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# Acute and chronic effects of MDMA on molecular mechanisms implicated in memory formation in rat hippocampus: Surface expression of CaMKII and NMDA receptor subunits

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**Abstract**

Acute 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy") treatment induces learning deficits in different animal models. In a passive avoidance learning task in rats, previous studies suggested a role for $Ca^{2+}$/calmodulin-dependent protein kinase II (CaMKII) and *N*-methyl-D-aspartate (NMDA) receptors in the acute learning impairment. As cognitive deficits by "ecstasy" in humans have been only reported in frequent recreational users, we examined whether a repeated MDMA treatment could induce in rats lasting molecular changes related to memory consolidation of passive avoidance. In rats with a pronounced 5-HT depletion by MDMA, the effect of another drug challenge was also examined. The surface expression in the hippocampus of NMDA receptor subunits, the scaffolding postsynaptic density protein PSD-95, phosphorylated CaMKII and protein phosphatase 1 (PP1) was measured. In rats repeatedly treated with MDMA (10 mg/kg) twice daily for 4 consecutive days, hippocampal 5-HT levels were markedly reduced 1 week later. At this time, neither learning performance was affected nor changes in membrane levels of NMDA receptor subunits, PSD-95, CaMKII and PP1 were found. In these rats, however, another drug challenge produced a rapid reduction in PSD-95 immunoreactivity and prevented the learning-specific increase in the NMDA receptor NR1 subunit and phosphorylated CaMKII. The results show no lasting change in learning-associated molecular events after a neurotoxic MDMA treatment. This drug only produces transient effects on early molecular events involved in memory consolidation, which do not appear to depend on endogenous 5-HT levels.


*Keywords:* MDMA; Ecstasy; Serotonin; Memory; NMDA receptor; CaMKII; Hippocampus

## 1. Introduction

3,4-Methylenedioxymethamphetamine (MDMA, "ecstasy") is a serotonergic neurotoxin inducing on acute treatment a rapid and marked reduction in serotonin (5-HT) levels in different rat brain regions (Stone et al., 1987). Multiple injections of MDMA result in neurodegeneration of 5-HT terminals and lasting 5-HT depletion (Battaglia et al., 1987; Stone et al., 1987). Cognitive deficits after the frequent recreational use of "ecstasy" have been reported in humans (Parrott and Lasky, 1998; McCann et al., 1999; Rodgers, 2000), and a correlation between memory impairment and altered 5-HT neuronal function has been found in some studies (Bolla et al., 1998; McCann et al., 1999; Reneman et al., 2000). However, the polydrug use of the subjects has been always recognised as a possible experimental confound (Cole and Sumnall, 2003). In different animal models of learning and memory, acute MDMA treatment also produces learning deficits that have been related in some cases (Sprague et al., 2003) but not in others (Ricaurte et al., 1993; Byrne et al., 2000; Taffe et al., 2002; Moyano et al., 2004b) to the neurotoxic effect of the drug on serotonergic terminals and the ensuing 5-HT depletion.

In previous studies from this laboratory, we found that acute administration of either MDMA or 3,4-methylenedioxyethamphetamine (MDEA, "eve") before the acquis-

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ition trial of a passive avoidance learning task impaired retention 24 h later (Barrionuevo et al., 2000; Moyano et al., 2004b). After repeated injections of MDMA or MDEA for 4 consecutive days, a pronounced 5-HT depletion was observed in the hippocampus 7 days later (Aguirre et al., 1997; Barrionuevo et al., 2000); however, no retention deficit was found at this time point in the 5-HT-depleted rats (Artaiz et al., 1996; Barrionuevo et al., 2000). Other behavioural studies have also shown that a neurotoxic regimen of MDMA does not result in a disruption of learning performance some weeks after completion of treatment (Ricaurte et al., 1993; Byrne et al., 2000; Winsauer et al., 2004). In close analogy, it is known that deficits in retention performance in the passive avoidance learning task by the serotonergic neurotoxin *p*-chloroamphetamine are not related to 5-HT depletion (Santucci et al., 1996). Rats treated with the 5-HT synthesis inhibitor, *p*-chlorophenylalanine (PCPA), do not either exhibit amnesic effects in this learning model (Misane et al., 1998; Moyano et al., 2004b) in spite of the pronounced 5-HT depletion. It is of note that, in our previous studies (Artaiz et al., 1996; Barrionuevo et al., 2000; Moyano et al., 2004b), acute administration of MDMA or MDEA 30 min before the acquisition session in rats with a previous drug-induced 5-HT depletion produced an amnesic effect similar to that observed in rats with normal 5-HT levels.

Even though other brain structures, such as the amygdala (Liang, 1999), may contribute to early memory processing of passive avoidance, the sequence of molecular events in the hippocampus associated to learning and retention of passive avoidance tasks appears to be strikingly similar to those underlying other forms of memory and also long-term potentiation (LTP) (reviewed in Izquierdo and Medina, 1997; Shobe, 2002). Abundant evidence indicates that *N*-methyl-D-aspartate (NMDA) receptors play a central role in the early stages of hippocampal-dependent memory storage and in LTP (reviewed by Riedel et al., 2003). In LTP induction, there is an early activation of NMDA receptors resulting in postsynaptic increase in $Ca^{2+}$ entry that activates $Ca^{2+}$-dependent enzymes such as $Ca^{2+}$/calmodulin-dependent protein kinase II (CaMKII), a basic substrate for early phase of memory formation (Fink and Meyer, 2002) that, after autophosphorylation at Thr286, remains in an active, $Ca^{2+}$-independent state and translocates to the postsynaptic density (PSD) where the enzyme binds, predominantly, to the NR2B subunit of the NMDA receptors (Shen and Meyer, 1999; Bayer et al., 2001). In the PSD, CaMKII can phosphorylate different postsynaptic proteins implicated in memory formation. The PSD is an structure whose stability is dependent, in part, of scaffolding proteins such as PSD-95, that is bound to NR2B and other proteins and participates in different functions, including NMDA receptor clustering at synapses (Kim et al., 1996) and inhibition of NMDA receptor internalization (Lavezzari et al., 2004).

The molecular mechanisms involved in the retention deficit induced by acute MDMA treatment are unclear. In our previous study (Moyano et al., 2004b), acute MDMA administration caused changes in molecular events in the hippocampus admittedly involved in learning consolidation. Specifically, the learning-induced increases in NMDA receptor NR1 subunit expression, membrane CaMKII and pCaMKII (Thr286) levels, and also in CaMKII enzyme activity were prevented by acute MDMA treatment. We wished to examine changes in NMDA receptor subunits, the scaffolding protein PSD-95, CaMKII and PP1 levels and CaMKII enzyme activity in the hippocampus of rats with a marked 5-HT depletion 1 week after a neurotoxic regimen of MDMA and trained or not on a passive avoidance task. In these 5-HT-depleted animals, the effect of another MDMA challenge was also studied.

## 2. Materials and methods

### 2.1. Animals

Male Wistar rats (Harlan, Barcelona, Spain) weighing 200–220 g were used. Animals were housed in plastic cages with free access to food and water and maintained in a temperature-controlled environment (21–23 °C) on a 12-h light/dark cycle. All procedures were in accordance with the guidelines established by the normative of the European Community of November 24, 1986 (86/609/EEC). This study was approved by the Ethical Committee of the University of Navarra (no. 016/01; March 6, 2001).

MDMA was given acutely (single dose of 10 mg/kg i.p.) or repeatedly (10 mg/kg bid for 4 consecutive days). In other experiments, 7 days after the last injection of the chronic treatment, rats received another dose of MDMA (10 mg/kg i.p.) or saline. For biochemical studies, non-trained rats were killed by decapitation 90 min after MDMA. Trained rats were treated with MDMA 30 min before the acquisition session and killed 1 h after training, i.e., 90 min after MDMA. The brains were immediately removed and the hippocampi were dissected, frozen on dry ice and stored at −80 °C.

### 2.2. Drugs and chemicals

MDMA.HCl was a gift from “Servicio de Restricción de Estupefacientes” (Dr. L. Domínguez, Madrid, Spain). Nonidet P-40, leupeptin and PMSF were from Roche Diagnostics (Germany). All other drugs and chemicals were from Sigma-Aldrich (USA). The source of antisera and radiochemicals is indicated in the corresponding sections.

### 2.3. Passive avoidance learning

This test was performed using a two-compartment (white/dark) passive avoidance apparatus, as described in previous studies from this laboratory (Otano et al., 1999;

Barrionuevo et al., 2000; Moyano et al., 2004a). The rat was placed in the illuminated area, and 3 s later, the door was raised. During 90 s, the animal explored the apparatus freely (habituation trial). After 10 min, the rat was placed again in the illuminated chamber. When the rat entered the dark compartment, a guillotine door was closed, and after 10 s, the animal was returned to its home cage. Sixty minutes later, the animal was placed again in the white chamber. When the rat entered the dark compartment, the guillotine door was closed again, and after 10 s, an inescapable 1.8 mA scrambled electrical foot shock was delivered for 3 s through the grid floor using a shock generator (training trial). The acute MDMA dose was given 30 min before the acquisition trial, and control rats received saline injections by the same route. A retention trial was given 24 h after the acquisition trial by replacing the rat in the illuminated compartment and measuring the response latency to re-enter the dark compartment using a cutoff time of 300 s. Biochemical studies were only performed in the early phase of memory consolidation, 1 h after the training trial.

### 2.4. Determination of 5-HT levels

The levels of 5-HT were measured in rat hippocampus by high-performance liquid chromatography with electrochemical detection, as described (Pérez-Otaño et al., 1991).

### 2.5. Production of protein extracts

To prepare tissue homogenates, the hippocampus was homogenized in ice-cold buffer containing 10 mM Tris–HCl (pH 7.4), 5 mM EDTA, protease inhibitors (0.1 mM PMSF, 1 mM EGTA, 5 μg/ml aprotinin, 5 μg/ml leupeptin) and phosphatase inhibitors (0.1 mM $Na_3VO_4$, 1 mM NaF) and centrifuged at 700 *g* for 10 min. The supernatant was centrifuged again at 10,000 *g* for 10 min at 4 °C and the pellet was resuspended in 10 mM Tris–HCl (pH 7.4) containing protease and phosphatase inhibitors. The protein concentration was determined (Bio-Rad protein assay) and the aliquots were frozen at −80 °C.

To obtain membrane-enriched proteins (P2 membrane proteins), the hippocampus was homogenized in ice-cold 10 mM Tris–HCl (pH 7.4)/5 mM EDTA buffer containing 320 mM sucrose, protease inhibitors (0.1 mM PMSF, 1 mM EGTA, 5 μg/ml aprotinin, 5 μg/ml leupeptin) and phosphatase inhibitors (0.1 mM $Na_3VO_4$, 1 mM NaF) and centrifuged at 700 *g* for 10 min. The supernatant was centrifuged again at 37,000 *g* for 40 min at 4 °C and the pellet (P2) was resuspended in 10 mM Tris–HCl in the presence of the indicated enzyme inhibitors (Dunah et al., 2000). The protein concentration was determined (Bio-Rad protein assay) and the aliquots were frozen at −80 °C.

Aliquots of total tissue homogenates or P2 membrane-enriched proteins were solubilized in non-denaturing conditions by adding 0.1 vol of 10% DOC in 500 mM Tris–HCl, pH 9.0, followed by incubation at 36 °C for 30 min. Then, 0.1 vol of a buffer containing 1% Triton X-100, 500 mM Tris–HCl (pH 9.0), and the protease and phosphatase inhibitors was added and the preparations were centrifuged at 37,000 *g* for 40 min at 4 °C. Supernatants were frozen at −80 °C. For pCaMKII (Thr286) determination, P2 membrane proteins were solubilized under denaturing conditions by adding 0.1 vol of 20% sodium dodecyl sulfate (SDS) containing 50% β-mercaptoethanol and boiled for 5 min. The denatured preparations were diluted 20-fold in 50 mM Tris–HCl (pH 7.4)/0.1% Triton X-100 buffer in the presence of protease and phosphatase inhibitors and centrifuged at 37,000 *g* for 10 min at 4 °C. Aliquots of the supernatant were frozen at −80 °C.

To obtain tissue lysates for the CaMKII enzyme activity assay, the hippocampus was homogenized in 5 vol ice-cold buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 0,25% sodium deoxicolate (DOC), protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM EGTA and 10 μg/ml leupeptin), phosphatase inhibitors (1 mM $Na_3VO_4$, 2 mM NaF and 2 mM $Na_4P_2O_7$), and incubated on ice for 30 min. The homogenate was centrifuged at 14,000 *g* for 20 min and the supernatant was aliquoted and frozen at −80 °C. Protein concentration was determined using the Bio-Rad protein assay with bovine serum albumin as standard.

### 2.6. Western blotting

Solubilized proteins from total tissue homogenates or P2 membrane-enriched proteins were separated onto SDS–polyacrilamide gels (7.5% for resolution of the NMDA receptor subunits, 10% for PSD-95 and pCaMKII). Samples were diluted in an equal volume of electrophoresis buffer and boiled for 5 min. Proteins were transferred to a nitrocellulose membrane (Hybond ECL; Amersham Biosciences) using a Trans-Blot SD semidry (Bio-Rad) system for 30 min at 12 V. The membranes were blocked with 5% milk, 0.05% Tween-20 in PBS for 1.5 h at room temperature, followed by overnight incubation with the following primary antibodies: rabbit polyclonal anti-NR1, anti-phospho-NR1(Ser897) and anti-NR2B (2 μg/ml each; Upstate Biotechnology), mouse monoclonal anti-PSD-95 (10 ng/ml; Upstate Biotechnology), rabbit polyclonal anti-phospho-CaMKII (Thr286) (2 μg/ml; Upstate Biotechnology) and rabbit polyclonal anti-PP1 (2 μg/ml; Upstate Biotechnology). The membranes were washed 3 times in PBS/Tween-20 at room temperature, and HRP-conjugated anti-rabbit or anti-mouse antibody (Dako; dilution 1:1500) was added and incubated for 60 min. Following two washes in PBS/Tween-20 and one in PBS alone, immunolabeled protein bands were detected using an enhanced chemiluminescence system (ECL Amersham Biosciences), following an autoradiographic

exposure to HyperfilmECL (Amersham Biosciences). The quantification of signals was determined by densitometry using the program ImageMaster I-D (Pharmacia).

### 2.7. Immunoprecipitation

Aliquots of P2 membrane-enriched proteins (100 μg) solubilized under non-denaturing conditions were diluted 10-fold with ice-cold immunoprecipitation buffer containing HEPES 50 mM (pH 7.1), 10% glycerol, 1.5% Triton X-100, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM NaF, 1 mM EGTA, 100 μM $Na_3VO_4$, 100 μM PMSF, 5 μg/ml aprotinin and 5 μg/ml leupeptin. Samples were pre-incubated for 2 h at 4 °C with 25 μl protein A/G Plus-agarose (Santa Cruz Biotechnology) and then centrifuged to remove proteins bound nonspecifically to agarose beads. The supernatants were incubated overnight at 4 °C with 4 μg of anti-NR2B and then 35 μl protein A/G Plus-agarose was added and the incubation continued at 4 °C for 2 additional h. Immunoprecipitates were isolated by centrifugation at 14,000 rpm for 5 min and were washed for 3 times with immunoprecipitation buffer. Bound proteins were eluted in electrophoresis buffer and boiled for 5 min. Immune complexes were analyzed by immunoblotting and the membranes were incubated with anti-PSD-95 antibody as above described.

### 2.8. $Ca^{2+}$/calmodulin kinase II enzyme activity assay

Analysis of total CaMKII activity was performed using the "CaMKII Assay Kit" (Upstate Biotechnology) with autocamtide as a selective peptide substrate, in the presence of calcium and calmodulin. The reaction was carried in MOPS assay buffer containing 5–20 μg of tissue lysate, 100 μM autocamtide, 1 mM $CaCl_2$, 8 ng/μl calmodulin, 0.4 μM PKA peptide inhibitor TYADFIASGRTGRRNAI, 0.4 μM PKC peptide inhibitor RFARKGALRQKNV and a $Mg^{2+}$/ATP mixture containing 20 nCi/μl [γ-$^{32}$P]-ATP (Amersham Biosciences). The mixture was incubated at 30 °C for 10 min, and the phosphorylated substrate was separated from residual [γ-$^{32}$P]-ATP using P81 phosphocellulose filters. The filters were rinsed 3 times with 0.75% phosphoric acid and once with acetone, and bound radioactivity was quantified in a scintillation counter using the BCS Scintillation Cocktail (Amersham Biosciences). Blanks to correct for non-specific binding of [γ-$^{32}$P]-ATP to the phosphocellulose paper were run in parallel, and CaMKII activity was expressed as pmol/min per μg protein.

### 2.9. Statistical analysis

In passive avoidance studies, nonparametric statistics was used (Kruskal–Wallis ANOVA followed by Mann–Whitney *U*-test). In biochemical studies, results were analyzed using two-way ANOVA (training × treatment) and post hoc comparisons were made using the Newman–Keuls test.

## 3. Results

### 3.1. Passive avoidance learning

On the training trial, latencies to enter the dark compartment were in the range of 0.9–27.5 s. In control animals, retention latencies measured 24 h later were close to the cutoff time of 300 s. Repeated pretreatment with MDMA 7 days before training did not significantly modify retention latency. In these rats, however, acute administration of another MDMA dose 30 min before the training trial strongly reduced retention latency 24 h later (Table 1).

### 3.2. NMDA receptor subunit levels

A neurotoxic treatment with MDMA (8 × 10 mg/kg), which caused 1 week later a marked reduction in hippocampal 5-HT levels, from 464.5 ± 29.1 to 187.1 ± 12.4 pg/mg wet tissue ($n = 6–8$, $P < 0.01$) did not result at this time in any change in NR1 levels in rat hippocampal homogenates (Fig. 1A). Likewise, this repeated MDMA treatment did not change membrane levels of NR1 and pNR1(Ser897) both in trained and untrained rats (Fig. 1B, C). Avoidance training produced 1 h later a significant increase in membrane levels of NR1 and pNR1, which was significantly prevented by acute administration of another MDMA dose (10 mg/kg) 30 min before training (Fig. 1B, C). The increase in NR1 and pNR1 membrane levels was not observed in rats receiving a non-contingent shock of the same intensity (not shown).

Serotonin depletion by repeated MDMA treatment did not either affect total or membrane NR2B levels (Fig. 2). In rats trained on the passive avoidance task 7 days after the neurotoxic MDMA treatment, another MDMA dose (10 mg/kg), injected 30 min before training, reduced NR2B immunoreactivity in membrane-enriched extracts (Fig. 2B).

Table 1
Effect of acute MDMA treatment on passive avoidance retention in rats pretreated or not with repeated doses of MDMA

| Pretreatment | Treatment | Latency (s) |
|---|---|---|
| Saline | Saline | 282 ± 17.14 |
| | MDMA | 23.8 ± 15.10* |
| MDMA (8 × 10 mg/kg) | Saline | 300 ± 0.00 |
| | MDMA | 19.3 ± 5.30* |

Pretreatment with MDMA given for 4 consecutive days (2 × 10 mg/kg/day), the last dose injected 7 days before the acquisition session. Acute MDMA treatment (10 mg/kg i.p.) given 30 min before the acquisition session. The retention trial was given 24 h after the acquisition session. Values are means ± S.E.M. of 8–10 animals.

* $p < 0.01$ vs. saline controls (Kruskal–Wallis ANOVA followed by Mann–Whitney *U*-test).

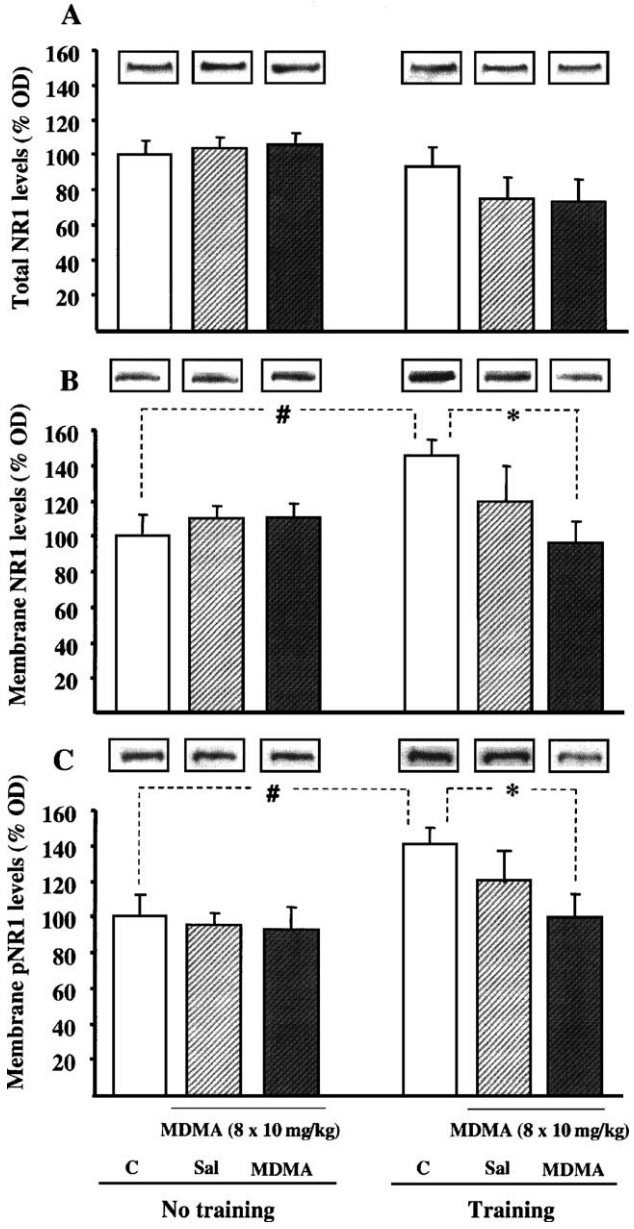


Fig. 1. NMDA receptor NR1 subunit levels in tissue homogenates (A), and NR1, pNR1(Ser897) levels in membrane-enriched protein extracts (B, C) from the hippocampus of rats pretreated with MDMA (8 × 10 mg/kg i.p.). Passive avoidance training session performed 7 days after the last MDMA injection. Rats killed 1 h after passive avoidance training. Additional MDMA treatment (single injection of 10 mg/kg i.p.) given 30 min before the acquisition session. In non-trained rats, acute MDMA treatment given 90 min before sacrifice. Values (means ± S.E.M.; $n = 10–16$) are expressed as percentage of optical density (OD) values of control non-trained rats. $^{\#}P < 0.05$ vs. control non-trained rats; $^{*}p < 0.05$ vs. control trained rats (two-way ANOVA followed by Newman–Keuls test). Representative scanned hybridized bands are shown at the upper part of the figures.

### 3.3. Levels of PSD-95 and PSD-95 associated to NR2B in membrane extracts

Serotonin depletion by the neurotoxic MDMA treatment did not induce any change in PSD-95 membrane levels. Another MDMA dose (10 mg/kg) given 7 days after the repeated treatment produced a decrease in PSD-95 levels both in untrained and trained animals (Fig. 3).

The levels of PSD-95 bound to NR2B were measured by immunoprecipitation. No significant changes were observed in animals with a marked 5-HT depletion by the neurotoxic MDMA treatment. When these animals received another dose of MDMA 30 min before training, a decrease in PSD-95 associated to NR2B in membrane-enriched extracts was found; such a decrease did not reach statistical significance in untrained animals (Fig. 4).

### 3.4. CaMKII levels and enzyme activity

Serotonin depletion in the hippocampus by the neurotoxic MDMA treatment (~60% reduction) did not significantly affect the immunoreactivity of membrane

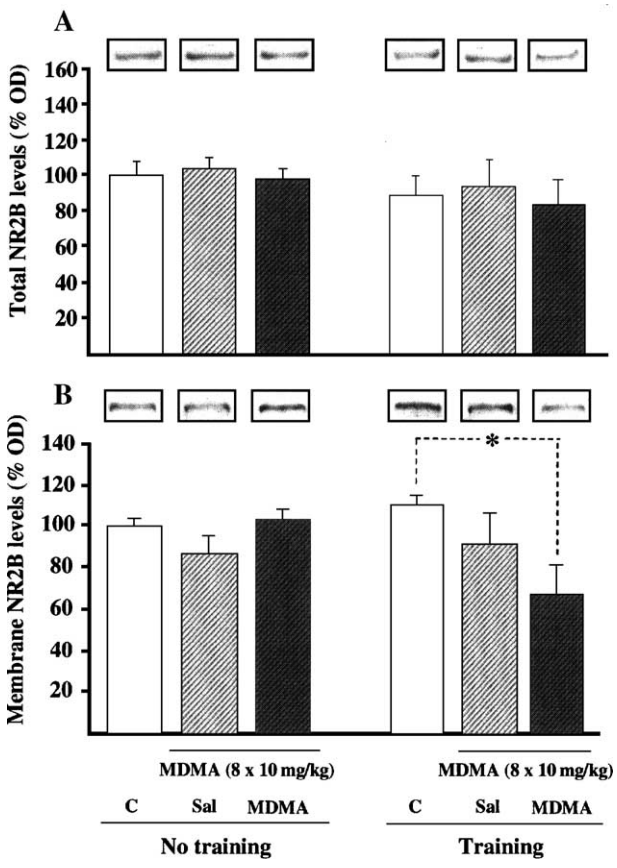


Fig. 2. NMDA receptor NR2B subunit levels in tissue homogenates (A) and membrane-enriched protein extracts (B) from the hippocampus of rats pretreated with MDMA (8 × 10 mg/kg i.p.). Passive avoidance training session performed 7 days after the last MDMA injection. Rats killed 1 h after passive avoidance training. Additional MDMA treatment (single injection of 10 mg/kg i.p.) given 30 min before the acquisition session. In non-trained rats, acute MDMA treatment given 90 min before sacrifice. Values (means ± S.E.M.; $n = 8–12$) are expressed as percentage of optical density (OD) values of control non-trained rats. $^{*}p < 0.05$ vs. control trained rats (two-way ANOVA followed by Newman–Keuls test). Representative scanned hybridized bands are shown at the upper part of the figures.

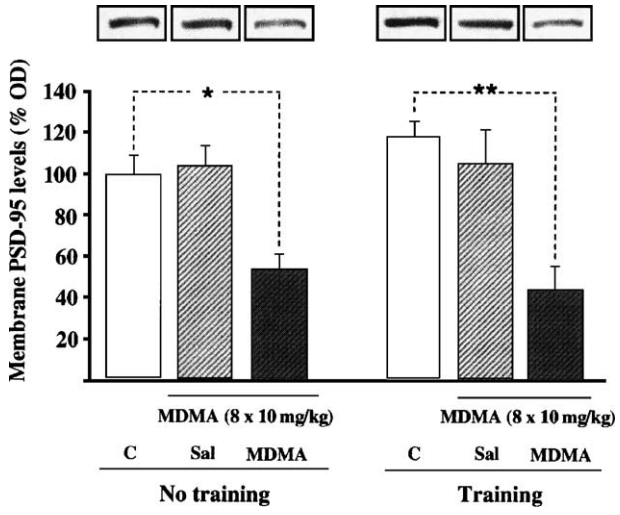


Fig. 3. PSD-95 levels in membrane-enriched protein extracts from the hippocampus of rats pretreated with MDMA (8 × 10 mg/kg i.p.). Passive avoidance training session performed 7 days after the last MDMA injection. Rats killed 1 h after passive avoidance training. Additional MDMA treatment (single injection of 10 mg/kg i.p.) given 30 min before the acquisition session. In non-trained rats, acute MDMA treatment given 90 min before sacrifice. Values (means ± S.E.M.; $n = 10–14$) are expressed as percentage of optical density (OD) values of control non-trained rats. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. the corresponding controls (two-way ANOVA followed by Newman–Keuls test). Representative scanned hybridized bands are shown at the upper part of the figure.

pCaMKII (Thr286) in control, non-trained rats; in these animals, another MDMA dose was also ineffective (Fig. 5). Passive avoidance training significantly increased

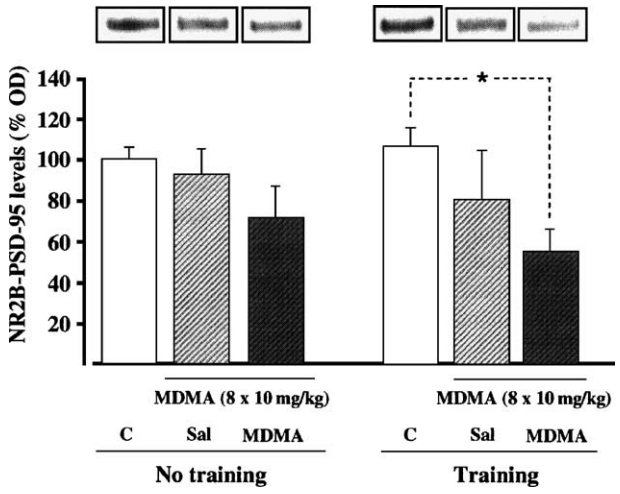


Fig. 4. Levels of PSD-95 bound to NR2B in membrane-enriched protein extracts from the hippocampus of rats pretreated with MDMA (8 × 10 mg/kg i.p.). Passive avoidance training session performed 7 days after the last MDMA injection. Rats killed 1 h after passive avoidance training. Additional MDMA administration (single injection of 10 mg/kg i.p.) given 30 min before the acquisition session. In non-trained rats, acute MDMA treatment given 90 min before sacrifice. Values (means ± S.E.M.; $n = 10–14$) are expressed as percentage of optical density (OD) values of control non-trained rats. $^{*}p < 0.05$ vs. control trained rats (two-way ANOVA followed by Newman–Keuls test). Representative scanned hybridized bands are shown at the upper part of the figure.

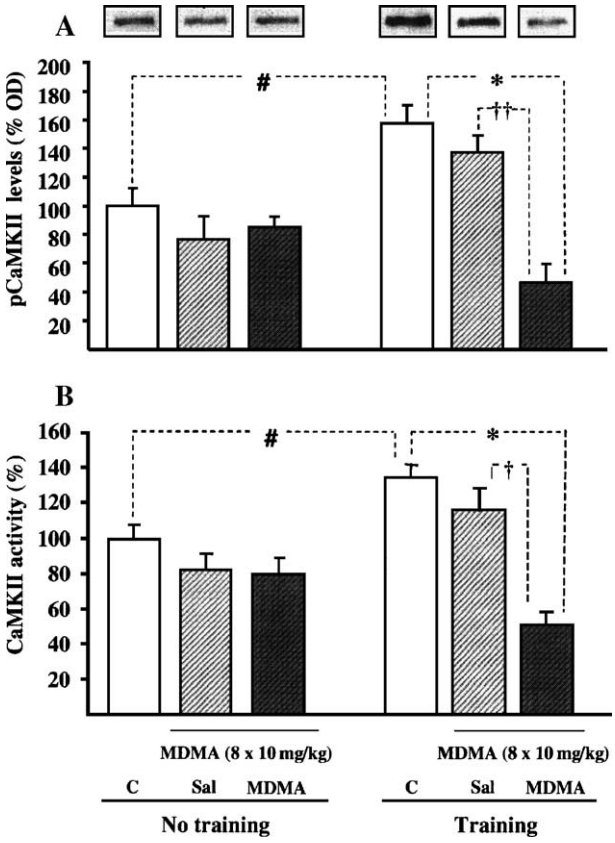


Fig. 5. Levels of pCaMKII (Thr286) in membrane-enriched protein extracts (A) and CaMKII enzyme activity in tissue lysates (B) from the hippocampus of rats pretreated with MDMA (8 × 10 mg/kg i.p.). Passive avoidance training session performed 7 days after the last MDMA injection. Rats killed 1 h after passive avoidance training. Additional MDMA treatment (single injection of 10 mg/kg i.p.) given 30 min before the acquisition session. In non-trained rats, acute MDMA treatment given 90 min before sacrifice. Values (means ± S.E.M.; $n = 7–10$) are expressed as percentage of optical density (OD) (A) or enzyme activity (B) values of control non-trained rats. $^{\#}P < 0.01$ vs. control non-trained rats; $^{*}p < 0.01$ vs. control trained rats; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ vs. rats pretreated 7 days before training with MDMA, 8 × 10 mg/kg i.p. (two-way ANOVA followed by Newman–Keuls test). Representative scanned hybridized bands for pCaMKII are shown.

pCaMKII levels in membrane-enriched protein extracts from the rat hippocampus. Such an increase, not observed in rats receiving a non-contingent shock (not shown), was not modified in 5-HT-depleted animals but was significantly prevented by another MDMA challenge given 30 min before training (Fig. 5A).

Similar results were found when CaMKII enzyme activity was measured in tissue lysates. An acute MDMA treatment, given 30 min before training, prevented the learning-associated increase in the enzyme activity (Fig. 5B).

### 3.5. PP1 levels

In non-trained animals, membrane PP1 levels did not change 1 week after the neurotoxic regimen of MDMA. At

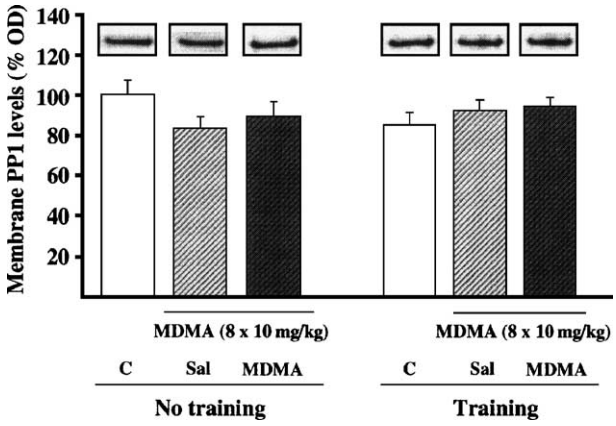


Fig. 6. Phosphatase 1 (PP1) levels in membrane-enriched protein extracts from the hippocampus of rats pretreated with MDMA (8 × 10 mg/kg i.p.). Passive avoidance training session performed 7 days after the last MDMA injection. Rats killed 1 h after passive avoidance training. Additional MDMA administration (single injection of 10 mg/kg i.p.) given 30 min before the acquisition session. In non-trained rats, acute MDMA treatment given 90 min before sacrifice. Values (means ± S.E.M.; $n = 7–10$) are expressed as percentage of optical density (OD) values of control non-trained rats. Representative scanned hybridized bands are shown at the upper part of the figure.

this time, another MDMA dose did not either produce any change in PP1 levels in trained or untrained rats (Fig. 6).

## 4. Discussion

A neurotoxic MDMA treatment causing 1 week later a marked 5-HT depletion in the hippocampus did not result in impaired retention performance of rats on a passive avoidance task and did not either induce any change in NMDA receptor subunits or CaMKII levels. In 5-HT depleted rats, another MDMA challenge induced retention impairment and prevented learning-associated changes in key molecules for learning consolidation such as pCaMKII (Thr286) and the NMDA receptor NR1 subunit.

Ionotropic NMDA receptors play important roles in synaptic plasticity and memory formation (Collingridge and Lester, 1989). This class of glutamate receptors is generally composed of two types of subunits, the NR1 subunit and the glutamate binding subunits NR2A-D (Hollmann and Heinemann, 1994), heteromeric combinations of NR1 with NR2A and NR2B being predominant in mature neurons (Tovar and Westbrook, 1999). NMDA receptors are localized to the postsynaptic density (PSD) where they are structurally organized in a large macromolecular signaling complex composed of kinases, phosphatases and scaffolding proteins (Kennedy, 2000; Sheng, 2001). A major scaffolding protein at the PSD is PSD-95, which enhances NMDA receptor clustering at synapses (Kim et al., 1996) and inhibits NR2-mediated internalization (Roche et al., 2001; Lavezzari et al., 2004). The NR2A subunit is highly synaptic whereas robust trafficking from synaptic to extrasynaptic sites has been reported for the NR2B subunit (Tovar and Westbrook, 1999; Lavezzari et al., 2004).

In animals trained on a passive avoidance learning task, we found an increase in hippocampal membrane expression of the NMDA receptor NR1 subunit, as already reported (Cammarota et al., 2000; Moyano et al., 2004b), and also in NR1 phosphorylated at Ser897, a PKA site (Tingley et al., 1997), a likely consequence of the early learning-associated increase in PKA enzyme activity (Bernabeu et al., 1997; Moyano et al., 2004a). Enhanced surface expression of pNR1 results in NMDA receptor activation and a subsequent increase in $Ca^{++}$ entry (Leonard and Hell, 1997). These learning-associated effects were not significantly modified 1 week after a neurotoxic MDMA treatment but were prevented by further administration of a single MDMA injection to the 5-HT depleted animals. In close parallelism, the neurotoxic MDMA treatment did not induce any retention deficit, but a subsequent single injection of MDMA markedly impaired retention performance. In keeping with the results of the present study, it was shown in a recent report (Winsauer et al., 2004) that the impairing effects of a neurotoxic regimen of MDMA on rate and accuracy in a multiple schedule of repeated acquisition were mostly limited to days on which rats received MDMA injections. These results, as well as the data of the present study and many others, could be attributed to state-dependent learning (e.g., Zarrindast and Rezayof, 2004). This possibility should be addressed in future studies by giving MDMA not only before training but also before the retention test.

The used schedule of MDMA administration also decreased hippocampal membrane NR2B levels. Reductions of NMDA subunits in the hippocampus are associated with deficits of LTP and spatial learning (Nihei et al., 2000; Clayton et al., 2002), so these findings suggest a role for NMDA receptors in the acute MDMA-induced retention deficit. It is known that an increase in NMDA receptor sensitivity is produced when NR1 subunits are incorporated into synapses (Scott et al., 2001). As the total hippocampal levels of the NR1 and NR2B subunits were not modified by avoidance learning or MDMA administration, it is to be supposed that the changes in membrane expression of NMDA receptor subunits are a consequence of increased trafficking to/from synapses.

On the basis of the possible internalization of NMDA receptor subunits as a determinant of the acute amnesic effect of MDMA, we studied PSD-95 membrane levels as well as PSD-95 associated to NR2B. In both cases, there was a pronounced decrease in trained animals with a previous MDMA-induced 5-HT depletion receiving another MDMA dose 30 min before the acquisition trial. PSD-95 has important functions at the synapses, such as organization of ionotropic glutamate receptors and, in general, regulation of synaptic transmission and plasticity (reviewed in Kim and Sheng, 2004). Although the regulation of NMDA receptor internalization is unclear, it seems that

PSD-95 could be implicated because it is the major scaffolding protein in the PSD and stabilizes ionotropic NMDA and non-NMDA receptors at the synapses (El-Husseini et al., 2000; Colledge et al., 2003). It should be noted that acute MDMA treatment of rats with normal hippocampal 5-HT content also markedly reduced the membrane levels of PSD-95 (experiments not shown) suggesting that MDMA rather produces a transient disorganization of membrane scaffolding proteins.

One of the molecular events more frequently linked to memory formation is the CaMKII activation by autophosphorylation at Thr286 after the early opening of the NMDA receptor channel and the transient postsynaptic entry of $Ca^{2+}$. The enzyme autophosphorylation, which is reversed by PP1, is fundamental for LTP induction and avoidance learning acquisition (Cammarota et al., 1998; Fink and Meyer, 2002). pCaMKII (Thr286) translocates to the PSD, where is the most abundant protein, and binds to the NMDA receptor, particularly to the NR2B subunit (Leonard et al., 1999). Subsequently, CaMKII phosphorylates different proteins involved in memory formation, including NMDA and AMPA receptor subunits (Barria et al., 1997).

In passive avoidance learning, like in LTP, an early increase in pCaMKII membrane levels and enzyme activity has been described (Cammarota et al., 1998; Moyano et al., 2004a), and a single dose of MDMA administered before the training trial prevented this increase (Moyano et al., 2004b). In the present study, 5-HT depletion by the neurotoxic MDMA regimen did not modify pCaMKII levels or enzyme activity, indicating that a lasting reduction of hippocampal 5-HT levels does not modify CaMKII function. It is known that 5-HT inhibits LTP induction in the hippocampus (Corradetti et al., 1992). MDMA-induced 5-HT depletion could modify the functional state of different 5-HT receptor subtypes with a positive or negative influence on passive avoidance and LTP, such as $5\text{-HT}_4$ and $5\text{-HT}_{1A}$ receptors respectively (Edegawa et al., 1998; Marchetti et al., 2004; Schiapparelli et al., 2005) resulting in no overt effect in these two models. In 5-HT-depleted animals, however, another MDMA challenge 30 min before training fully reversed the learning-specific increase in pCaMKII membrane levels and CaMKII activity. Since PP1 levels were not modified by MDMA treatment, the present results rather suggest that the effect of MDMA on CaMKII activity and pCaMKII membrane levels in 5-HT-depleted rats subjected to avoidance training can be related to the reduced NMDA receptor subunit levels which are some of the principal CaMKII binding partners in the PSD (Colbran, 2004). Besides, the possible NMDA receptor internalization could be provoking a low synaptic activity and, consequently, $Ca^{2+}$ inflow would be diminished and CaMKII activation would be prevented.

In non-trained animals, the molecular modifications previously found after a single dose of MDMA in rats with normal hippocampal 5-HT levels, such as reduced membrane NR1 and NR2B subunit levels and increased PP1 levels (Moyano et al., 2004b) were not observed in 5-HT-depleted animals. Unexpectedly, different mechanisms seem to be involved in the effects of MDMA on the molecular markers studied in untrained animals, normal levels of hippocampal 5-HT being a determinant factor. Conversely, the molecular changes in 5-HT-depleted animals treated with MDMA 30 min before the acquisition trial were quite similar to those previously observed in rats with normal 5-HT levels (Moyano et al., 2004b) suggesting that 5-HT-independent molecular mechanisms account for the observed learning deficit.

The long-term behavioural consequences of exposure of laboratory animals to high MDMA doses are controversial and many studies have reported no remarkable drug effects (e.g., Cole and Sumnall, 2003). The present study did not reveal any cognitive deficit in rats 1 week after a neurotoxic MDMA treatment. No learning-specific molecular change was either found. It seems that passive avoidance impairment and the associated biochemical changes induced by MDMA are limited to an early phase after drug administration and do not depend on endogenous 5-HT levels.

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