr>
<td><strong>GPx striatum</strong></td>
<td>15.12 ± 4.633</td>
<td>19.19 ± 1.771</td>
<td>19.43 ± 6.354</td>
</tr>
<tr>
<td><strong>GR frontal cortex</strong></td>
<td>24.87 ± 4.694</td>
<td>19.40 ± 2.402</td>
<td>21.34 ± 1.679</td>
</tr>
<tr>
<td><strong>GR hippocampus</strong></td>
<td>25.20 ± 5.389</td>
<td>29.69 ± 6.013</td>
<td>27.36 ± 1.037</td>
</tr>
<tr>
<td><strong>GR striatum</strong></td>
<td>21.73 ± 6.285</td>
<td>25.68 ± 3.485</td>
<td>29.46 ± 4.759</td>
</tr>
<tr>
<td><strong>SOD frontal cortex</strong></td>
<td>1.74 ± 0.479</td>
<td>2.10 ± 0.301</td>
<td>1.33 ± 0.109</td>
</tr>
<tr>
<td><strong>SOD hippocampus</strong></td>
<td>2.09 ± 0.611</td>
<td>0.82 ± 0.409</td>
<td>0.28 ± 0.220</td>
</tr>
<tr>
<td><strong>SOD striatum</strong></td>
<td>1.56 ± 0.491</td>
<td>1.44 ± 0.416</td>
<td>0.89 ± 0.434</td>
</tr>
</tbody>
</table>

*P < 0.05 compared with control group.

### TABLE II. Semiquantitative Analysis: Staining Pattern Within Each Sample Was Categorized as Absent (–), Weak (+), Moderate (++), Intense (+++), Intense (++++)

<table>
<thead>
<tr>
<th></th>
<th>Group III (24 hr)</th>
<th>Group II (16 hr)</th>
<th>Group I (6 hr)</th>
<th>Control group (IV)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anti-MDMA</strong></td>
<td>+ + + + + +</td>
<td>+ + + + + +</td>
<td>+ + + + + + + +</td>
<td>– – – – – – – – – – –</td>
</tr>
<tr>
<td><strong>TrpH</strong></td>
<td>+ + + + + +</td>
<td>+ + + + + +</td>
<td>+ + + + + + + +</td>
<td>– – – – – – – – – – –</td>
</tr>
<tr>
<td><strong>VMAT2</strong></td>
<td>+ + + + + +</td>
<td>+ + + + + +</td>
<td>+ + + + + + + +</td>
<td>– – – – – – – – – – –</td>
</tr>
<tr>
<td><strong>HSP 70</strong></td>
<td>+ + + + + +</td>
<td>+ + + + + +</td>
<td>+ + + + + + + +</td>
<td>– – – – – – – – – – –</td>
</tr>
<tr>
<td><strong>Apoptosis (TUNEL)</strong></td>
<td>+ + + + + +</td>
<td>+ + + + + +</td>
<td>+ + + + + + + +</td>
<td>– – – – – – – – – – –</td>
</tr>
<tr>
<td><strong>GAP-43</strong></td>
<td>– – – – – –</td>
<td>– – – – – –</td>
<td>– – – – – – – –</td>
<td>– – – – – – – – – – –</td>
</tr>
<tr>
<td><strong>β-APP</strong></td>
<td>– – – – – –</td>
<td>– – – – – –</td>
<td>– – – – – – – –</td>
<td>– – – – – – – – – – –</td>
</tr>
<tr>
<td><strong>GFAP</strong></td>
<td>– – – – – –</td>
<td>– – – – – –</td>
<td>– – – – – – – –</td>
<td>– – – – – – – – – – –</td>
</tr>
</tbody>
</table>
fatal cases, in which the use of the same antibody allowed to show a marked immunoreactivity corresponding to neurons in all cortical regions, basal ganglia, hypothalamus, hippocampus, cerebellar vermis, and white matter (De Letter et al., 2003). Our study, although confirming the topographic distribution of MDMA, revealed a different location of the substance with time, with a markedly positive reaction in the basal ganglia and thalamus in rats sacrificed at 6 hr (group I) after treatment, whereas a weaker uniform positivity was seen for the frontal cortex, striatum, hippocampus, and thalamus in the second (16 hr) and third (24 hr) groups.

It is well known that MDMA reduced the activity of TrypH, the rate-limiting enzyme in the synthesis of the neurotransmitter serotonin, converting 5-hydroxytryptophan to serotonin. In the literature, the deficit in activities of TrypH occurred in the rat neostriatum, hippocampus, hypothalamus, and cortex. The negative expression of anti-TrypH in our study was explained by the MDMA-induced serotonergic deficit probably being due to adaptive changes of gene expression and function of proteins, expression of a metabolic state of quiescence or exhaustion, rather than a neurotoxic damage. Moreover, the scientific work refers only to radioimmunologic dosage, without providing any data on tissue expression of antibody itself, in reference to the administration of MDMA.

A strong positive reaction to the anti-VMAT2 was observed. Although there are no reports on the tissue expression (by immunohistochemical reaction) of VMAT-2 after administration of MDMA, the findings from our study are in agreement with those reported in the literature. After administration of methamphetamine, there is a fast (1–48 hr) decrease in cytoplasmic function of the transporter VMAT2 corresponding to the striatum, associated with a reduced cytoplasmic reactivity.
(Chu et al., 2008). In our study, this finding is well defined in relation to the times at which the rats were sacrificed.

High doses of MDMA lead to an excessive sympathetic activation, and hyperthermia can induce a physiological dysregulation sufficient to produce nonspecific neuronal damage, involving not only the serotonergic cells (Baumann et al., 2007), as evaluated from the response obtained by immunohistochemistry using antibodies to HSP27, HSP70, and HSP90. The literature includes only one study conducted on rats treated with MDMA in different doses and sacrificed after 3 days, which revealed a positive reaction to HSP27, located at all cortical areas and hippocampus (Adori et al., 2006). We observed a cortical positivity for HSP70, located in the most superficial layers, after 6 hr and gradually increasing after 16 and 24 hr. A constant negative reactivity was noted in response to HSP90 and -27.

We did not find a positive reaction for GAP43, a marker of synaptic plasticity and a key regulator in normal pathfinding and arborization of serotonergic axons during early brain development. GAP43 is frequently used as a marker for sprouting, because it is located in growth cones, is maximally present during nervous system development, and is reinduced in injured and regenerating neural tissues. The role of GAP43, still to be precisely defined, may be connected to intracellular regulation events, which allows the growing axon to adapt to the changing environmental framework. Thus, negative expression could be due to the neurodegenerative timing (Casoli et al., 2004). We found no B-APP expressivity (a transmembrane glycoprotein type 1 expressed preferentially in the brain in various isoforms and used by many authors as an immunohistochemical marker of the axonal damage in different etiopathogenesis), indicative of the absence of early axonal damage.

Fig. 4. Immunohistochemical visualization of anti-VMAT2. A: Strong positive reaction in the thalamus areas at 6 hr (B). C: Group II (16 hr) showed a weak but uniform positive cytoplasmic reaction in the basal ganglia. D: Confocal laser scanning microscopy: Group III (24 hr) showed a marked positive nuclear reaction (in green) in the deep layers.
Massive doses of MDMA are associated with high levels of GFAP in different brain areas, but this increase is not related to the degree of serotonergic depletion. Positivity to GFAP, expression of reactive gliosis, and astrocytic response resulting from neuronal damage are sometimes located only in areas corresponding to the hippocampus (Adori et al., 2006), but after repeated dosing with methamphetamine, not MDMA, and after longer times from sacrifice. In other studies, no glial reaction was reported after administration of MDMA, implying an important distinction between the effects of methamphetamine and those of MDMA (Baumann et al., 2007). Our results are indicative of the absence of reactive astrocytes (Sharma and Ali, 2008).

Finally, in agreement with the literature, although referring only to an immunohistochemical reaction for caspases 3 and 1 (Capela et al., 2007b), we found a gradually increase in apoptosis (TUNEL) from 6 hr to 24 hr after MDMA administration (Neri et al., 2007). A dosage effect is suggested by the fact that at lower doses the effects of MDMA may be repaired by factors such as HSP70 (Fornai et al., 2004). At higher doses, repair mechanisms may be overcome, and irreversible damage results (Granado et al. 2008).

In conclusion, although oxidative stress and the production of ROS are already established in the early hours after administration of a single dose of MDMA (3 and 6 hr) or in the so-called acute or reversible phase, there are very few morphologic changes in the brain, except for sporadic and limited hemorrhage, a gradual and progressive neuronal cytoplasmic hypereosinophilia, and an increase in apoptotic phenomenon in the same locations where it has been shown that there is more action of MDMA. Our study has demonstrated that oxidative stress is responsible for MDMA-selective neurotoxicity from the early stages, with gradually increasing neuronal alterations demonstrated with the most common immunohistochemical methods.

Fig. 5. A: Low-magnification view of HSP70 immunolabeling located in the most superficial layer was revealed after 6 hr (B). C: In group II (16 hr) and in rats sacrificed after 24 hr (D), positivity was localized exclusively in the cortex but with a more extended involvement of the cortical layers.
Fig. 6. A: A marked positivity was revealed in the superficial layers of rats sacrificed after 24 hr. Weak apoptotic reaction in the deep layers of rats sacrificed after 16 hr (C). B: Marked positivity was revealed in the superficial layers of rats sacrificed after 24 hr. D: Confocal laser scanning microscopy shows a marked positive nuclear reaction (in red apoptotic bodies) after 24 hr.

**TABLE III. MDMA and MDAA Plasma Levels**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total rats</th>
<th>Number</th>
<th>MDMA (μg/ml)</th>
<th>MDAA (μg/ml)</th>
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<tbody>
<tr>
<td>6 Hours 1</td>
<td>1</td>
<td></td>
<td>0.368</td>
<td>0.032</td>
</tr>
<tr>
<td>6 Hours 2</td>
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<tr>
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<td>4</td>
<td>0.153</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>0.448</td>
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</tr>
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<td>16 Hours 8</td>
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REFERENCES


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* I'm fine with my help