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ORIGINAL INVESTIGATION

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NMDA receptor subunit and CaMKII changes in rat hippocampus induced by acute MDMA treatment: a mechanism for learning impairment

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Abstract *Rationale:* Cognitive deficits have been reported in recreational 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy") users. In rats and other animal species, acute MDMA administration produces an impairment in passive avoidance and other learning tasks. Different studies have shown that this learning deficit is not strictly related to the pronounced serotonin (5-HT) depletion induced by the drug. *Objectives:* This study was aimed at determining if acute MDMA administration induces in the rat hippocampus early molecular changes related to memory impairment in a passive avoidance task. The membrane expression of key molecules in memory consolidation, such as the NR1 and NR2B subunits of the *N*-methyl-D-aspartate (NMDA) receptor, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and protein phosphatase 1 (PP1) was measured. Some of these studies were also performed after 5-HT depletion induced by the 5-HT synthesis inhibitor *p*-chlorophenylalanine (PCPA). *Methods:* Neurochemical studies were performed in rats treated with MDMA and killed 90 min later and also in rats subjected to passive avoidance 30 min after MDMA treatment. Western blotting was used for measuring the levels of NMDA receptor subunits, CAMKII and PP1. Enzyme activity assays were also performed. *Results:* In hippocampal membrane extracts, passive avoidance training increased NMDA receptor NR1 subunit expression as well as CaMKII levels and phosphorylated CaMKII. In untrained rats, MDMA reduced NR1 and NR2B protein levels, membrane CaMKII levels and enzyme activity, and enhanced PP1 levels and activity. In trained rats, MDMA prevented the learning-specific increase in NR1 subunit expression and membrane CaMKII/pCaMKII levels. After pronounced 5-HT depletion by PCPA, MDMA impaired passive avoidance retention to a similar extent and also prevented the training-associated changes in NR1 levels and CaMKII activity. *Conclusions:* Diminished function of hippocampal CaMKII and reduced levels of synaptic NMDA receptor subunits appear to be involved in the impairment of passive avoidance learning induced in rats by acute MDMA treatment.

Keywords MDMA · Ecstasy · Serotonin · Learning · Memory · NMDA receptor · CaMKII · PP1 · Hippocampus

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Introduction

3,4-Methylenedioxymethamphetamine (MDMA, "ecstasy") is a popular recreational drug with a selective neurotoxic effect on serotonin (5-HT) nerve terminals in a variety of animal species, including probably humans. The pharmacology of MDMA is still enigmatic, and many aspects of the cellular and molecular mechanisms involved in MDMA actions remain to be elucidated (for recent reviews on MDMA, see Cole and Sumnall 2003; Green et al. 2003; Lyles and Cadet 2003).

Subtle but significant long-term deficits in cognitive performance have been associated with the frequent recreational use of "ecstasy" (Bolla et al. 1998; McCann et al. 1999; Morgan 1999; Rodgers 2000; Verkes et al. 2001). In different animal models of learning and memory, chronic MDMA treatment also produces learning deficits that do not appear strictly related to the neurotoxic effect of the drug on serotonergic terminals and the ensuing 5-HT depletion (Frederick et al. 1995; Byrne et al. 2000; Taffe et al. 2002). In previous studies from this laboratory (Artaiz et al. 1996; Barrionuevo et al. 2000), it was found that acute administration of either MDMA or 3,4-methylenedioxyethamphetamine (MDEA, "eve") before the acquisition trial of a passive avoidance learning task impaired retention 24 h later. By giving repeated injections of MDMA or MDEA for 4 consecutive days, a profound 5-HT depletion in different brain regions,

including the hippocampus, was observed 7 days later (Aguirre et al. 1997; Barrionuevo et al. 2000). No retention deficit was, however, found at this time point in the 5-HT-depleted rats (Artaiz et al. 1996; Barrionuevo et al. 2000). These results were in keeping with another report (Santucci et al. 1996) showing that deficits in retention performance in the passive avoidance and radial maze arm tasks by the serotonergic neurotoxin *p*-chloroamphetamine were not related to 5-HT depletion but rather, as suggested by the authors, to excessive 5-HT release. It is of note that in our previous studies in rats with extensive MDMA or MDEA-induced 5-HT depletion (Artaiz et al. 1996; Barrionuevo et al. 2000), a new drug challenge again produced an impairment in retention performance.

On the basis of the short-term retention deficit induced by acute MDMA treatment, it appeared of interest to study the effect of this neurotoxin on early molecular events involved in the formation of aversive memory in the rat hippocampus. Even though other brain structures, such as the amygdala, may contribute to early memory processing of passive avoidance, the sequence of molecular events in the hippocampus associated with learning and retention of passive avoidance tasks appears to be strikingly similar to those underlying other forms of learning and also long-term potentiation (LTP) (reviewed in Izquierdo and Medina 1997; Shobe 2002). The role of glutamate NMDA receptors in the early stages of hippocampal-dependent memory storage and in LTP has been extensively documented (e.g. review 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 key molecule in subsequent events related to synaptic potentiation (Fink and Meyer 2002). NMDA receptors are not only the source for Ca^{2+} entry but also the postsynaptic adapter sites for CaMKII through direct binding of the enzyme to the NR1 and NR2B subunits (Leonard et al. 1999). The phosphorylation/activation of CaMKII is regulated by phosphatases, in particular protein phosphatase 1 (PP1), an enzyme with an inhibitory role in memory formation (Genoux et al. 2002).

This study explores the possibility of an effect of MDMA on crucial molecular mechanisms involved in passive avoidance learning that could account for the short-term retention deficit induced by this drug. We sought for early changes in the membrane expression of the NMDA receptor NR1 and NR2B subunits, and for the expression and activity of the enzymes CaMKII and PP1. To examine the influence of available 5-HT on these changes, experiments were also performed in rats pretreated with the 5-HT synthesis inhibitor *p*-chlorophenylalanine (PCPA).

Materials and methods

Animals and drug treatment

Male Wistar rats (Harlan, Barcelona, Spain) weighing 200–220 g were used. Three animals were allocated three to a cage with free access to food and water and maintained in a temperature-controlled environment (21–23°C) on a 12-h light/dark cycle with lights on at 0800 hours. 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).

A single dose of MDMA (10 mg/kg IP) was given 90 min before the animals were killed. In rats subjected to avoidance training, the acquisition trial was performed 30 min after drug treatment. In biochemical studies, the rats were killed by decapitation 1 h after training (i.e. 1 h after the footshock of the acquisition trial). The corresponding controls received identical pharmacological treatments, but were not subjected to passive avoidance training. In another control group, rats were directly placed on the grid floor and were killed 1 h after receiving an identical footshock to the trained rats. The brains were immediately removed and the hippocampi were dissected, frozen on dry ice and stored at −80°C.

PCPA was given for 3 consecutive days at a daily dose of 100 mg/kg IP. Behavioural and biochemical studies were performed 24 h after the last PCPA injection.

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.

Passive avoidance learning

The experiments were performed using a two-compartment (white/dark) passive avoidance apparatus, as described in previous studies from this laboratory (Artaiz et al. 1995; Otano et al. 1999; Barrionuevo et al. 2000). 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). Ten minutes later, 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 compartment. When the rat entered the dark chamber, the guillotine door was closed again and, after 10 s, an inescapable 2 mA scrambled electrical footshock was delivered for 3 s through the grid floor using a shock generator (acquisition trial). A retention trial was given 24 h after the acquisition trial by placing the rat in the illuminated compartment and measuring the response latency to re-enter the dark compartment using a cut-off time of 300 s. MDMA was given 30 min before the acquisition trial, and control rats received saline injections by the same route. Animals were assigned at random to the different treatments.

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).

Production of total and membrane protein extracts

Total protein extracts

The hippocampus was homogenized in 5 vol ice-cold buffer (B1) containing 50 mM Tris–HCl (pH 7.7), 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.

Membrane protein extracts

To obtain a membrane-enriched protein fraction, the hippocampus was homogenized in ice-cold 10 mM Tris–HCl (pH 7.4)/5 mM EDTA buffer (B2) 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 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 the membrane-enriched protein extract were solubilized in non-denaturing conditions by adding 0.1 volumes 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 500 mM Tris–HCl (pH 9.0), 1% Triton X-100 (B3) and the indicated protease and phosphatase inhibitors, as in buffer B2, was added and the preparations were centrifuged at 37,000 *g* for 40 min at 4°C. Aliquots of the supernatant were frozen at −80°C.

Other aliquots were solubilized in denaturing conditions by adding 0.1 vol 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 X100 buffer (B4) in the presence of the protease and phosphatase inhibitors, as indicated in buffer B2, and centrifuged at 37,000 *g* for 10 min at 4°C. Aliquots of the supernatant were frozen at −80°C.

Western blotting

Total protein extract, 20 μg, or membrane-enriched protein extract solubilized in either denaturing or non-denaturing conditions were separated onto SDS-polyacrylamide gels (7.5% for resolution of the NMDA receptor subunits, 10% for CaMKII and pCaMKII, 12% for PP1). Samples were diluted in an equal volume of electrophoresis buffer (B5: 0.2 M Tris–HCl, pH 7.4, 15% glycerol, 5% SDS, 8% β-mercaptoethanol, 200 μg/ml bromophenol blue) 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: mouse monoclonal anti-CaMKII (1:10,000; Chemicon), rabbit polyclonal anti-phospho-CaMKII(Thr286) (2 μg/ml; Upstate Biotechnology), rabbit polyclonal anti-PP1 (2 μg/ml; Upstate Biotechnology), rabbit polyclonal anti-NR1 and anti-NR2B (2 μg/ml each; 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, except for PP-1, which was diluted 1:3000) 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 chemiluminiscence system (ECL Amersham Biosciences), following an autoradiographic exposure to HyperfilmECL (Amersham Biosciences). The quantification of the signals was determined by densitometry using the program ImageMaster I-D (Pharmacia).

Enzyme assays

Ca^{2+}/calmodulin kinase II assay

Analysis of CaMKII activity was performed using the CaMKII Assay kit (Upstate Biotechnology) with autocamtide as selective peptide substrate in the presence of calcium and calmodulin. The reaction was carried in MOPS assay buffer containing 30–40 μg of total protein extract, 100 μM autocamtide, 1 mM $CaCl_2$, 8 ng/μl calmodulin, 0.4 μM PKA peptide inhibitor TYADFIASGRTGR-RNAI, 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 the 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.

Protein phosphatase 1 (PP1) assay

Analyses were performed using the Protein Serine/Threonine Phosphatase (PSP) Assay Kit (BioLabs), which quantifies the release of inorganic phosphate from a labelled protein The substrate used was myelin basic protein (MyBP), labelled with [γ-^{32}P]-ATP following the kit recommendations. Total protein extract, 10 μg, prepared without phosphatase inhibitors, was added to 30 μl of assay buffer containing 2 nM okadaic acid and 50 μM FK-506 as PP2A and calcineurin inhibitors, respectively. After a preincubation for 5 min at 30°C, the reaction was started by adding 10 μl of substrate and incubated for 10 min at 30°C. The reaction was terminated by adding 200 μl of cold 20% trichloroacetic acid and, after centrifugation at 12,000 *g*, radioactivity was counted in the supernatant using the BCS Scintillation Cocktail (Amersham Biosciences). Activity of PP1 was expressed as pmol/min per μg protein.

Results

Passive avoidance learning

On the acquisition trial, latencies to enter the dark chamber were in the range of 1.4–24.6 s in control animals and also after any drug treatment. MDMA (10 mg/kg), administered 30 min before the acquisition trial, significantly reduced retention latency 24 h later (Table 1). Pretreatment with the 5-HT synthesis inhibitor PCPA did not significantly modify retention latency. In PCPA-pretreated rats, MDMA also markedly reduced retention latency 24 h later (Table 1).

5-HT levels

A single injection of MDMA (10 mg/kg) produced a significant reduction in hippocampal 5-HT levels 90 min later, from 519.4±52.5 (controls) to 274.0±23.4 pg/mg

Table 1 Effect of MDMA on passive avoidance retention in rats pretreated or not with *p*-chlorophenylalanine (PCPA)

Treatment	Latency (s)
Saline	260.8±32.4
MDMA	14.8±3.2*
PCPA + Saline	198.0±45.4
PCPA + MDMA	10.3±2.0*

MDMA (10 mg/kg IP) given 30 min before the acquisition session. PCPA (100 mg/kg IP) given for 3 consecutive days; the last dose injected 24 h before the acquisition session. The retention trial was given 24 h after the acquisition session. Values are means ± SEM of 8–10 animals. *P<0.01 vs saline controls (Kruskal–Wallis ANOVA followed by Mann–Whitney U-test).

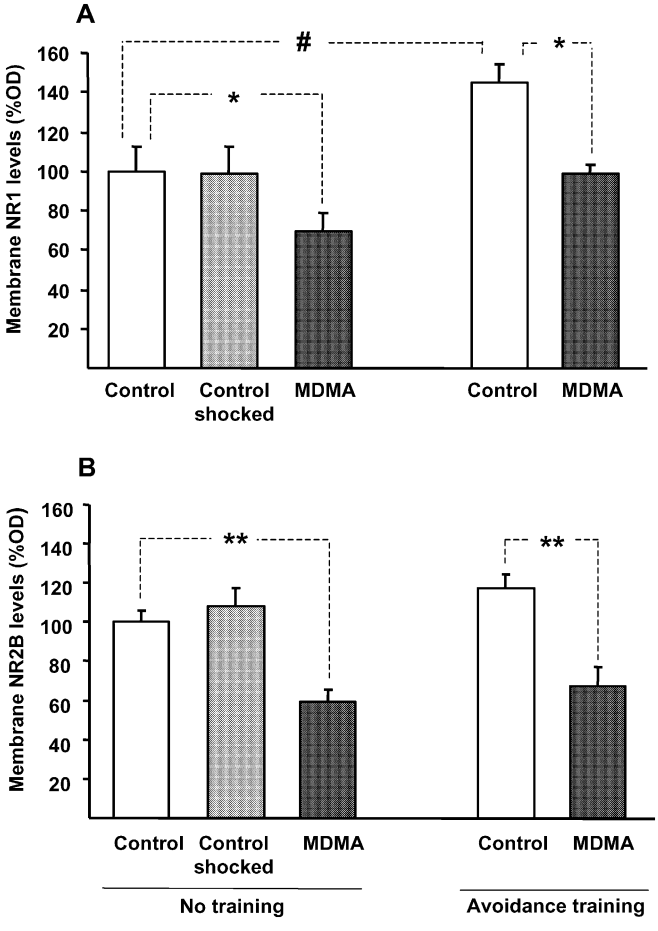

Fig. 1 NMDA receptor NR1 (**a**) and NR2B (**b**) subunit levels in membrane-enriched protein extracts from hippocampus of rats killed 1 h after passive avoidance training and of the corresponding non-trained controls. MDMA (10 mg/kg IP) given 30 min before the acquisition session or the non-contingent shock. In non-shocked rats, MDMA given 90 min before death. Values (means ± SEM; n=8–12) 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, **P<0.01 vs the corresponding control rats (Student's t-test)

wet tissue (means ± SEM; n=6; P<0.01, Student's t-test). One day after acute MDMA treatment, 5-HT loss was more pronounced (mean ± SEM=167.1±20.4; n=6; P<0.01).

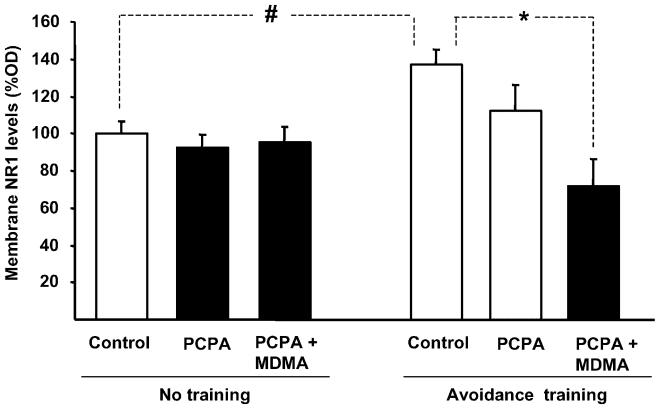

Fig. 2 Effect of MDMA on NR1 subunit levels in membrane-enriched protein extracts from hippocampus of rats pretreated with *p*-chlorophenylalanine (PCPA, 100 mg/kg IP for 3 consecutive days). Passive avoidance training session performed 24 h after the last PCPA injection. Rats killed 1 h after passive avoidance training. MDMA (10 mg/kg IP) given 30 min before the acquisition session. In non-trained rats, MDMA given 90 min before death. Values (means ± SEM; n=8–12) 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 the corresponding control rats (Student's t-test)

Repeated administration of the 5-HT synthesis inhibitor, PCPA (100 mg/kg IP for 3 consecutive days), markedly reduced 5-HT levels in rat hippocampus 24 h after the last dose, from 463.6±32.5 (controls) to 40.8± 2.2 pg/mg wet tissue (means ± SEM; n=6; P<0.01, Student's t-test).

NMDA receptor subunit levels

Passive avoidance training induced a significant increase in the membrane expression of the NMDA receptor NR1 subunit but not of the NR2B subunit (Fig. 1a,b). This increase in membrane NR1 levels was not observed in rats receiving a non-contingent shock (Fig. 1a). MDMA treatment reduced basal NR1 membrane levels and fully prevented the learning-associated increase in NR1 protein expression (Fig. 1a). Membrane NR2B basal levels were also significantly reduced by MDMA both in trained and untrained rats (Fig. 1b).

In rats with a pronounced PCPA-induced depletion (>90%) of hippocampal 5-HT, the learning-associated increase in NR1 membrane levels was also fully prevented by MDMA (Fig. 2).

CaMKII levels and enzyme activity

Neither passive avoidance training nor MDMA administration modified total CaMKII immunoreactivity in rat hippocampus (Fig. 3a). In membrane-enriched extracts, passive avoidance training induced a significant increase in CAMKII levels (∼40%) that was not observed in shocked untrained rats (Fig. 3b). Acute MDMA (10 mg/

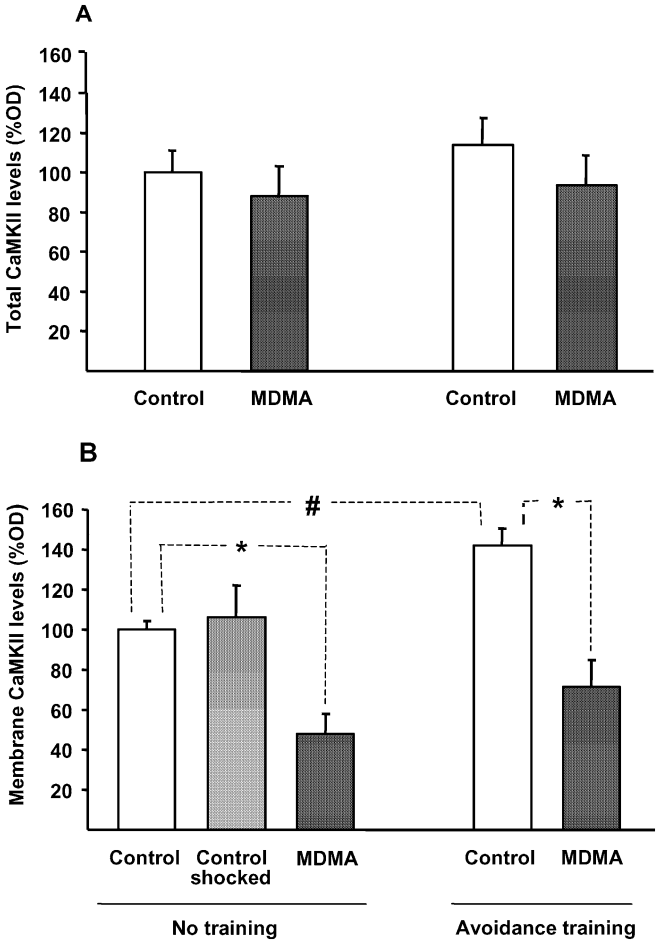

Fig. 3 Ca^{2+}/calmodulin kinase II (CaMKII) levels in total (**a**) and membrane-enriched (**b**) protein extracts from hippocampus of rats killed 1 h after passive avoidance training and of the corresponding non-trained controls. MDMA (10 mg/kg IP) given 30 min before the acquisition session or the non-contingent shock. In non-shocked rats, MDMA given 90 min before death. Values (means ± SEM; n=8–12) are expressed as percentage of optical density (OD) values of control non-trained rats. (P<0.01 vs control non-trained rats; *P<0.01 vs the corresponding control rats (Student's t-test)

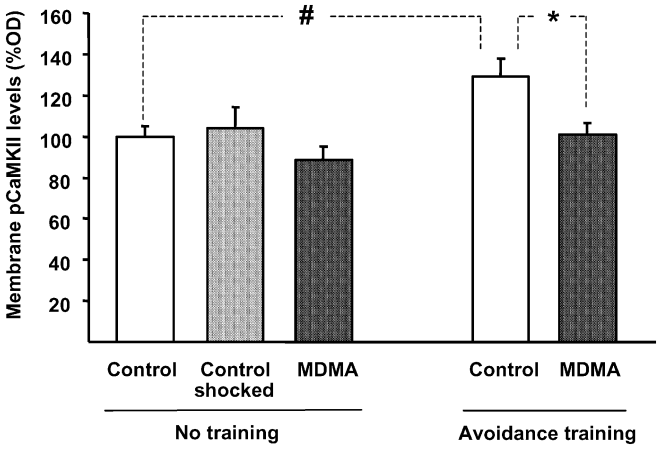

Fig. 4 Levels of pCaMKII (Thr-286) in membrane-enriched protein extracts from the hippocampus of rats killed 1 h after passive avoidance training and of the corresponding non-trained controls. MDMA (10 mg/kg IP) given 30 min before the acquisition session or the non-contingent shock. In non-shocked rats, MDMA given 90 min before death. Values (means ± SEM; n=10–14) are expressed as percentage of optical density (OD) values of control non-trained rats. (p<0.01 vs control non-trained rats; *P<0.05 vs trained control rats (Student's t-test)

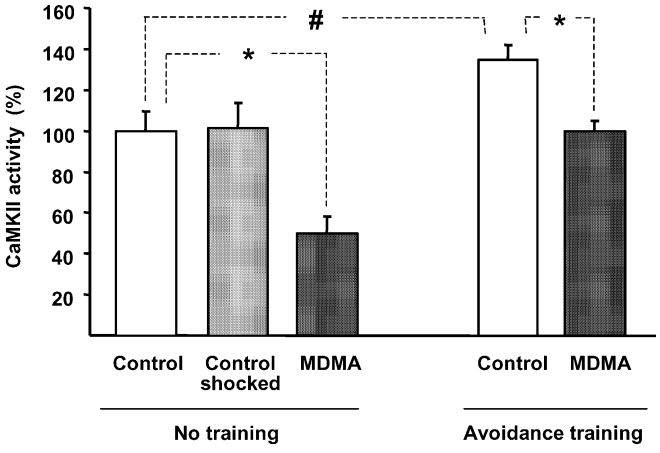

Fig. 5 Ca^{2+}/calmodulin kinase II (CaMKII) activity in total protein extracts from the hippocampus of rats killed 1 h after passive avoidance training and of the corresponding non-trained controls. MDMA (10 mg/kg IP) given 30 min before the acquisition session or the non-contingent shock. In non-shocked rats, MDMA given 90 min before death. Values (means ± SEM; n=8–12) are expressed as percentage of enzyme activity of control non-trained rats. (P<0.01 vs control non-trained rats; *P<0.01 vs the corresponding control rats (Student's t-test)

kg) produced a significant reduction in membrane CAMKII basal levels and fully reversed the learning-associated increase in CAMKII levels (Fig. 3b).

pCaMKII(Thr-286) levels were also measured in membrane extracts prepared under denaturing conditions. Phosphorylated protein immunoreactivity was also increased (~32%) after passive avoidance training and not after a non-contingent footshock. As shown in Fig. 4, administration of MDMA significantly prevented the effect of training.

In enzyme activity assays, CaMKII activity in the hippocampus of control untrained rats was 1.36± 0.13 pmol/min per μg protein (mean ± SEM; n=9). This enzyme activity was significantly increased by training (mean ± SEM=1.84±0.11; n=11; P<0.01) and was not modified by a non-contingent shock. Total enzyme activity was significantly reduced by MDMA, both in untrained and trained rats (Fig. 5). In rats pretreated with PCPA and trained on the passive avoidance task, there was also a significant effect of MDMA on CaMKII activity, from 1.97±0.12 pmol/min per μg protein in trained controls to 1.58±0.08 (means ± SEM; n=10–11; P<0.05, Student's t-test).

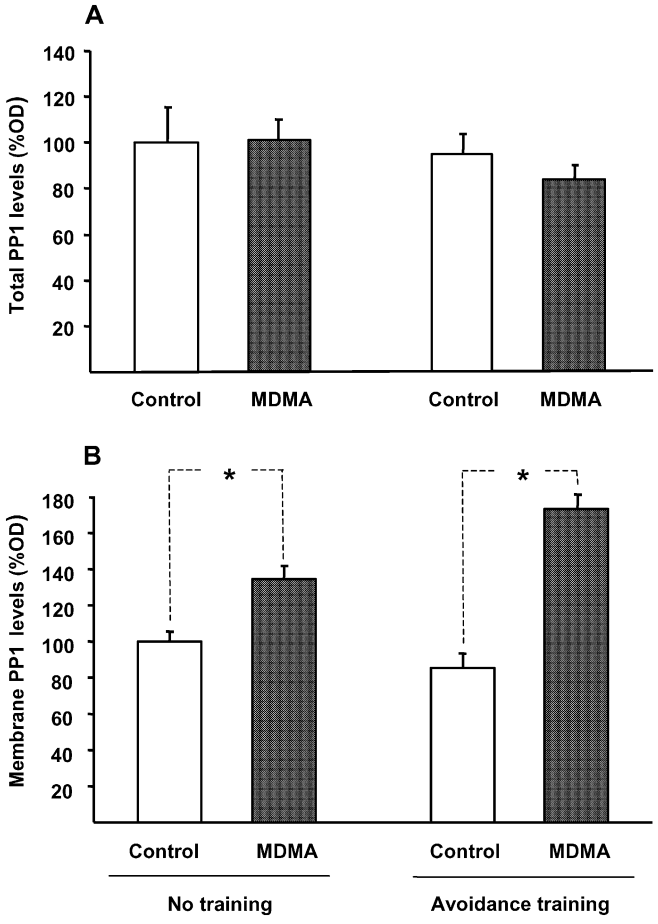

Fig. 6 Phosphatase 1 (*PP1*) levels in total (**a**) and membrane-enriched (**b**) protein extracts from the hippocampus of rats killed 1 h after passive avoidance training and of the corresponding non-trained controls. MDMA (10 mg/kg IP) given 30 min before the acquisition session. In non-trained rats, MDMA given 90 min before death. Values (means ± SEM; n=12–15) are expressed as percentage of optical density (*OD*) values of control non-trained rats. *P<0.01 vs the corresponding trained or untrained control rats (Student's t-test)

PP1 levels and enzyme activity

Passive avoidance training did not induce any significant effect on PP1 immunoreactivity, either in total nor in membrane-enriched protein extracts (Fig. 6a,b). No significant increase in PP1 levels was either found in total extracts after MDMA (Fig. 6a). Membrane PP1 levels were significantly increased by MDMA, both in untrained and trained animals, the effect being much more marked (~103% increase) in the latter case (Fig. 6b).

In enzyme activity assays, the method was initially validated with the phosphatase inhibitor okadaic acid (1 μM) and different PP1 concentrations to determine the linear range of dephosphorylation. As above, avoidance training did not produce any change in PP1 enzyme activity that was enhanced by MDMA, either in untrained or trained animals (Fig. 7).

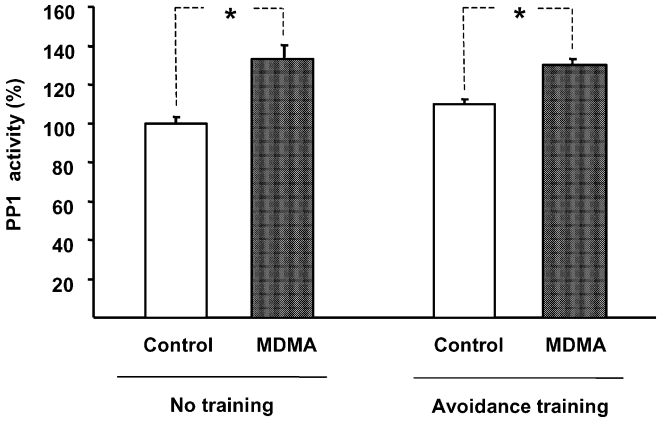

Fig. 7 Phosphatase 1 (PP1) activity in total protein extracts from the hippocampus of rats killed 1 h after passive avoidance training and of the corresponding non-trained controls. MDMA (10 mg/kg IP) given 30 min before the acquisition session. In non-trained rats, MDMA given 90 min before death. Values (means ± SEM; n=8–12) are expressed as percentage of enzyme activity of control non-trained rats. *P=0.01 vs the corresponding control rats (Student's t-test)

Discussion

The molecular events leading to MDMA-induced cognitive dysfunction are unknown. The present results suggest that acute MDMA treatment induces several changes in early molecular mechanisms that account for the consolidation of aversive memory in the rat. MDMA reduced the synaptic membrane expression of the NMDA receptor subunits, NR1 and NR2B, and of CaMKII, in the rat hippocampus. Conversely, MDMA increased the membrane expression of the phosphatase PP1. All of these changes may contribute to an impaired learning retention.

In this study, acute MDMA produced a 5-HT loss in the hippocampus that was already significant 90 min after treatment and was more marked when rats were killed 1 day after treatment. The acute 5-HT depleting effect of MDMA appears to be more pronounced and longer-lasting in the rat hippocampus than in the frontal cortex, where MDMA may increase CRE-binding and tryptophan hydroxylase mRNA expression, leading to a quicker recovery of 5-HT levels (Frechilla et al. 1999). The same MDMA dose caused a severe retention impairment in a passive avoidance task that was not, however, observed 1 week after a repeated MDMA treatment producing a profound and lasting hippocampal 5-HT depletion (Artaiz et al. 1996). Qualitatively identical effects on passive avoidance performance had also been found after repeated administration of MDEA, a MDMA analogue (Barrionuevo et al. 2000). Other mechanisms involved in memory consolidation were then investigated.

NMDA receptors are crucial for synaptic plasticity and memory. NMDA receptor antagonists block LTP induction and different forms of hippocampal-dependent memory (e.g. Riedel et al. 2003). Reductions of NMDA receptor NR1 subunit in the hippocampus are associated with deficits of LTP and spatial learning (Nihei et al.

2000). Diminished hippocampal NR2B subunit expression abolishes LTP and impairs spatial learning (Clayton et al. 2002). Conversely, overexpression of NMDA receptor NR2B subunit leads to enhanced activation of NMDA receptors and to superior learning and memory ability in different tasks (Tang et al. 1999). Following induction of hippocampal LTP, increases in the expression of the NR2B subunit in the dentate gyrus have been reported (reviewed in Loftis and Janowsky 2003). In the present study, we found that passive avoidance training increased the hippocampal membrane expression of the NMDA receptor NR1 subunit. This increase was learning-specific, i.e. was not affected by a non-contingent shock. The rapid increase in NR1 subunit expression in hippocampal membranes of rats trained on an inhibitory avoidance task is in keeping with a previous report (Cammarota et al. 2000). In the latter study, no change was found, as in the present report, on NR2B subunit expression. In general, the elevation of NR2B subunit expression is delayed after LTP induction (Loftis and Janowsky 2003), although transient increases in the expression of the NR2B subunit in hippocampal LTP have been also reported (Thomas et al. 1994; Williams et al. 2003). Interestingly, MDMA not only reduced basal levels of NR1 and NR2B subunits, but also prevented the enhanced expression in trained rats of the NR1 subunit, suggesting a more specific involvement of this NMDA receptor subunit in the retention impairment induced by the drug. A similar effect of MDMA on NR1 subunit expression was found in trained rats after pretreatment with PCPA, a tryptophan hydroxylase inhibitor (Jequier et al. 1967), that produced a marked hippocampal 5-HT depletion. PCPA pretreatment did not alter by itself passive avoidance retention, as previously reported (Misane et al. 1998), and also did not modify the retention impairment induced by a subsequent MDMA challenge. Given the very small remaining amount of endogenous 5-HT after PCPA (<10% of control), these findings suggest that the acute effects of MDMA on learning-specific molecular events can hardly be interpreted in terms of 5-HT release. The hyperthermic response in rats to MDMA, reported in most studies (Green et al. 2003), could perhaps influence NMDA receptor subunit expression, which may be down-regulated by prolonged heat stress (cited in Loftis and Janowsky 2003). While the effect of MDMA-induced hyperthermia on NMDA receptors cannot be really excluded, the consistent prevention by MDMA, in animals depleted or not of 5-HT, of the training-associated increase in NR1 expression appears to argue against this possibility.

CaMKII is an essential biochemical substrate for the early phase of memory formation (Fink and Meyer 2002). Intracellular calcium elevation after appropriate stimuli causes enzyme autophosphorylation followed by translocation to the postsynaptic density, where it binds to the NR1 and NR2B subunits of the NMDA receptor (Leonard et al. 1999; Shen and Meyer 1999). This binding locks CaMKII in an active, calcium/calmodulin-independent state (Bayer et al. 2001) and provides spatial control of phosphorylation of different postsynaptic proteins implicated in memory formation, particularly the GluR1 subunit of the AMPA receptor (Barria et al. 1997). CaMKII dephosphorylation by PP1 leads to the dissociation of CaMKII from the postsynaptic density (Yoshimura et al. 1999). In keeping with previous studies (Cammarota et al. 1998), we found that passive avoidance training increased CaMKII levels in membrane extracts without any change in total expression, suggesting a CaMKII translocation to postsynaptic sites. The increase in phosphorylated CaMKII and in total enzyme activity was also learning-specific, i.e. was not influenced by a non-contingent shock. Acute MDMA treatment reduced membrane CaMKII levels and catalytic activity, both in trained and untrained animals, but pCaMKII levels were only reduced by MDMA in trained animals, suggesting that this drug exerts a negative influence on memory formation through a reduced CaMKII phosphorylation/activation. In untrained animals, MDMA reduced CaMKII enzyme activity but did not modify membrane pCaMKII levels. It is known that pCaMKII (Thr-286) is in a Ca^{2+}-independent state (Hudmon and Schulman 2002), whereas both Ca^{2+}-dependent and independent CaMKII activity was measured in the present study. The results reported in this study are still at a descriptive stage, and we can only suggest that the acute 5-HT releasing effect of MDMA is not involved, or is involved only to a limited extent, in the retention deficit induced by the drug. Interestingly, a reduced CaMKII activity in rat hippocampus has been reported after acute treatment with methamphetamine, another neurotoxic amphetamine derivative (Suemaru et al. 2000). This methamphetamine effect was also found by a challenge after a 4-week abstinence from chronic methamphetamine administration. In the present study with PCPA-pretreated animals, we found that MDMA reduced the learning-associated increase in CaMKII activity, and our preliminary results (not shown) appear to indicate that acute MDMA also reduces membrane CaMKII levels in rats with a 5-HT depletion previously induced by repeated MDMA treatment.

PP1 activity inhibition through endogenous PP1 inhibitors, such as Inhibitor-1 (Endo et al. 1996), is necessary to maintain an active state of CaMKII at the postsynaptic location (Shobe 2002) and enhanced PP1 activity leads to memory deficits (Genoux et al. 2002). MDMA treatment did not modify total PP1 levels in total protein extracts from rat hippocampus, but significantly increased enzyme activity and, importantly, produced a considerable increase in membrane PP1 levels in trained animals and a much more moderate increase in PP1 membrane levels in untrained rats. Such a learning-associated elevation in PP1 levels by MDMA results in lower pCaMKII levels, and the reduced CaMKII function should disrupt the ensuing later events involved in memory formation.

As reported in previous studies (see Introduction), the retention impairment induced by MDMA and other 5-HT neurotoxins does not strictly depend on reduced 5-HT

levels in the forebrain. The results here described also suggest that the effect of MDMA does not possibly, or does not solely, depend on endogenous 5-HT release and provide a molecular mechanism for the deficit in passive avoidance performance induced by this drug. These effects should probably be taken into consideration at the time of deciphering other enigmatic behavioural and neurochemical effects of MDMA. Here, we have only measured molecular changes at very early stages of memory consolidation that result in retention impairment one day later. The long-term significance of the present findings obviously remains to be established.

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