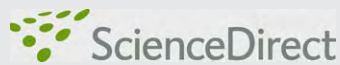


available at www.sciencedirect.comwww.elsevier.com/locate/brainres**BRAIN
RESEARCH**

Research Report

Association of caffeine to MDMA does not increase antinociception but potentiates adverse effects of this recreational drug

Jordi Camarasa¹, David Pubill¹, Elena Escubedo*

Unitat de Farmacologia i Farmacognòsia, Facultat de Farmàcia, Universitat de Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain

ARTICLE INFO

Article history:

Accepted 28 June 2006

Keywords:

MDMA
Caffeine
Rat
Neurotoxicity
Antinociception

Abbreviations:

DA, dopamine
DAT, dopamine transporter
5-HT, serotonin
SERT, serotonin transporter
MDMA, 3,4-methylenedioxy-
methamphetamine or ecstasy
METH, methamphetamine

ABSTRACT

Ecstasy (MDMA) street tablets often contain several other compounds in addition to MDMA, particularly caffeine. Then, it becomes necessary to study the consequences of caffeine plus MDMA combination. MDMA (1 mg/kg) elicited an analgesic response both at the spinal and supraspinal levels. However, when associated, MDMA and caffeine did not show any synergistic interaction. When caffeine was administered prior to MDMA, a potentiation of locomotor activity was observed, which consisted in an increase in maximal values and in a prolonged time of activity. In the neurotoxicity studies, a hyperthermic effect of MDMA was observed. Although caffeine alone failed to alter body temperature, it potentiated MDMA-induced hyperthermia. This association also significantly increased MDMA lethality (from 22% to 34%). Following administration of MDMA to rats, there was a persistent decrease in the number of serotonin transporter sites in the cortex, striatum and hippocampus, which was potentiated by caffeine co-treatment. This MDMA toxicity in rats was accompanied by a transient dopaminergic impairment in the striatum, measured as decreased [³H]WIN35428 binding sites, by 31% 3 days after treatment, which was not modified by caffeine. A transient down-regulation of 5-HT₂ receptors occurred in the cortex of MDMA-treated rats, whose recovery was slowed by co-treatment with caffeine. In conclusion, the association of MDMA with caffeine does not generate any beneficial effects at the antinociceptive level. The acute effects stemming from this association, in tandem with the final potentiation of serotonergic terminals injury, provide evidence of the potentially greater long-term adverse effects of this particular recreational drug combination.

© 2006 Elsevier B.V. All rights reserved.

1. Introduction

3,4-Methylenedioxymethamphetamine (MDMA, ecstasy) is a recreational drug that achieved popularity in the United States about two decades ago, but which continues to be highly popular in Europe, particularly at dance parties (“raves”).

Ecstasy street tablets from the illicit market often contain several other compounds in addition to MDMA, particularly caffeine in varying amounts. In a recent paper, [Klingler et al. \(2005\)](#) determined that about 10% of all pills analyzed were supplemented with this xanthine derivative. Moreover, beverages containing caffeine (“energy drinks”) are frequently

* Corresponding author. Fax: +34 934035982.

E-mail address: eescubedo@ub.edu (E. Escubedo).

¹ J. Camarasa and D. Pubill contributed equally to this work.

consumed while taking ecstasy in order to reduce drowsiness and fatigue.

The acute effects of MDMA in the central nervous system are complex, with several molecular sites of action. MDMA has major effects on serotonin (5-HT) pathways since it causes an acute and rapid increase in extracellular 5-HT in the striatum, frontal-cortex and hippocampus via the release of 5-HT and the inhibition of its uptake (Green et al., 2003). In addition, dopamine (DA) and noradrenalin have also been implicated. MDMA binds to all three of the monoamine presynaptic transporters, exhibiting its highest affinity for the 5-HT transporter (SERT). It also binds to several classical receptors, its highest affinity being for 5-HT₂, α 2-adrenergic, M1 and H1 receptors. MDMA itself induces DA release (Schmidt et al., 1987; Steele et al., 1987; White et al., 1994; Cadoni et al., 2005), although the acute increase in serotonergic neurotransmission that concomitantly occurs markedly amplifies the concentration of extracellular DA (Gudelsky and Nash, 1996) through an activation of postsynaptic 5-HT_{2A/2C} receptors (Sprague et al., 1998).

Administration of a neurotoxic regimen of MDMA to rats results in inhibition of tryptophan hydroxylase, decreased cerebral tissue concentrations of 5-HT and 5-hydroxy-indoleacetic acid (Shankaran and Gudelsky, 1998; Wallace et al., 2001). A neurotoxic damage to presynaptic serotonergic nerve endings also occurs (Battaglia et al., 1987, 1988).

While the effects of repeated administration of high doses of MDMA on 5-HT nerve fibers and terminals have been studied extensively, little is known about its effects on dopaminergic and serotonergic receptor density. Psychosis, and especially paranoid psychosis, has been the most frequent disorder associated with MDMA use in humans (McGuire et al., 1994). Since DA and 5-HT changes have been associated with psychiatric diseases, it would be interesting to determine the possible relationship between modifications of D₂ and 5-HT₂ receptor density and the degree of neurotoxicity in a regimen schedule of MDMA in rats that simulates chronic ingest.

On the other hand, caffeine is a very popular psychostimulant among young adults. This drug acts as a nonselective A₁ and A₂ adenosine receptor antagonist and as a phosphodiesterase inhibitor. It increases DA release from striatal nerve terminals (Okada et al., 1997). Le Donne and Sonsalla (1994) pointed out that activation of adenosine A₁ receptors can protect against methamphetamine (METH)-induced neurotoxicity in mice. Furthermore, METH-induced decrements in neostriatal DA content and tyrosine hydroxylase activity in mice were potentiated by concurrent treatment with caffeine.

At the antinociceptive level, O'Regan and Clow (2004), in a study performed in humans, suggested that MDMA, at least in the short term, may cause serotonin-mediated alterations in pain sensitivity. Moreover, the authors also demonstrated an association between pain tolerance and MDMA usage. Crisp et al. (1989) studying the antinociceptive effect of MDMA in rats, suggested that the antinociceptive properties of MDMA may contribute to the popularity of this compound as a recreational drug. In addition, caffeine is used as an adjuvant analgesic for various types of pain such as headache, dental and postoperative pain, etc.

The primary aim of the present paper was to investigate the relationship between MDMA-induced neurotoxicity and the potential D₂ and 5-HT₂ receptor density regulation and, secondly, we sought to evaluate the effects of this relationship in MDMA plus caffeine-treated animals in order to better understand the possible consequences of a chronic MDMA consumption pattern in tandem with caffeine. An additional goal of the present study was to determine and compare the analgesic efficacy of MDMA and caffeine in mice, using different animal models of acute (thermal or chemical stimuli) or chronic (formalin) pain. The possible interaction (potentiation) between these compounds at the antinociceptive level, and their interaction with the dopaminergic or serotonergic system were also studied.

2. Results

2.1. Antinociceptive effect

2.1.1. Effects of MDMA and caffeine in the writhing test
MDMA elicited a dose-dependent analgesic response as demonstrated by a significant inhibition of abdominal contractions in mice receiving acetic acid with respect to control-treated (saline+acetic acid) animals. Caffeine showed a slight antinociceptive effect (about 20%) only at the highest dose assayed (10 mg/kg) (Fig. 1).

When associated, caffeine (10 mg/kg) plus MDMA (1 mg/kg), had a cumulative antinociceptive effect. The number of abdominal stretching movements was significantly reduced compared with animals treated with MDMA or caffeine alone (see Fig. 1). At the lowest dose (1 mg/kg), caffeine did not modify the MDMA effect (number of abdominal stretching movements: 36.75 ± 1.32 , $n=6$ CAF+MDMA vs. 36.25 ± 1.03 , $n=8$ MDMA).

When given alone at a dose up to 2 mg/kg, methysergide, a nonselective 5-HT receptor antagonist, had no effect on abdominal stretching movements and failed to modify the effect of MDMA (1 mg/kg) or caffeine (10 mg/kg).

When given at doses of 0.1 and 0.5 mg/kg haloperidol, a nonselective DA receptor antagonist, reduced stretching abdominal movements: 44.55 ± 5.73 , $n=6$ ($P < 0.05$, vs. saline) and 30.05 ± 3.96 , $n=7$ ($P < 0.01$), respectively. However, at this dose range locomotor impairment was evident. A subsequent dose of 0.05 mg/kg haloperidol, which did not interfere with measurements, was chosen. At this dose, the compound modified neither the effect of MDMA (1 mg/kg) nor of caffeine (10 mg/kg) (Fig. 1).

2.1.2. Effects of MDMA and caffeine in the hot-plate test

In this test, MDMA and caffeine induced a significant increase in the pain threshold. Table 1 shows the latency time, measured before and 20 min after drug administration.

The association of caffeine (10 mg/kg but not 1 mg/kg) plus MDMA (1 mg/kg) elicited an antinociceptive response similar to that obtained with MDMA or caffeine alone. Under our conditions, methysergide (2 mg/kg) did not modify the basal pain threshold. Furthermore, methysergide pretreatment significantly antagonized the analgesic response induced by MDMA (5 mg/kg) but not by caffeine (5 mg/kg). Haloperidol at a

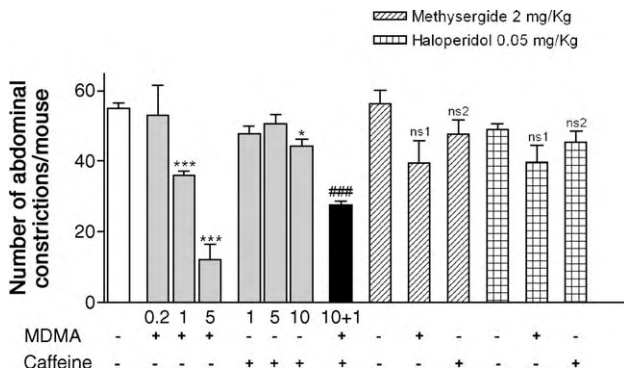


Fig. 1 – Antinociceptive effect (mouse writhing test) of MDMA (0.2–5 mg/kg, s.c.) and caffeine (1–10 mg/kg, s.c.), given alone or in combination: caffeine (10 mg/kg) plus MDMA (1 mg/kg) and the effect of methysergide or haloperidol pretreatment. *P<0.05 and *P<0.001 vs. saline; ###P<0.001 vs. MDMA or caffeine given alone; ns1, nonsignificant vs. MDMA 1 mg/kg; ns2, nonsignificant vs. caffeine 10 mg/kg. Each value represents the mean ± SEM (vertical lines) (n=6–8).**

dose of 0.05 mg/kg did not modify significantly the basal pain threshold. This compound failed to antagonize the analgesic response elicited either by MDMA (5 mg/kg) or by caffeine (10 mg/kg) (Table 1).

2.1.3. Effects of MDMA and caffeine in the formalin test

As expected, the subcutaneous plantar administration of 0.5% formalin solution into the hind paw induced an acute, immediate pain response (i.e., licking and flinching of the injected paw), which lasted about 5 min (first phase). Subsequently, this response disappeared for about 5–10 min

and then recurred, lasting about 25 min (second phase). In our experiments, the peak period of the second phase occurred from 20 to 25 min.

When MDMA was administered subcutaneously 15 min prior to the formalin injection, it dose-dependently inhibited the formalin-induced biphasic response (see Table 2). Interestingly, the effect of this compound was present in both phases. Caffeine, only at the highest dose tested (10 mg/kg) was able to slightly inhibit the first phase, whereas the second phase was unaffected. When associated, caffeine (10 mg/kg) did not affect the antinociceptive response elicited by MDMA (1 mg/kg) alone.

Methysergide, at the lowest dose (0.5 mg/kg) not affecting either animal behavior or formalin response, significantly antagonized the analgesic response induced by MDMA (1 mg/kg) in both phases. Conversely, this compound did not antagonize the analgesic response induced by caffeine (10 mg/kg). Moreover, haloperidol (0.05 mg/kg) did not antagonize the effect of MDMA (1 mg/kg) or caffeine (10 mg/kg) in any of both phases (Table 2).

2.2. Effects of MDMA and caffeine in spontaneous locomotor activity

Subcutaneous administration of MDMA elicited a dose-dependent locomotor stimulant response. A dose of 5 mg/kg was chosen for further studies. Caffeine also elicited a dose-dependent stimulant effect (Table 3). At the same dose, caffeine stimulated locomotor activity more than MDMA. When caffeine (10 mg/kg, but not 1 mg/kg) was administered prior to MDMA (5 mg/kg), a significant potentiation in

Table 1 – Antinociceptive effect of MDMA and caffeine in the mouse hot-plate test and the effect of methysergide or haloperidol pretreatment—results are expressed as mean ± SEM corresponding to prior drug administration (before) and 20 min after the subcutaneous injection of the drug (after)

Treatment (s.c.)	Dose (mg/kg)	Licking/jumping latency (in seconds)	
		Before	After
Saline	0	4.60 ± 0.28	5.76 ± 0.23
MDMA	1	4.64 ± 0.78	10.34 ± 1.91*
MDMA	5	4.69 ± 1.33	18.14 ± 2.82***
Caffeine	5	4.28 ± 0.80	8.74 ± 0.97*
Caffeine	10	4.96 ± 0.58	11.25 ± 0.92***
Caffeine + MDMA	10 + 1	5.61 ± 0.74	10.55 ± 2.07 ^{ns1}
Methysergide	2	5.57 ± 0.88	4.48 ± 1.08
Methysergide + MDMA	2 + 5	5.00 ± 1.05	11.31 ± 1.48 [#]
Methysergide + Caffeine	2 + 5	5.62 ± 1.15	9.29 ± 0.89 ^{ns2}
Haloperidol	0.05	4.59 ± 0.31	9.08 ± 2.08
Haloperidol + MDMA	0.05 + 5	4.77 ± 0.41	17.15 ± 7.48
Haloperidol + Caffeine	0.05 + 5	4.85 ± 0.47	12.21 ± 2.67

*P<0.05; ***P<0.001 in comparison with saline treated mice; #P<0.05 vs. MDMA 5 mg/kg; ns1, nonsignificant vs. MDMA 1 mg/kg; ns2, nonsignificant vs. caffeine 5 mg/kg. The number of animals in each group ranged from 6 to 8 with the exception of saline which numbered 12.

Table 2 – Dose-dependent antinociceptive effects of MDMA and caffeine in the formalin test in mice and the effect of methysergide or haloperidol pretreatment

Treatment (s.c.)	Dose (mg/kg)	Time licking or biting (in seconds)	
		First phase	Second phase
Saline/vehicle	0	93.48 ± 7.68	56.06 ± 8.75
MDMA	0.2	71.75 ± 15.71	30.48 ± 11.51
MDMA	1	51.29 ± 6.28**	22.70 ± 4.7**
MDMA	5	4.45 ± 2.08***	0.78 ± 0.78***
Caffeine	5	73.04 ± 7.51	61.92 ± 7.40
Caffeine	10	62.41 ± 6.15*	39.43 ± 9.96
Caffeine + MDMA	10 + 1	48.03 ± 4.19 ^{***ns}	23.4 ± 7.02 ^{***ns}
Methysergide	0.5	86.58 ± 5.30	58.04 ± 12.06
Methysergide + MDMA	0.5 + 1	77.09 ± 5.33 ^{##}	54.82 ± 6.04 ^{##}
Methysergide + Caffeine	0.5 + 10	64.99 ± 8.78	53.95 ± 8.47
Haloperidol	0.05	82.44 ± 5.26	60.14 ± 7.91
Haloperidol + MDMA	0.05 + 1	61.28 ± 6.67	28.05 ± 4.69
Haloperidol + Caffeine	0.05 + 10	70.08 ± 4.32	42.22 ± 5.09

Results are expressed as mean ± SEM and represent the time (in seconds) spent in paw licking or biting response during the first (0–5 min) and the second phase (20–25 min).

*P<0.05; **P<0.01; ***P<0.001 in comparison with the first and second phases of saline treated mice; ##P<0.01 vs. MDMA 1 mg/kg; ns, nonsignificant vs. MDMA 1 mg/kg. The number of animals in each group ranged from 6 to 8 with the exception of saline which numbered 22.

locomotor activity was observed, which was due to the increase in maximal values and to a prolonged time of activity (Table 3 and Fig. 2).

Similarly, to explore the association of MDMA with another potent stimulant of locomotor activity that increases DA release (in nucleus accumbens), methamphetamine (METH) was used. METH induced a dose-dependent stimulation of locomotor activity, which contrary to MDMA, was prevented by pretreatment with haloperidol (area under the curve, saline: 72887 ± 6909 ; METH 1 mg/kg: $258815 \pm 37016^{**}$ and Haloperidol 0.1 mg/kg+METH 1 mg/kg: $145155 \pm 24021^{***\#}$; $^{**}P < 0.01$, $^{***}P < 0.001$ vs. saline $\#P < 0.05$ vs. METH).

When MDMA (5 mg/kg) was associated with METH (0.2 mg/kg), a very similar potentiation to caffeine+MDMA was observed (saline: 72887 ± 6909 ; MDMA: $99363 \pm 6496^*$, METH: $125085 \pm 8770^*$; METH+MDMA: $465191 \pm 94456^{**}$ # \$\$; $^*P < 0.05$ and $^{**}P < 0.01$ vs. saline, $\#P < 0.05$ vs. METH; $^{$$$}P < 0.01$ vs. MDMA, $n = 8$ in each group).

2.3. Neurotoxicity assessment

2.3.1. Lethality

Co-treatment with caffeine and MDMA increased the incidence of death from 22.22% to 34.09%. Analysis using Fischer's exact test revealed that caffeine significantly increased lethality in MDMA-treated rats ($P = 0.041$).

2.3.2. Hyperthermia

When the ambient temperature was raised to 27 ± 1 °C, MDMA exerted a hyperthermic effect. Body weight, the degree of hyperthermia and its maintenance were closely related. At this ambient temperature, an initial body weight < 250 g was associated with a better survival index for MDMA-treated groups. Thus, only rats weighing 180–220 g at the beginning of the treatment were used and housed individually. Measuring body temperature 1 h after the first dose of MDMA, the hyperthermic response was significantly increased by co-treatment with caffeine (37.2 ± 0.1 °C saline, 37.5 ± 0.3 °C caffeine, 38.4 ± 0.2 °C ** MDMA, 39.6 ± 0.3 °C $^{***\#}$ CAF+MDMA, $n = 7-14$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. saline; $\#P < 0.01$ vs. MDMA).

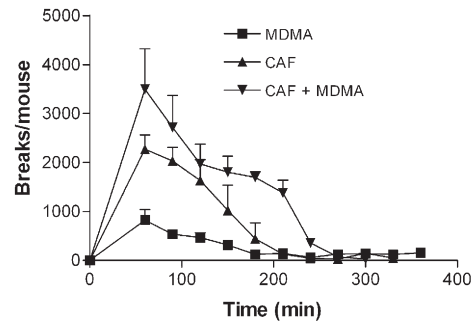


Fig. 2 – Time course profile of the potentiation of MDMA-induced increase of mouse spontaneous locomotor activity by caffeine. MDMA (5 mg/kg, s.c.), caffeine (CAF, 10 mg/kg, s.c.) and its association. Vertical axis shows the number of interruption counts/animal, in a 10 min-block and monitored over 360 min. The first 30 min of registered counts was discarded. All data points reflect the mean \pm SEM of 9 mice.

2.3.3. Body weight

Animals were weighed the first day of treatment and 24 h after the last dose. Saline-treated animals increased their body weight by 29.2 ± 4.2 g ($n = 10$) during this time interval. The group of MDMA-treated animals experimented a weight loss of 13.1 ± 2.2 g ($n = 12$, $P < 0.001$ vs. saline group) that was potentiated in animals also receiving caffeine (loss of body weight of 16.2 ± 2.9 g, $n = 14$, $P < 0.001$ vs. MDMA group). Caffeine alone also reduced the increase in body weight experienced by the saline group (8.6 ± 2.2 g, $n = 11$, $P < 0.001$ vs. saline group).

2.3.4. Quantification of dopamine and serotonin uptake sites [3 H]Paroxetine binding was measured in the cortex, hippocampus and striatum in rats. MDMA-treated animals sacrificed 3 days post-treatment showed a significant decrease in [3 H]paroxetine binding that was very similar in both the cortex and hippocampus (58% and 57%, respectively). This persisted in MDMA-treated animals sacrificed 7 days post-treatment (52% cortex and 48% hippocampus) (Figs. 3A and 3B). 5-HT

Table 3 – Dose-dependent effects of MDMA and caffeine treatment in spontaneous locomotor activity in mice

Drug (dose, mg/kg)	Total AUC	Maximum breaks/mouse	Hyperlocomotion present (min)
Saline	72887 ± 6909	829 ± 113	–
MDMA (1)	$60,950 \pm 14,972$	697 ± 187	–
MDMA (5)	$99,363 \pm 6496^*$	840 ± 213	120
MDMA (10)	$202,195 \pm 46,387^{**}$	$1683 \pm 444^*$	150
Caffeine (1)	$121,745 \pm 20,124$	$1605 \pm 105^*$	60
Caffeine (5)	$189,740 \pm 4583^*$	$2017 \pm 229^{**}$	120
Caffeine (10)	$267,780 \pm 55,371^{**}$	$2269 \pm 292^{***}$	150
Caffeine (10)+MDMA (5)	$457,505 \pm 42,121^{***\#}$	$3493 \pm 478^{***\#}$	240
Haloperidol (0.1)+MDMA (5)	$83,687 \pm 7150^{ns}$	737 ± 301	120

Results are expressed as mean \pm SEM ($n = 9$) and represent the measurement of the area under the curve over the period 30 to 360 min (total AUC), the maximum number of breaks/animal and the time that a significant hyperlocomotor effect was still present (in minutes). The number of animals in each group was 9 with the exception of saline which numbered 14. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. saline; $\#P < 0.05$ vs. caffeine 10 mg/kg; ns, nonsignificant vs. MDMA 5 mg/kg.

terminal injury, which was measured as the loss in [3 H]paroxetine binding in the striatum, was progressive, increasing nonsignificantly from days 3 to 7 days after treatment (36% to 49%) (Fig. 3C). Caffeine did not affect 5-HT uptake site density but did increase 5-HT terminal loss induced by MDMA when measured 7 days after treatment (76% cortex, 71% hippocampus; 73% striatum, $P < 0.05$ vs. MDMA respectively) (Fig. 3).

On the other hand, MDMA decreased the abundance of DA uptake sites (measured as specific [3 H]WIN 35428 binding) by 31% ($100 \pm 4.5\%$ saline group vs. $68.2 \pm 16.3\%$ MDMA group, $P < 0.05$) 3 days after treatment. Co-treatment with caffeine and MDMA did not potentiate the injury to DA terminals induced by MDMA ($63.5 \pm 16.6\%$ CAF+MDMA group, n.s. vs. MDMA group). We observed no decrease in [3 H]WIN 35428 binding sites 7 days post-treatment in any group.

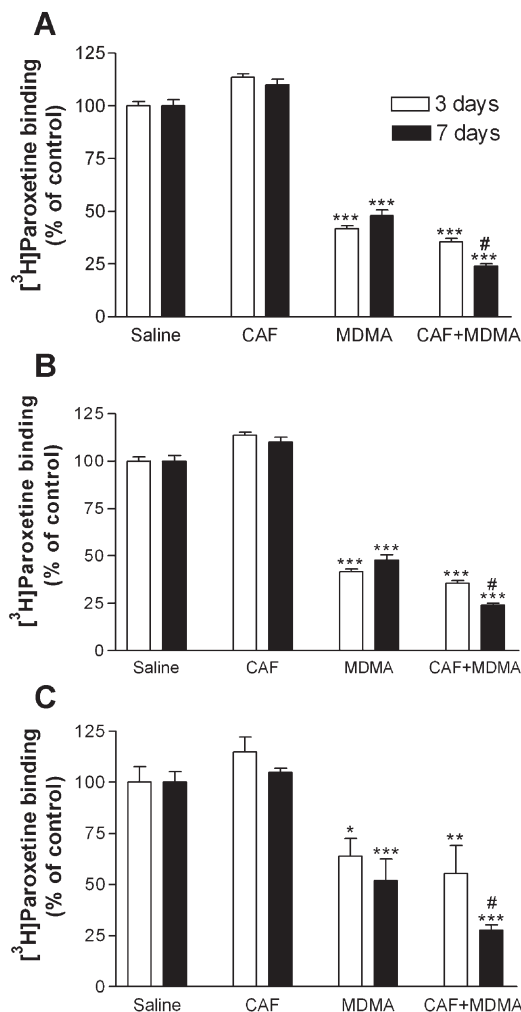


Fig. 3 – Effect of treatment with MDMA or caffeine given alone (see Experimental procedures for dose schedule), or in combination, on the density of serotonin uptake sites in rat cortex (A), hippocampus (B) and striatum (C). Animals were sacrificed at 3 (open bars) or 7 (solid bars) days after treatment. Values are expressed as means \pm SEM of those obtained from at least five animals in each group. * $P < 0.05$; *** $P < 0.001$ vs. saline and # $P < 0.05$ vs. MDMA at 7 days.

Table 4 – Effect of MDMA, caffeine (CAF) and its association (see Experimental procedures for dose schedule), in the affinity of [3 H]spiperone for dopaminergic D_2 receptors in striatal membranes (specific saturation binding experiments, saline B_{max} 198 ± 14 fmol/mg) and of [3 H]ketanserin for the serotonergic 5-HT $_2$ receptors in cortical membranes (specific saturation binding experiments, saline B_{max} 663 ± 22 fmol/mg). Animals were sacrificed at 7 days post-treatment

Treatment	[3 H]spiperone binding		[3 H]ketanserin binding	
	K_D (pM)	n	K_D (nM)	n
Saline	117 ± 30	5	0.92 ± 0.07	5
MDMA	122 ± 45	3	1.02 ± 0.12	3
CAF	127 ± 34	4	1.15 ± 0.10	4
CAF+MDMA	130 ± 47	3	1.10 ± 0.12	3

Results are expressed as mean \pm SEM.

2.3.5. Quantification of dopamine (D_2) and serotonin (5-HT $_2$) receptors

The density of dopaminergic D_2 receptors was determined in striatal membranes by measuring the specific binding of [3 H]spiperone. When saturation experiments were carried out, we could demonstrate that neither MDMA nor caffeine treatment modified the K_D value of [3 H]spiperone (Table 4). Binding to a single concentration (150 pM) of [3 H]spiperone was then determined. [3 H]spiperone binding to D_2 receptors was significantly modified in MDMA-treated animals. Three days after treatment, the percentage of binding had increased while 7 days post-treatment it tended to return to basal levels. Co-treatment with caffeine prevented this initial up-regulation of D_2 receptor binding induced by MDMA (3 days after treatment). Treatment with caffeine alone had no effect on [3 H]spiperone binding (see Fig. 4A).

The density of serotonergic 5-HT $_2$ receptors was determined in cortical membranes by measuring the specific binding of [3 H]ketanserin. As above, saturation experiments were carried out, and similarly, no modifications in [3 H]ketanserin affinity (K_D values) were produced by any treatment (see Table 4). Binding experiments with a single concentration (1 nM) of [3 H]ketanserin were then performed.

In the MDMA-treated group, [3 H]ketanserin binding was initially reduced by about 40% (3 days after treatment), tending to recover later (7 days after treatment). This tendency was very similar in caffeine-plus MDMA-treated animals, although caffeine seemed to impair recovery to basal values (saline). Caffeine alone had no effect on [3 H]ketanserin binding (see Fig. 4B).

3. Discussion

MDMA, at least in the short-term and in humans, may cause 5-HT-mediated alterations in pain sensitivity (O'Regan and Clow, 2004). The antinociceptive properties of MDMA may contribute to the popularity of this compound as a recreational drug (Crisp et al., 1989).

Pain sensations are relayed from the periphery to the brain and, at the level of the spinal cord, ascending pain signals are

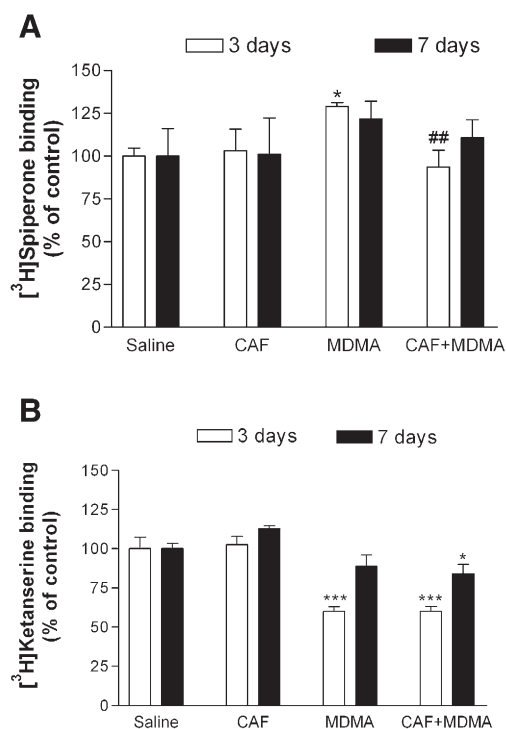


Fig. 4 – (A) Effect of treatment with MDMA or caffeine given alone (see Experimental procedures for dose schedule), or in combination on the density of dopamine D₂ receptors in rat striatal membranes. Animals were sacrificed at 3 (open bars) or 7 (solid bars) days after treatment. Values are expressed as means ± SEM of those obtained from at least five animals in each group. **P*<0.05 vs. saline and ##*P*<0.01 vs. MDMA at 3 days. (B) Effect of treatment with MDMA or caffeine given alone (see Experimental procedures for dose schedule), or in combination on the density of serotonergic 5-HT₂ receptors in rat cortical membranes. Animals were sacrificed at 3 (open bars) or 7 (solid bars) days after treatment. Values are expressed as means ± SEM of those obtained from at least five animals in each group. **P*<0.05 and **P*<0.001 vs. saline.**

modulated by afferent signals from touch receptors. The dorsal horn of the spinal cord is innervated by serotonergic neurons, which are involved in the modulation of nociceptive transmissions (Wang and Nakai, 1994). Under normal conditions, 5-HT is implicated in numerous processes from mood to sleep, and the raphe magnus nucleus has been postulated to constitute the descending pathway for pain mediation.

Caffeine is used as an adjuvant analgesic for various types of pain. This drug acts as a nonselective antagonist of adenosine receptors. It has intrinsic antinociceptive properties in threshold tests. It is unclear how caffeine produces its analgesic effect, but it is likely to be via adenosine receptor antagonism (Fredholm et al., 1999). As a consequence of this antagonism, Ghelardini et al. (1997) suggested that this drug increased the extracellular levels of acetylcholine in the hippocampus of freely moving rats, thus inducing antinociception. Recently, Godfrey et al. (2006) described an analgesic effect of caffeine at low doses and concluded this resulted from its antagonistic A_{2A} properties.

In the present paper, we have used three experimental models to determine analgesic properties: the writhing test (a nonspecific acute pain test), the hot-plate test (a thermal threshold test) and the formalin test (a tonic pain test with an inflammatory component). The hot-plate test subsequently measures responses to thermal stimulations, whereas the writhing and formalin tests evaluate responses to chemical stimulations. The pain pathways that these tests focus on vary as well. The writhing test mainly focuses on changes at or below the spinal cord level, whereas the formalin and hot-plate tests emphasize changes above the spinal cord level. The temperature of the thermal stimulus in the hot-plate was 54 °C, a factor that might explain the differences in the observed effects. The higher temperature ensures a supraspinal component to this antinociceptive test (Godfrey et al., 2006).

The analgesic effects of MDMA are already present at 1 mg/kg in the three assayed tests. In the present study, the analgesic effectiveness of MDMA was better in the formalin test than in writhing and hot-plate tests. This suggests that the analgesic effects of MDMA are exerted both at the spinal and supraspinal levels, mainly in front of chemical stimulations. This is supported by the fact that, in the formalin test, the second phase, which represents more central pain processing mechanisms, showed a slightly higher sensitivity to MDMA.

We have demonstrated that DA receptors are not involved in the analgesic effects of both MDMA and caffeine. The fact that methysergide (a nonselective antagonist of 5-HT₁, 5-HT₂ and 5-HT₇ receptors) prevented the effect of MDMA in the more specific analgesic tests, but not that of caffeine, indicates a central serotonergic mechanism for the antinociceptive action of MDMA.

Caffeine showed a slight antinociceptive effect. It has been demonstrated that this drug has got adjuvant analgesic properties when combined with nonsteroidal anti-inflammatory drugs (Laska et al., 1984). However, when associated, MDMA and caffeine did not show any synergistic interaction.

MDMA exerted an acute hyperlocomotive effect. Several studies using agonists and antagonists found that this hyperactivity, which is initiated by the release of endogenous 5-HT, results from the activation of 5-HT_{1B} receptors (Rempel et al., 1993; McCreary et al., 1999). However, the enhanced extracellular 5-HT induced by MDMA can, by acting on 5-HT₂ receptors, produce an increase in DA release, which also may contribute to the hyperlocomotion effect of MDMA (Yamamoto et al., 1995). In fact, 5-HT₂ receptor blockade with MDL 100,907 has been reported to attenuate MDMA-induced increases in extracellular concentrations of striatal DA (Schmidt et al., 1992), and also to significantly reduce MDMA-stimulated locomotion without affecting basal locomotion levels (Kehne et al., 1996).

In our experiments, caffeine had a stimulant effect on locomotor activity, which was present albeit at low doses. The stimulant effect of caffeine on locomotor activity is related to central dopaminergic neurotransmission, and can be attributed to its blockade of adenosine A₂ receptors in central dopaminergic presynaptic sites (Kim and Palmiter, 2003). From studies with haloperidol and GBR 12909 (a well-known DAT inhibitor) (Powell et al., 2001), it is evident that an increase in dopaminergic activity induced by caffeine is

mainly involved in its locomotor stimulant effect (Ferre et al., 1992).

When associated, caffeine and MDMA potentiated locomotor activity. This effect does not seem to be an effect on kinetics, since the hyperlocomotion response is present during the same time-interval. In this context, it could be hypothesized that the blockade of adenosine A₂ receptors by caffeine potentiates the DA release induced by MDMA. The administration of caffeine plus MDMA would subsequently induce an increase in dopaminergic activity. An increase in dopamine release in the mesolimbic system may precipitate purposive movement, increasing motivation and alertness, reducing appetite, and inducing insomnia, acute effects that are often seen when ingesting ecstasy pills.

The hyperthermic response to MDMA is always present at high ambient temperature and is a consequence of the 5-HT and DA release induced by MDMA (Colado et al., 2004; Saadat et al., 2005). The neurotoxic damage caused by substituted amphetamines requires a combination of acute hyperthermia and increased neurotransmitter mobilization. Induction of only one of these effects is not sufficient to cause serotonergic nerve terminal degradation (O'Loinsigh et al., 2001). Accordingly, in the present paper, the treatment of animals for neurotoxic studies was carried out at a high ambient temperature, and a hyperthermic effect of MDMA was observed. Although caffeine alone failed to alter body temperature, it significantly increased the hyperthermic response to MDMA. Hyperthermia and cardiovascular effects induced by MDMA are signs of an acute toxicity that leads to lethality. In our experiments, the MDMA-treated group showed a lethality of 22%, which significantly increased by pretreatment with caffeine. Most likely, the more pronounced hyperthermia observed in co-treated animals played a role in this higher toxicity.

Centrally acting appetite suppressant drugs stimulate brain catecholamine and 5-HT pathways in the hypothalamus. MDMA as fenfluramine, another amphetamine derivative used as an anorectic in clinical trials, mediates its effects through serotonergic mechanisms in the lateral hypothalamus (Conductier et al., 2005). The maximal anorectic effect of MDMA was especially apparent 24 h after completing treatment. Animals suffered a body weight loss of about 6–7%, which rose to 8% when rats were also treated with caffeine. It must be pointed that caffeine alone also had an effect on body weight: while saline-treated animals increased in body weight about 15%, the caffeine-treated group increased in body weight only about 4%. Most likely, the activity of caffeine on locomotion explains this difference.

The fact that 5-HT concentration can be influenced by tryptophan hydroxylase activity, which is modified by MDMA administration, suggests that [³H]paroxetine (a ligand of 5-HT transport sites in neurons) binding might be a more accurate indication of 5-HT nerve ending loss (Green et al., 2003) following chronic MDMA treatment. There are many papers reporting that [³H]paroxetine binding is reduced 7 days after neurotoxic regimen of MDMA (Battaglia et al., 1987; Pubill et al., 2003). In the present study, administration of a neurotoxic regimen of MDMA to rats resulted in a persistent decrease in the number of SERT sites in the cortex, striatum and hippocampus. In the CAF+MDMA

group, the final injury in 5-HT terminals was greater than in MDMA-treated animals for all areas assayed. Thus, we could then establish that the classical neurotoxic lesion induced by MDMA is potentiated by caffeine co-treatment. This is in agreement with the results obtained by McNamara et al. (2006), who used a different dosage regimen, room temperature and time of sacrifice, and measured the injury through 5-HT and 5-HIAA levels.

From our results, it can be speculated that the increase in DA release induced by CAF+MDMA treatment enhances the availability of DA for transport into the depleted 5-HT terminals (Sprague and Nichols, 1995). Once concentrated within the 5-HT terminal, DA can be deaminated by MAO-B, yielding oxidative products that selectively destroy the 5-HT terminal (Sprague et al., 1998).

We have previously reported that adenosine can play a neuroprotective role in METH-induced injury (Escubedo et al., 1998). Accordingly, in this paper, we demonstrate that a blocker of adenosine receptors potentiates the neurotoxicity of another amphetamine derivative as MDMA. Although no direct relationship has been established between the magnitude of hyperthermia and the extension of serotonergic injury, the hypothesis that increased neurotoxicity of MDMA in the presence of caffeine is due to the enhanced temperature response cannot be ruled out.

In contrast to the rapid DA release induced by MDMA, no persistent MDMA-induced loss in dopaminergic terminals was observed in rats. In a previous paper from our group, we reported that MDMA-induced neurotoxicity in rats was accompanied by a dopaminergic lesion in the striatum, quantitatively similar to that induced by METH although unlike METH, it reversed itself 7 days post-treatment. This is in agreement with our present results since [³H]WIN35428 binding sites decreased by 31% in MDMA group, 3 days post-treatment, but returned to control levels 7 days after treatment. Caffeine administration together with MDMA did not modify any of these parameters.

As far as cortical 5-HT₂ receptors are concerned, a transient down-regulation of 5-HT₂ receptors occurs in the cortex of MDMA-treated rats. Although the same tendency in 5-HT₂ receptors population could be noted, when animals were sacrificed 7 days after treatment, no statistical significance was observed. This initial down-regulation of 5-HT₂ receptors, similarly described by other authors (Scheffel et al., 1992; Reneman et al., 2002), could represent an acute consequence to the initial increase in released 5-HT (Sveen et al., 2004). Co-treatment with caffeine slowed the recovery of MDMA-induced down-regulation.

An up-regulation of D₂ receptors was found in MDMA-treated animals 3 days after treatment. This could represent a response to the transient DA terminal injury, since as dopaminergic impairment reverses, D₂ receptor density returns to control levels. Surprisingly, no increase in D₂ receptors was detected in the CAF+MDMA group. A possible explanation for this difference could be that caffeine induces supplemental DA release from functional terminals, thereby maintaining the dopaminergic input without need of increasing receptor density.

In conclusion, the association of MDMA with caffeine (voluntarily in the form of “energy drinks” simultaneously

with ecstasy, or unwittingly from adulterated ecstasy pills) does not generate any beneficial effects at the antinociceptive level. However, the stimulant effect observed in animals is potentiated when both drugs are administered together, contributing to the popularity of these stimulants as recreational drugs. It should be noted, however, that lethality, loss of body weight and hyperthermia are also potentiated. The acute effects stemming from this association, in tandem with the final potentiation of serotonergic terminals injury, provide evidence of the potentially greater long-term adverse effects of this particular recreational drug combination.

4. Experimental procedures

4.1. Animals

Experimental protocols for the use of animals in this study were approved by the Animal Ethics Committee of the University of Barcelona under the supervision of the Autonomous Government of Catalonia, following the guidelines of the European Communities Council (86/609/EEC). Efforts were made to minimize suffering and reduce the number of animals used. Except for treatments involving neurotoxicity assessment, animals were housed at 22 ± 1 °C under a 12-h light/dark cycle with free access to food (standard laboratory diet, PANLAB SL, Barcelona, Spain) and drinking water. Adult male Swiss CD-1 mice (Charles River, Barcelona, Spain) weighing 22 to 30 g were used in the antinociceptive and locomotor activity experiments. To assess neurotoxicity, adult male Sprague–Dawley rats weighing 180–220 g (Harlan Ibérica, Barcelona, Spain) were used.

4.2. In vivo mice experiments

The animals were divided into four treatment groups: saline (vehicle+vehicle), CAF (caffeine+vehicle), MDMA (vehicle+MDMA) and CAF+MDMA (caffeine+MDMA). Caffeine was administered i.p. or s.c. 20 min before vehicle or MDMA.

Caffeine was suspended in a mixture of sodium carboxymethylcellulose (0.5%)+Tween 80 (0.1%) in distilled water. Haloperidol (given subcutaneously) was dissolved in ethanol at a concentration of 5 mg/ml and subsequent dilutions were made in saline. Formalin was prepared by diluting a formaldehyde solution (37%) with saline to obtain the concentration of 0.5%. All other drugs were dissolved in saline (NaCl 0.9%) and injected in a volume of 4 ml/kg.

4.3. Antinociceptive effect

The animals were randomly assigned to one of four treatment groups. The number of animals in each group ranged from 6 to 8 with the exception of the saline group, which included more animals.

4.3.1. Writhing test

This test was performed according to the method of [Koster et al. \(1959\)](#). Briefly, each mouse was isolated and placed in an

individual acrylic observation cage (25×20×25 h cm). Methysergide or haloperidol pretreatment was performed 10 min before MDMA or caffeine administration. MDMA or caffeine (s.c.) was administered 15 min prior to the acetic acid injection. At this time, mice were injected with 10 ml/kg of 0.6% (v/v) acetic acid (i.p.). The cumulative number of writhing responses was recorded for 10 min, starting 5 min after acetic acid injection. For scoring purposes, we defined a writhing response as a contraction of the abdominal muscles followed by a stretching of at least one hind limb.

4.3.2. Hot-plate test

Supraspinally, mediated nociception was tested according to the method of [O'Callaghan and Holtzman \(1976\)](#) using the hot-plate analgesymeter LE 7406 (PANLAB, SL, Barcelona, Spain). Mice were placed in the apparatus, which was thermostatically maintained at 54 ± 0.5 °C in a precision water bath. Hot-plate latency was defined as the interval time (in seconds) between placement of the mouse onto the hot-plate and the instant a nociceptive response was elicited (e.g., licking a forepaw, or hopping off the plate). Each mouse was subjected to two trials. A time latency value was recorded immediately prior to drug administration (pre-test value) and 20 min after (post-test value) treatment. Mice were preselected, and animals with a pre-test value of more than 10 s were excluded from the study (8%). Drugs or the appropriate vehicle were administered subcutaneously. A cut-off time of 25 s was established. Animals not responding at this time were removed from the hot-plate and given a score of 25 s.

4.3.3. The formalin test

This was performed according to the modified method suggested by [Shibata et al. \(1989\)](#). Briefly, a low concentration of formalin (0.5%) was used. Since licking the forepaw as an indication of pain response may be confused with the normal grooming response, we observed the hind paw of mice. Subplantar injection of formalin results in flinching, licking or a biting behavior during the early acute phase, which resembles acute pain, followed by a quiescent interphase, and then a second delayed phase representing chronic pain. The acute phase is believed to correspond to the peripheral pain pathways, whereas the second phase represents more central pain processing mechanisms. In our study, both phases of the formalin test were well delineated and quantifiable.

The test was performed in an isolated room with an ambient temperature of 23–24 °C to assess a robust second phase. The observation chamber was a rectangular acrylic transparent cage (30×20×20 h cm). Beneath the floor, a mirror at a 45° angle was mounted to allow clear observation of the paws of the animal. Each mouse was placed in the observation chamber 15 min (at the time of drug administration) before the formalin injection to acclimatize the animal to the new environment. This is necessary to obtain biphasic and reproducible responses. Mice were gently restrained and 25 µl of 0.5% sterile formalin was injected subcutaneously into the right hind paw, using a micro syringe with a 29-gauge needle. Each animal was returned to the chamber and the pain response was

recorded continuously for a period of 35 min using the CompuLet 5 system (PANLAB SL, Barcelona, Spain) and associated software. The total time (in seconds) spent in licking and/or biting responses in the administered paw during each 5 min block was recorded as an indicator of pain response. The recording of the early phase (first phase) started immediately and lasted for 5 min. The late phase (second phase) started about 15–20 min after the formalin injection.

4.4. Spontaneous locomotor activity

Prior to experimentation, all mice ($n=9$ /group) received two habituation sessions (48 and 24 h before testing) that were intended to reduce the novelty and stress associated with handling and injection. During these sessions, each mouse was given a subcutaneous injection of saline and placed in a Plexiglas cage. This cage constituted the activity box that was later placed inside a frame system of two sets of 16 infrared photocells (LE8811, PANLAB, SL, Barcelona, Spain) mounted according to the x, y axis coordinates and 1.5 cm above the wire mesh floor. Occlusions of the photo beams were recorded and sent to a computerized system (SedaCom32, PANLAB, SL, Barcelona, Spain). The interruption counts, over a 10 min-block, were used as a measure of horizontal locomotor activity. The locomotor activity of each mouse was monitored for 360 min. All experiments were conducted between 9:00 a. m. and 3:00 p.m. On the testing day, the animals received the drug treatment and were immediately placed in the activity box. Registration of horizontal locomotor activity then began, although the first 30 min of registered counts was discarded. Results are expressed as: (a) area under the curve, which was measured as the total changes from baseline at each recording interval over 30 to 360 min; (b) as maximum breaks/mouse; and (c) the maximal interval time that significant hyperlocomotion was present.

4.5. In vivo treatments for neurotoxicity assessment

The animals received a neurotoxic dosage regimen of MDMA that is thought to be equivalent to a chronic administration (Battaglia et al., 1988; Pubill et al., 2003) of 20 mg/kg b.i.d. for 4 days. This was the dose used for both MDMA and caffeine. Animals were housed one per cage and randomly assigned to one of four groups (6–8 animals/group): saline, caffeine, MDMA or caffeine+MDMA as described above. Caffeine was administered i.p. (1 ml/kg) 30 min before vehicle or MDMA, respectively. MDMA was administered s.c. (1 ml/kg) at 9 h and 16 h for 4 consecutive days.

The animals were allowed to acclimatize to an environmental temperature of 27 ± 1 °C before receiving the first dose of MDMA and were kept under these conditions until 1 h after the last dose. Thereafter, they were returned to normal housing conditions (22 ± 1 °C). Body temperature was measured using a lubricated and flexible rectal probe inserted 2.5 cm into the rectum and attached to a digital thermometer (0331 PANLAB, SL, Barcelona, Spain) 1 h after the first injection of MDMA. If rectal temperature rose above 41 °C, animals were kept on ice for 5 min. Body weight was registered 24 h after the last dose of MDMA.

4.5.1. Tissue membrane preparation

Three days or 1 week following the last treatment, animals were killed by decapitation under isoflurane anesthesia and brains were removed rapidly from the skull and the striatum, the hippocampus and the cortex were quickly excised, dissected out, frozen on dry ice and stored at -80 °C until later use. When required, samples were thawed and homogenized in 10 vol. buffer: 5 mM Tris–HCl, 320 mM sucrose and protease inhibitors (aprotinin 4.5 $\mu\text{g}/\mu\text{l}$, 0.1 mM PMSF and 1 mM sodium orthovanadate), pH 7.4, with a Polytron homogenizer. The homogenates were centrifuged at $15,000 \times g$ for 30 min at 4 °C. The resulting pellets were washed twice and the final pellets were resuspended in the appropriate buffer and stored at -80 °C for use in radioligand binding experiments. Protein content was determined using the Bio-Rad protein reagent (Bio-Rad Labs. Inc., Hercules, CA, USA), according to the manufacturer's specifications.

4.5.2. Binding experiments

Variations in the density of striatal, hippocampal and cortical SERT were quantified by measuring the specific binding of 0.05 nM [^3H]paroxetine after incubation with 150 μg protein at 25 °C for 2 h in a Tris–HCl buffer (50 mM, pH 7.4) containing 120 mM NaCl and 5 mM KCl to a final volume of 1.6 ml. Clomipramine (100 μM) was used to determine nonspecific binding.

Modifications in the density of dopamine transporters (DAT) in striatal membranes were measured by [^3H]WIN 35428 equilibrium binding assays. Membranes were resuspended in phosphate-buffered 0.32 M sucrose, pH 7.9 at 4 °C to a concentration of 1 $\mu\text{g}/\mu\text{l}$. Binding assays were performed in borosilicate glass tubes containing 200 μl of [^3H]WIN 35428 dilution in phosphate-buffered 0.32 M sucrose (final radioligand concentration: 5 nM) and 50 μl of membranes. Incubation was conducted for 2 h at 4 °C. Nonspecific binding was determined in the presence of 30 μM bupropion.

As affinity (K_D) values do not differ significantly in control and treated rats (Escubedo et al., 1998), a single concentration of the radioligand was used for [^3H]paroxetine and [^3H]WIN 35428 binding.

The density of dopamine D_2 receptors was determined in striatal membranes by specific binding of [^3H]spiperone. Saturation experiments were carried out and the specific binding of [^3H]spiperone (10–750 pM) was determined after incubation with 200 μg protein at 25 °C for 3 h in a Tris–HCl buffer (50 mM, pH 7.4) containing 120 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , ascorbic acid 0.01%, bacitracin 0.007%, 0.05 mM PMSF, 0.1 mM EDTA, 10 μM pargiline and 0.1 μM ketanserin to a final volume of 3 ml. Butaclamol (2 μM) was used to determine nonspecific binding. As no variations in K_D were assessed, further binding experiments to a single concentration (150 pM) of [^3H]spiperone were performed.

The density of serotonergic 5-HT $_2$ receptors was measured in cortical membranes by determining the specific binding of [^3H]ketanserin. Saturation experiments were also carried out. The specific binding of [^3H]ketanserin (0.125–8 nM) was measured after incubation with 100 μg protein at 37 °C for 30 min in a Tris–HCl buffer (50 mM, pH 7.4) containing protease inhibitors to a final volume of 0.5 ml. Methysergide (10 μM) was used to determine nonspecific

binding. As no variations in K_D were assessed, binding to a single concentration (1 nM) of [3 H]ketanserin was used.

All incubations were finished by rapid filtration under vacuum through GF-51 glass fiber filters (Schleicher and Schuell, Dassel, Germany). Tubes and filters were washed rapidly 3 times with 4 ml ice-cold buffer and the radioactivity in the filters was measured by liquid scintillation spectrometry. Specific binding was defined as the difference between the radioactivities measured in the absence (total binding) and presence (nonspecific binding) of an excess of nonlabeled ligand.

4.6. Drugs and reagents

Drugs and reagents were obtained from the following sources: 3,4-Methylenedioxy-methamphetamine hydrochloride was provided by the National Health Laboratory (Barcelona, Spain). Caffeine, haloperidol, methamphetamine hydrochloride and methysergide maleate were purchased from Sigma-Aldrich. Glacial acetic acid and formaldehyde were from Panreac (Barcelona, Spain). [3 H]ketanserin, [3 H]paroxetine, [3 H]spiperone and [3 H]WIN 35428 ((-)-2-beta-carbomethoxy-3-beta-(4-fluorophenyl)tropane 1,5-naphthalenedisulfonate) from Perkin-Elmer Life Sciences (Boston, MA, USA). All buffer reagents were of analytical grade.

4.7. Data analysis

Results are given as the mean \pm SEM (standard error of the mean). One-way ANOVA, followed by Tukey's test, was used to verify the significance between means. *P* values less than 0.05 were considered significant. To compare results from hot-plate experiments (both before and after), paired *t* tests were used. Data were analyzed with InStat (GraphPad) software. Lethality data were analyzed using Fisher's exact test. K_D values were calculated by using the RADLIG 4.0 program Biosoft, Cambridge, UK.

Acknowledgments

We are grateful to Drs. M. Amat, J. Bosch and N. Llor for spectral analysis of MDMA demonstrating its chemical purity; to N. Caballero and L. Campins for technical assistance and to Linguistic Advice Service of the University of Barcelona for revising the language of the manuscript.

This work was supported by grants from Plan Nacional sobre Drogas 2002, Ministerio de Educación y Ciencia (SAF2005-0573), Generalitat de Catalunya (SGRE00793) and FIS (PI050486).

REFERENCES

- Battaglia, G., Yeh, S.Y., O'Hearn, E., Molliver, M.E., Kuhar, M.J., De Souza, E.B., 1987. 3,4-Methylenedioxyamphetamine and 3,4-methylenedioxyamphetamine destroy serotonin terminals in rat brain: quantification of neurodegeneration by measurement of [3 H]paroxetine-labeled serotonin uptake sites. *J. Pharmacol. Exp. Ther.* 242, 911-916.
- Battaglia, G., Yeh, S.Y., De Souza, E.B., 1988. MDMA-induced neurotoxicity: parameters of degeneration and recovery of brain serotonin neurons. *Pharmacol. Biochem. Behav.* 29, 269-274.
- Cadoni, C., Solinas, M., Pisanu, A., Zernig, G., Acquas, E., Di Chiara, G., 2005. Effect of 3,4-methylenedioxyamphetamine (MDMA, "ecstasy") on dopamine transmission in the nucleus accumbens shell and core. *Brain Res.* 1055, 143-148.
- Colado, M.I., O'Shea, E., Green, A.R., 2004. Acute and long-term effects of MDMA on cerebral dopamine biochemistry and function. *Psychopharmacology (Berlin)* 173, 249-263.
- Conductier, G., Crosson, C., Hen, R., Bockaert, J., Compan, V., 2005. 3,4-N-methylenedioxyamphetamine-induced hypophagia is maintained in 5-HT1B receptor knockout mice, but suppressed by the 5-HT2C receptor antagonist RS102221. *Neuropsychopharmacology* 30, 1056-1063.
- Crisp, T., Stafinsky, J.L., Boja, J.W., Schechter, M.D., 1989. The antinociceptive effects of 3,4-methylenedioxyamphetamine (MDMA) in the rat. *Pharmacol. Biochem. Behav.* 34, 497-501.
- Escubedo, E., Guitart, L., Sureda, F.X., Jimenez, A., Pubill, D., Pallas, M., Camins, A., Camarasa, J., 1998. Microgliosis and down-regulation of adenosine transporter induced by methamphetamine in rats. *Brain Res.* 814, 120-126.
- Ferre, S., Fuxe, K., von Euler, G., Johansson, B., Fredholm, B.B., 1992. Adenosine-dopamine interactions in the brain. *Neuroscience* 51, 501-512.
- Fredholm, B.B., Bättig, K.C., Holmen, J.A., Nehlig, A.D., Zvartau, E. E., 1999. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacol. Rev.* 51, 83-133.
- Ghelardini, C., Galeotti, N., Bartolini, A., 1997. Caffeine induces central cholinergic analgesia. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 356, 590-595.
- Godfrey, L., Yan, L., Clarke, G.D., Ledent, C., Kitchen, I., Hourani, S. M., 2006. Modulation of paracetamol antinociception by caffeine and by selective adenosine A(2) receptor antagonists in mice. *Eur. J. Pharmacol.* 531, 80-86.
- Green, A.R., Mechan, A.O., Elliott, J.M., O'Shea, E., Colado, M.I., 2003. The pharmacology and clinical pharmacology of 3,4-methylenedioxyamphetamine (MDMA, "ecstasy"). *Pharmacol. Rev.* 55, 463-508.
- Gudelsky, G.A., Nash, J.F., 1996. Carrier-mediated release of serotonin by 3,4-methylenedioxyamphetamine: implications for serotonin-dopamine interactions. *J. Neurochem.* 66, 243-249.
- Kehne, J.H., Ketteler, H.J., McCloskey, T.C., Sullivan, C.K., Dudley, M.W., Schmidt, C.J., 1996. Effects of the selective 5-HT2A receptor antagonist MDL 100,907 on MDMA-induced locomotor stimulation in rats. *Neuropsychopharmacology* 15, 116-124.
- Kim, D.S., Palmiter, R.D., 2003. Adenosine receptor blockade reverses hypophagia and enhances locomotor activity of dopamine-deficient mice. *Proc. Natl. Acad. Sci. U. S. A.* 100, 1346-1351.
- Klingler, W., Heffron, J.J., Jurkat-Rott, K., O'Sullivan, G., Alt, A., Schlesinger, F., Bufler, J., Lehmann-Horn, F., 2005. 3,4-Methylenedioxyamphetamine (ecstasy) activates skeletal muscle nicotinic acetylcholine receptors. *J. Pharmacol. Exp. Ther.* 314, 1267-1273.
- Koster, R., Anderson, M., de Beer, E.J., 1959. Acetic acid for analgesic screening. *Fred. Proc.* 18, 412.
- Laska, E.M., Sunshine, A., Mueller, F., Elvers, W.B., Siegel, C., Rubin, A., 1984. Caffeine as an analgesic adjuvant. *J. Am. Med. Assoc.* 251, 1711-1718.
- Le Donne, K.T., Sonsalla, P.K., 1994. Protection against methamphetamine-induced neurotoxicity to neostriatal dopaminergic neurons by adenosine receptor activation. *J. Pharmacol. Exp. Ther.* 271, 1320-1326.
- McCreary, A.C., Bankson, M.G., Cunningham, K.A., 1999. Pharmacological studies of the acute and chronic effects of

- (+)-3,4-methylenedioxymethamphetamine on locomotor activity: role of 5-hydroxytryptamine(1A) and 5-hydroxytryptamine(1B/1D) receptors. *J. Pharmacol. Exp. Ther.* 290, 965–973.
- McGuire, P.K., Cope, H., Fahy, T.A., 1994. Diversity of psychopathology associated with use of 3,4-methylenedioxymethamphetamine ('Ecstasy'). *Br. J. Psychiatr.* 165, 391–395.
- McNamara, R., Kerans, A., O'Neill, B., Harkin, A., 2006. Caffeine promotes hyperthermia and serotonergic loss following co-administration of the substituted amphetamines, MDMA ('Ecstasy') and MDA ('Love'). *Neuropharmacology* 50, 69–80.
- O'Callaghan, J.P., Holtzman, S.G., 1976. Prenatal administration of morphine to the rat: tolerance to the analgesic effect of morphine in the offspring. *J. Pharmacol. Exp. Ther.* 197, 533–544.
- O'Loinsigh, E.D., Boland, G., Kelly, J.P., O'Boyle, K.M., 2001. Behavioural, hyperthermic and neurotoxic effects of 3,4-methylenedioxymethamphetamine analogues in the Wistar rat. *Prog. Neuro-Psychopharmacol. Biol. Psychiatry* 25, 621–638.
- O'Regan, M.C., Clow, A., 2004. Decreased pain tolerance and mood in recreational users of MDMA. *Psychopharmacology (Berlin)* 173, 446–451.
- Okada, M., Kiryu, K., Kawata, Y., Mizuno, K., Wada, K., Tasaki, H., Kaneko, S., 1997. Determination of the effects of caffeine and carbamazepine on striatal dopamine release by in vivo microdialysis. *Eur. J. Pharmacol.* 321, 181–188.
- Powell, K.R., Iuvone, P.M., Holtzman, S.G., 2001. The role of dopamine in the locomotor stimulant effects and tolerance to these effects of caffeine. *Pharmacol. Biochem. Behav.* 69, 59–70.
- Pubill, D., Canudas, A.M., Pallas, M., Camins, A., Camarasa, J., Escubedo, E., 2003. Different glial response to methamphetamine- and methylenedioxymethamphetamine-induced neurotoxicity. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 367, 490–499.
- Rempel, N.L., Callaway, C.W., Geyer, M.A., 1993. Serotonin1B receptor activation mimics behavioral effects of presynaptic serotonin release. *Neuropsychopharmacology* 8, 201–211.
- Reneman, L., Endert, E., De, B.K., Lavalaye, J., Feenstra, M.G., de Wolff, F.A., Booij, J., 2002. The acute and chronic effects of MDMA ('ecstasy') on cortical 5-HT_{2A} receptors in rat and human brain. *Neuropsychopharmacology* 26, 387–396.
- Saadat, K.S., O'Shea, E., Colado, M.I., Elliott, J.M., Green, A.R., 2005. The role of 5-HT in the impairment of thermoregulation observed in rats administered MDMA ('ecstasy') when housed at high ambient temperature. *Psychopharmacology (Berlin)* 179, 884–890.
- Scheffel, U., Lever, J.R., Stathis, M., Ricaurte, G.A., 1992. Repeated administration of MDMA causes transient down-regulation of serotonin 5-HT₂ receptors. *Neuropharmacology* 31, 881–883.
- Schmidt, C.J., Levin, J.A., Lovenberg, W., 1987. In vitro and in vivo neurochemical effects of methylenedioxymethamphetamine on striatal monoaminergic systems in the rat brain. *Biochem. Pharmacol.* 36, 747–755.
- Schmidt, C.J., Fadayel, G.M., Sullivan, C.K., Taylor, V.L., 1992. 5-HT₂ receptors exert a state-dependent regulation of dopaminergic function: studies with MDL 100,907 and the amphetamine analogue, 3,4-methylenedioxymethamphetamine. *Eur. J. Pharmacol.* 223, 65–74.
- Shankaran, M., Gudelsky, G.A., 1998. Effect of 3,4-methylenedioxymethamphetamine (MDMA) on hippocampal dopamine and serotonin. *Pharmacol. Biochem. Behav.* 61, 361–366.
- Shibata, M., Ohkubo, T., Takahashi, H., Inoki, R., 1989. Modified formalin test: characteristic biphasic pain response. *Pain* 38, 347–352.
- Sprague, J.E., Nichols, D.E., 1995. The monoamine oxidase-B inhibitor L-deprenyl protects against 3,4-methylenedioxymethamphetamine-induced lipid peroxidation and long-term serotonergic deficits. *J. Pharmacol. Exp. Ther.* 273, 667–673.
- Sprague, J.E., Everman, S.L., Nichols, D.E., 1998. An integrated hypothesis for the serotonergic axonal loss induced by 3,4-methylenedioxymethamphetamine. *Neurotoxicology* 19, 427–441.
- Steele, T.D., Nichols, D.E., Yim, G.K., 1987. Stereochemical effects of 3,4-methylenedioxymethamphetamine (MDMA) and related amphetamine derivatives on inhibition of uptake of [3H] monoamines into synaptosomes from different regions of rat brain. *Biochem. Pharmacol.* 36, 2297–2303.
- Sveen, M.L., Knudsen, G.M., Aznar, S., 2004. No effect of MDMA (ecstasy) on cell death and 5-HT_{2A} receptor density in organotypic rat hippocampal cultures. *Neurosci. Lett.* 362, 6–9.
- Wallace, T.L., Gudelsky, G.A., Vorhees, C.V., 2001. Alterations in diurnal and nocturnal locomotor activity in rats treated with a monoamine-depleting regimen of methamphetamine or 3,4-methylenedioxymethamphetamine. *Psychopharmacology (Berlin)* 153, 321–326.
- Wang, Q.P., Nakai, Y., 1994. The dorsal raphe: an important nucleus in pain modulation. *Brain Res. Bull.* 34, 575–585.
- White, S.R., Duffy, P., Kalivas, P.W., 1994. Methylenedioxymethamphetamine depresses glutamate-evoked neuronal firing and increases extracellular levels of dopamine and serotonin in the nucleus accumbens in vivo. *Neuroscience* 62, 41–50.
- Yamamoto, B.K., Nash, J.F., Gudelsky, G.A., 1995. Modulation of methylenedioxymethamphetamine-induced striatal dopamine release by the interaction between serotonin and gamma-aminobutyric acid in the substantia nigra. *J. Pharmacol. Exp. Ther.* 273, 1063–1070.