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ORIGINAL INVESTIGATION

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3,4-Methylenedioxymethamphetamine induces monoamine release, but not toxicity, when administered centrally at a concentration occurring following a peripherally injected neurotoxic dose

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Abstract *Rationale:* There is good evidence that 3,4-methylenedioxymethamphetamine (MDMA)-induced neurotoxicity results from free radical formation. However, it is unclear whether it is the presence of MDMA or a metabolite in the brain that initiates this process. *Objective:* We wished to measure the concentration of MDMA in the brain following peripheral administration of neurotoxic doses and examine the effect on acute monoamine release and the subsequent neurotoxic loss in 5-hydroxytryptamine (5-HT) content when a high concentration of MDMA was infused into cerebral tissue. *Methods:* Selectively placed microdialysis probes were used to determine both the concentration of MDMA in the brain following peripheral injection and the degree of 5-HT release. Monoamines in dialysate and tissue were measured with standard HPLC techniques. *Results:* MDMA, administered intraperitoneally, at doses of 10 and 15 mg/kg, which produce neurodegeneration, resulted in an estimated cerebral extracellular concentration of MDMA of 11 and 20 µM, respectively. When MDMA (100–400 µM) was perfused through a selectively placed microdialysis probe it dose-dependently increased 5-HT release in the hippocampus and dopamine release in the striatum. Seven days after perfusion of MDMA the concentration of 5-HT and its metabolite, 5-hydroxyindoleacetic acid was unchanged in the ipsilateral side of the brain of normothermic rats and also in the brains of animals made hyperthermic to mimic the acute effect of MDMA given peripherally. In contrast, perfusion with 5,7-dihydroxytryptamine (400 µM) markedly decreased the cerebral 5-HT content. A second probe, also placed in the hippocampus at a distance of 1 mm from the main probe, revealed that during the perfusion of MDMA (400 µM) the estimated extracellular concentration of MDMA in the hippocampus was between 10.4 and 19.5 µM, i.e. in the range of concentrations observed after systemic injection of neurotoxic doses of MDMA. *Conclusions:* These data demonstrate that MDMA when injected directly into the brain produces 5-HT release but no neurotoxicity, suggesting that it must be metabolised peripherally in order to produce compounds that induce free radical formation and neurotoxicity in the brain.

Keywords 3,4-Methylenedioxymethamphetamine · Microdialysis · 5-Hydroxytryptamine · Neurotoxicity · Hyperthermia · Dopamine · 5,7-Dihydroxytryptamine

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Introduction

The recreationally used drug 3,4-methylenedioxymethamphetamine (MDMA or 'ecstasy') administered systemically has two distinct neurochemical effects in the brain of rats. The first action consists of an inactivation of tryptophan hydroxylase and a rapid and dramatic release of 5-hydroxytryptamine (5-HT) and dopamine (DA) from neuronal stores, resulting in substantial depletion of these monoamines (Schmidt 1987; Colado and Green 1994; Colado et al. 1999). Simultaneously, signs associated with the 5-HT behavioural syndrome and hyperthermia are evident (Slikker et al. 1989; Colado et al. 1993). The second action of MDMA is that of producing a long-term degeneration of 5-HT nerve terminals in several areas of the brain. The degeneration has been demonstrated both histologically (O'Hearn et al. 1988; Molliver et al. 1990) and biochemically and is reflected in a substantial decrease in the concentration of 5-HT and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), and a decrease in the density of 5-HT uptake sites labelled with [^{3}H]-paroxetine (Sharkey et al. 1991; Hewitt and Green 1994; Colado et al. 1997).

The mechanisms involved in producing this neurodegeneration are not fully understood at present, but recent

data indicate that a major mechanism by which MDMA induces damage to 5-HT-containing neurones in rat brain is by increasing the formation of hydroxyl free radicals (Colado and Green 1995; Colado et al. 1997; Yeh 1999), and that these are formed in 5-HT nerve endings where MDMA penetrates via the 5-HT transporter system (Gudelsky and Nash 1996). Using intracerebral microdialysis, it has been shown that systemic administration of MDMA increases the formation of 2,3-dihydroxybenzoic acid from salicylate in hippocampal and striatal dialysates, indicating an increased production of hydroxyl radicals (Colado et al. 1997; Shankaran et al. 1999). This effect on hydroxyl formation was absent in rats with a lesion of the 5-HT nerve terminals (Colado et al. 1997).

Whether increased free radical formation following peripheral administration of MDMA results from a direct action of the parent compound in the nerve terminal is unclear. There are only two studies reporting on the long-term effects of MDMA after its administration directly in the brain. The first (Molliver et al. 1986) reported that direct injection of MDMA into the brain failed to produce neurotoxicity. However, since this was only an abstract report, experimental details are sparse. The second report demonstrated that an intraraphe microinjection of MDMA in rats did not produce neurotoxicity to 5-HT terminals (Paris and Cunningham 1991). However, this result is not surprising since systemic administration of MDMA leaves the 5-HT cell bodies in the raphe nucleus intact (Battaglia et al. 1991; Lew et al. 1996).

In order to evaluate the contribution of the peripheral metabolism of systemically administered MDMA to its neurotoxic actions, we have now undertaken a study to examine the long-term effect of local application of MDMA on 5-HT nerve terminals in the brain. Initial experiments measured the cerebral content of MDMA following peripheral administration of neurotoxic doses. MDMA was then administered in a range of concentrations similar to, or higher than, the estimated cerebral extracellular concentration reached after systemic administration of neurotoxic doses of MDMA by perfusing MDMA through a microdialysis probe implanted in the hippocampus. We investigated both the acute and long-term effects of the drug on the release and metabolism of 5-HT and DA when administered via this route. To confirm the correct placement of the probe and the likely diffusion of compounds injected in this way, two experiments were performed. In the first, a neurotoxic dose of 5,7-dihydroxytryptamine (5,7-DHT) was also infused and its effect on hippocampal 5-HT content examined. In the second experiment a second probe was placed at a defined distance from the main probe to determine the concentration of MDMA at a site remote from the perfusion site.

A further weakness of previous studies on the effect of centrally administered MDMA has been that locally administered MDMA would probably not have induced hyperthermia, in contrast to the effect of the peripherally administered drug. It is clear that acute MDMA-induced hyperthermia plays a key role in enhancing the long-term neurotoxic effect of the drug (Farfel and Seiden 1995a, b; Malberg et al. 1996; Colado et al. 1998, 1999). We have, therefore, undertaken some studies where animals were made hyperthermic during the time of infusion of drug into the brain.

Some of these results were given in preliminary form to a meeting of the British Pharmacological Society (Esteban et al. 1999).

Materials and methods

Animals and drug administration

All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication number 85–23, revised 1985).

Male Dark Agouti rats (175–200 g; Interfauna) were used. They were housed in groups of five, in conditions of constant temperature (21±2°C) and a 12-h light/dark cycle (lights on: 07.00 a.m.) and given free access to food and water. MDMA (obtained from the Ministry of Health, Spain) was dissolved in saline (0.9% NaCl) and given IP in a volume of 1 ml/kg or directly applied to the brain through the microdialysis probe. Doses are reported in terms of the base.

Implantation of a microdialysis probe in the hippocampus

Rats were anaesthetised with pentobarbitone (Euta-Lender; 40 mg/kg) and secured in a Kopf stereotaxic frame with the tooth bar at –3.3 mm below the interaural zero. The dialysis probe (3.5 mm×240 µm; Cuprophan) was implanted in the right hippocampus according to the following coordinates: +2.2 mm from the interaural line, –4.3 mm lateral and –8.0 mm below the surface of the brain (König and Klippel 1963). Probes were secured to the skull as described by Baldwin et al. (1994).

A second probe was also implanted in the hippocampus of a further group of rats. In these animals we used a double probe, which was constructed so that shafts and tips were parallel and separated by a distance of 1 mm. In this double probe one of the tips (used for MDMA perfusion) was placed according to the same coordinates used in the implantation of a single probe, and the second tip (used for recovering MDMA) was at a distance of 1 mm from the first one in the rostral direction (+3.2 mm from the interaural line). As the hippocampus extends 2 mm in the anteroposterior direction (König and Klippel 1963), the tip used for drug perfusion would be placed at the centre of the hippocampus and the other one at its border. The correct placement of the probes was verified by dye perfusion on test animals prior to proceeding with experimental groups.

Systemic administration of MDMA and measurement of MDMA in the hippocampus dialysate

The day after probe implantation, probes were perfused at a rate of 1 µl/min with artificial CSF (aCSF). All microdialysis experiments showed in this work were performed in awake and freely moving rats. After a 60-min resting period samples were collected every 30 min. Three basal samples were first collected and the rats then injected IP with several doses of MDMA (5, 10 and 15 mg/kg). Previous results have demonstrated that MDMA at the doses of 10 and 15 mg/kg produces, 1 week later, a marked depletion of several 5-HT parameters reflecting neurotoxicity (O'Shea et al. 1998).

MDMA was measured in the dialysate by HPLC and electrochemical detection. The mobile phase consisted of KH_2PO_4 (0.025 M) and CH_3CN (26%), and was adjusted to pH 2.5 with phosphoric acid, filtered and degassed. Dimethyloctylamine was

added at a concentration of 100 µl/l to improve peak shape (Colado et al. 1995). The flow rate was 1 ml/min.

The HPLC system consisted of a pump (Waters 510) linked to a manual sample injector (Loop 20 µl; Rheodyne), a stainless steel reversed-phase column (Spherisorb ODS2, 5 µm, 150×4.6 mm) with a precolumn and a coulometric detector (Coulochem 5100A) with a 5011 analytical cell. The working electrode potential was set at 850 mV with 5 µA gain. The current produced was monitored by using a computer data handling system (AXXIOM 747).

Evaluation of probe recovery in vitro

In order to estimate the extracellular concentration of MDMA in the hippocampus after its IP administration, we assessed the ability of the probes to recover MDMA in vitro from solutions containing different concentrations of the drug. Although the percentage recovery of MDMA obtained by in vitro calibration of the microdialysis probes underestimate the extracellular brain concentration, this method shows good reproducibility and establishes a linear relationship between the concentrations of low molecular compounds in the dialysate and in the surrounding medium (Hamberger et al. 1991). Probes were connected to the microdialysis system set-up as in the in vivo experiments, immersed in and perfused with aCSF at a flow of 1 µl/min. Samples were collected every 30 min. After a 1-h resting period, three basal samples were collected and then probes were placed in Eppendorf tubes containing progressively more concentrated standard solutions of MDMA (25, 100, 200 and 400 µM). For each MDMA concentration 3×30 min samples were collected. At the end of each period, a volume of 30 µl of the standard solution of MDMA was directly collected and analysed with the corresponding dialysis samples. The in vitro recovery was determined as $[(C_o \times 100)/C_i]$, where C_o and C_i are the MDMA concentrations of the collected sample and the standard solution, respectively.

Perfusion of MDMA through the probe in the hippocampus in normothermic and hyperthermic rats

A parallel microdialysis study was conducted perfusing MDMA (100, 200, and 400 µM) through the probe for 2.5 h in a range of concentrations adequate to release to the extracellular space a concentration of the drug similar to or 4 times higher than that obtained after IP administration of MDMA (15 mg/kg). MDMA (400 µM) was also perfused for 6 h in normo- and hyperthermic rats.

The ability of the probe to deliver MDMA in vivo was also examined. For that, MDMA 400 µM was perfused through the probes in situ in the hippocampus. Dialysates were collected every 30 min during a 2.5-h perfusion period. The in vivo delivery was determined as $[(C_i - C_o) \times 100/C_i]$, where C_i and C_o are the MDMA concentrations in the input and output solutions of the corresponding intervals.

During the perfusion time with MDMA, rectal temperature was measured using a digital readout thermocouple (type K thermometer; Portec) which had a resolution of ±0.1°C and accuracy of ±0.2°C. This was attached to a CAC-005 rodent sensor which was inserted 2.5 cm into the rectum, the rat being lightly restrained by holding in the hand. A steady readout was obtained within 10 s of probe insertion.

In one experiment, rats were perfused with MDMA and had their rectal temperature kept elevated to near that seen in rats given an IP injection of MDMA (15 mg/kg). This was achieved by placing the rats in a cage with a Harvard homeothermic blanket (model 50–7087) covering the base.

Implantation of a microdialysis probe in the striatum

Rats were anaesthetised with sodium pentobarbitone (Euta-Lender; 40 mg/kg, IP) and secured in a Kopf stereotaxic frame with the tooth bar at –3.3 mm below the interaural zero. The dialysis probe (3.5 mm×240 µm; Cuprophan) was implanted in the right striatum according to the following coordinates: +7.9 mm from the interaural line, –2.5 mm lateral and –8.0 mm below the surface of the brain (König and Klippel 1963). Probes were secured to the skull as described by Baldwin et al. (1994). The next day probes were perfused with MDMA 400 µM for 6 h.

Measurement of monoamines and their metabolites in the hippocampal and striatal dialysate

Twenty-four hours after implantation, probes were perfused with aCSF (KCl: 2.5 mM; NaCl: 125 mM; $MgCl_2.6H_2O$: 1.18 mM; $CaCl_2.2H_2O$: 1.26 mM) at a rate of 1 µl/min and samples collected from the freely moving animals at 30-min intervals in tubes containing 5 µl of a solution composed of $HClO_4$ (0.01 M), cysteine (0.2%) and sodium metabisulphite (0.2%). The first 60-min sample was discarded and the next three 30-min baseline samples collected before starting MDMA perfusion.

5-HT, DA and their metabolites were measured in the dialysate by HPLC and electrochemical detection. The mobile phase consisted of KH_2PO_4 (0.05 M), octanesulphonic acid (0.16 mM), EDTA (0.1 mM) and methanol (16%), and was adjusted to pH 3 with phosphoric acid, filtered and degassed. The flow rate was 1 ml/min.

The HPLC system consisted of a pump (Waters 510) linked to a manual sample injector (Loop 20 µl; Rheodyne), a stainless steel reversed-phase column (Spherisorb ODS2, 5 µm, 150×4.6 mm) with a precolumn and a coulometric detector (Coulochem 5100A) with a 5011 analytical cell. The working electrode potential was set at 400 mV with 500 nA gain. The current produced was monitored by using a computer data handling system (AXXIOM 747).

Measurement of monoamines and their metabolites in the hippocampal and striatal tissue

Seven days after dialysis experiment rats were killed by cervical dislocation and decapitation, the brains rapidly removed and the right and left hippocampus and striatum dissected out on ice. Tissue was homogenised and 5-HT, 5-HIAA and DA measured by HPLC. The average weight for the hippocampus and striatum was 43 and 30 mg, respectively. A group of rats was killed immediately after stopping MDMA perfusion (400 µM, 6 h) and the content of 5-HT and 5-HIAA was determined in the right and left hippocampus.

The mobile phase and flow of the pump were the same as mentioned above and the working electrode potential was set at 0.85 V. The HPLC system consisted of a pump (Waters 510) linked to an automatic sample injector (Loop 200 µl; Waters 712 WISP), a stainless steel reversed-phase column (Spherisorb ODS2, 5 µm, 150×4.6 mm) with a precolumn and an amperometric detector (Waters M460). The current produced was monitored by using an integrator (Waters M745).

Perfusion of 5,7-DHT through the probe in the hippocampus

In order to confirm that a compound infused down the probe would lesion 5-HT nerve terminals in the hippocampus, the known 5-HT neurotoxin 5,7-DHT (400 µM) was perfused through the probe for 6 h. 5,7-DHT was dissolved in aCSF containing 0.1% ascorbic acid as antioxidant. To prevent damage to noradrenergic neurons, animals perfused with vehicle and 5,7-DHT were injected with desipramine (25 mg/kg, IP) 30 min before starting perfusion. During the microdialysis experiment, rectal temperature was measured as detailed above. Seven days later, rats were killed and the concentration of 5-HT and 5-HIAA in the right and left hippocampus determined by HPLC using the method described above.

Statistics

Statistical analyses of the microdialysis experiments were performed using the statistical computer package BMDP/386 Dynamic (BMDP Statistical Solutions). Data were analysed by two-way analysis of variance (ANOVA) with repeated measures (program 2V) or, where missing values occurred, an unbalanced repeated measure model (program 5V) was used. Both used treatment as the between-subjects factor and time as the repeated measure. ANOVA was performed on both pretreatment and posttreatment data. Temperature data were also analysed by two-way ANOVA with repeated measures. Data from monoamine experiments were analysed by the one-way ANOVA followed by the Tukey multiple comparison test when a significant *F* value was obtained (GraphPad Prism, version 3).

Results

The extracellular concentration of MDMA following systemic administration of the drug

A single injection of MDMA (5, 10 and 15 mg/kg, IP) produced a rapid and dose-dependent increase in the concentration of MDMA in the hippocampal dialysate. The effect was maximal between 30 and 60 min, reaching a peak of 2.50±0.17 µM (*n*=6) and 4.35±0.42 µM (*n*=9) following, respectively, the neurotoxic doses of 10 and 15 mg/kg MDMA. The content then declined progressively to below detectable levels 6.5 h later (Fig. 1).

The in vitro probe recoveries of solutions containing different concentrations of MDMA were 22.4±1.7% (5) for 25 µM, 24.8±0.7% (5) for 100 µM, 23.4±0.5% (5) for 200 µM and 20.8±0.6% (5) for 400 µM. We can therefore assume that probes are recovering approximately 22% of the extracellular concentration of MDMA. Thus a concentration of 2.50 and 4.35 µM in the hippocampal dialysate reflects an estimated extracellular brain concentration of around 11 and 20 µM, respectively (Fig. 1).

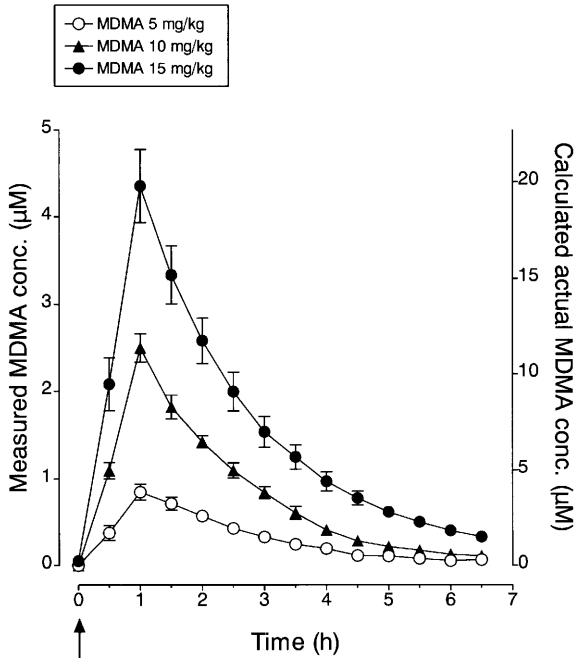

Fig. 1 Measured 3,4-methylenedioxymethamphetamine (MDMA) concentration in the hippocampal dialysate (*left axes*) and calculated actual MDMA concentration in the extracellular space of the hippocampus (*right axes*) following IP injection of MDMA (5, 10 and 15 mg/kg). MDMA injection is indicated by the *arrow*. The actual MDMA concentration was calculated taking into account the previously known recovery efficiency of the probes. Each value is the mean ± SEM, *n*=6–9

The extracellular concentration of MDMA in the brain following its administration by microdialysis

The concentration of MDMA that is delivered to the extracellular space of the brain during the perfusion of MDMA 400 µM is shown in Fig. 2. If we consider the difference between the MDMA concentration of the perfusate and the dialysate, the percentage of MDMA reaching the extracellular space during the perfusion period can be estimated to be around 20%. These data suggest therefore that during the perfusion with MDMA (100, 200 and 400 µM) the concentration of the drug diffusing to the extracellular space is between 20 and 80 µM in the immediate area of the probe.

In order to determine the concentration that MDMA (400 µM) reaches in the extracellular space during the perfusion period (6 h), a second probe was implanted in the hippocampus and perfused with aCSF during the same time period. The concentration of MDMA recovered by the second probe was between 2.29 and 4.28 µM (Fig. 3). These values reflect an estimated extracellular concentration of MDMA between 10.4 and 19.5 µM (Fig. 3), i.e. in the range of that found after peripheral administration of MDMA 10 and 15 mg/kg (Fig. 1).

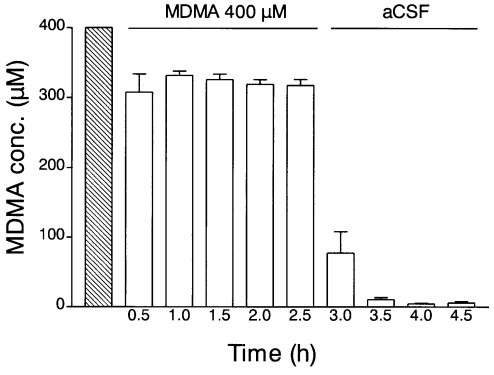

Fig. 2 Concentration of MDMA in the hippocampal dialysate of rats perfused during 2.5 h with artificial CSF (aCSF) containing MDMA 400 µM. The *shaded bar* represents the concentration of MDMA in the perfusate. *Bars* represent the mean ± SEM for the corresponding 30-min interval after MDMA perfusion, *n*=5

Acute effect of MDMA microdialysed in the hippocampus on 5-HT release

The perfusion of MDMA (100, 200 and 400 µM) during 2.5 h through the microdialysis probe produced a dose-

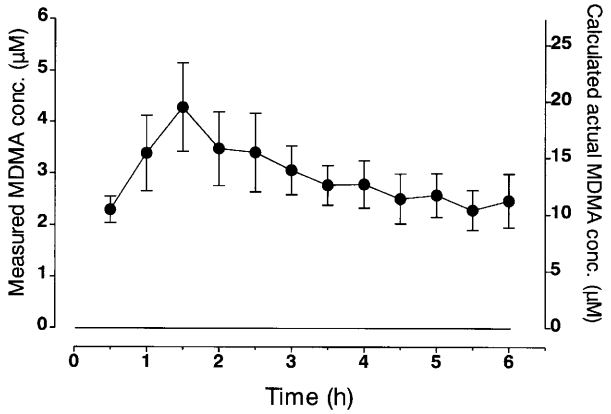

Fig. 3 Measured MDMA concentration in the hippocampal dialysate (*left axes*) and calculated actual MDMA concentration in the extracellular space of the hippocampus (*right axes*) during the perfusion of MDMA 400 μM (6 h, indicated by the *bar*). The actual MDMA concentration was calculated taking into account the previously known recovery efficiency of the probes. These data were obtained by implanting a doubled probe in the hippocampus. Each value is the mean ± SEM, *n*=11

dependent increase in the concentration of 5-HT in the hippocampal dialysate. 5-HT release peaked between 30 and 60 min after MDMA perfusion and declined thereafter reaching basal levels 60 min after reperfusion with aCSF (Fig. 4a). MDMA perfusion did not produce any statistically significant change in the content of 5-HIAA in the dialysate except at the highest dose (400 μM; Fig. 4b). This concentration produced a decrease in 5-HIAA levels which was maintained after MDMA perfusion ceased (Fig. 4b).

During the time of perfusion there was no difference in the rectal temperature of the animals (data not shown).

Acute effect of MDMA microdialysed in the hippocampus on 5-HT content

MDMA (400 μM) microdialysed during 6 h produced a marked decrease of 5-HT content in the right and left hippocampus (ipsi- and contralateral, respectively, to the perfusion) compared with that observed following aCSF perfusion. The depletion was significantly higher in the side perfused with MDMA (right vs left hippocampus; Fig. 5a). The content of 5-HIAA was not modified in either side by MDMA perfusion (Fig. 5b).

Long-term effect of MDMA microdialysed in the hippocampus on 5-HT content

No difference was observed in the concentration of 5-HT and 5-HIAA in the hippocampus between rats perfused during 2.5 h with MDMA (100, 200 or 400 μM) or aCSF 7 days earlier (Table 1).

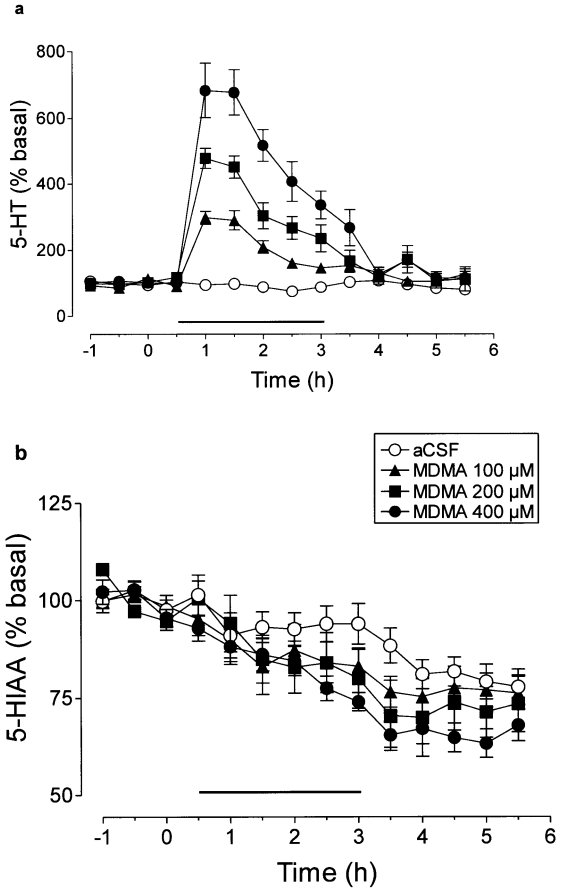

Fig. 4 Changes in the levels of (**a**) 5-hydroxytryptamine (5-HT) and (**b**) 5-hydroxyindoleacetic acid (5-HIAA) in the hippocampal dialysate of rats perfused with aCSF or MDMA (100, 200 and 400 μM) during 2.5 h. The *horizontal bar* represents the period of perfusion. Values are expressed as percentage of the mean of three measurements before drug perfusion. Each value is the mean ± SEM of eight to ten rats. **a** There was no difference in the basal levels of 5-HT in the dialysate. MDMA perfusion produced a significant difference in the levels of 5-HT [$F(3,36)=56.49$, $P<0.001$] which was dose-dependent [for MDMA 100 μM vs aCSF: $F(1,20)=59.35$, $P<0.001$; MDMA 200 μM vs MDMA 100 μM, $F(1,17)=13.78$, $P<0.001$; MDMA 400 μM vs MDMA 200 μM: $F(1,16)=11.10$, $P<0.001$]. **b** There was no difference in the basal levels of 5-HIAA in the dialysate. MDMA perfusion produced a decrease in 5-HIAA content only at the highest concentration perfused [$F(1,20)=8.06$, $P<0.01$]. Basal levels were for 5-HT (pg/μl): aCSF (0.218±0.017, *n*=10), MDMA 100 μM (0.221±0.016, *n*=10), MDMA 200 μM (0.191±0.024, *n*=9), MDMA 400 μM (0.175±0.018, *n*=9) and for 5-HIAA (pg/μl): aCSF (58.2±5.4, *n*=12), MDMA 100 μM (46.3±3.2, *n*=10), MDMA 200 μM (57.3±6.4, *n*=8), MDMA 400 μM (51.9±4.4, *n*=9)

5-HT content in the hippocampus of rats kept at normal and high ambient temperature and perfused with MDMA for 6 h

In order to assay both the effect produced by a long-lasting perfusion with MDMA as well as influence of hyperthermia on the long-term effect of MDMA, an experiment was performed microdialysing MDMA (400 μM) through the probe for 6 h in rats whose rectal temperature was maintained in a similar range to that seen in rats

Table 1 Concentration of 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in the right and left hippocampus 7 days after perfusion with artificial CSF (aCSF) or 3,4-methylenedioxymethamphetamine (MDMA; 100, 200 and 400 µM) through the probe for 2.5 h. Results shown as mean ± SEM (*n*)

Treatment	5-HT (ng/g tissue)		5-HIAA (ng/g tissue)	
	Right	Left	Right	Left
aCSF	315±12 (12)	376±11 (12)	478±17 (12)	421±14 (12)
MDMA 100 µM	311±18 (10)	365±17 (10)	449±17 (10)	431±16 (10)
MDMA 200 µM	309±23 (8)	363±25 (7)	415±11 (8)	434±14 (8)
MDMA 400 µM	297±14 (7)	366±20 (7)	460±21 (7)	432±18 (7)

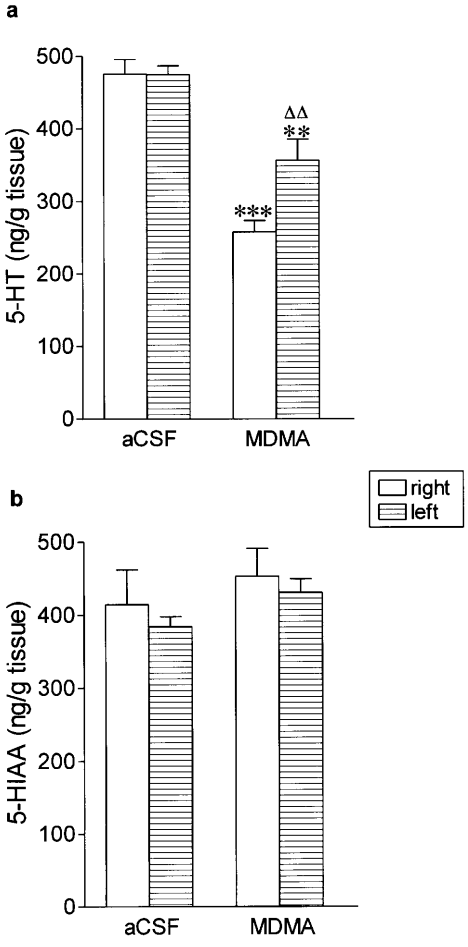

Fig. 5 Concentration of (**a**) 5-HT and (**b**) 5-HIAA in the right and left hippocampus after perfusion with aCSF or MDMA 400 µM through the probe for 6 h. Animals were killed immediately after stopping dialysis. Results shown as mean ± SEM, *n*=11. Different from its corresponding site of aCSF-perfused rats: ***P*<0.01, ****P*<0.001. Different from the right hippocampus of MDMA-perfused rats: ΔΔ*P*<0.01

given MDMA IP. This was achieved by placing the rats in a cage with a homeothermic blanket covering the base during the perfusion time. In that way the rectal temperature of the rats was increased by approximately 2°C (data not shown). Seven days later no change was observed in the hippocampal 5-HT concentration of rats kept either normothermic or hyperthermic (Table 2).

Table 2 Concentration of 5-HT and 5-HIAA in the right and left hippocampus 7 days after perfusion with aCSF or MDMA (400 µM) through the probe for 6 h in normothermic and hyperthermic rats. Results shown as mean ± SEM (*n*)

Treatment	5-HT (ng/g tissue)		5-HIAA (ng/g tissue)	
	Right	Left	Right	Left
aCSF	368±11 (9)	401±13 (9)	500±33 (9)	465±33 (9)
MDMA 400 µM				
Normothermic	368±10 (9)	424±14 (9)	438±16 (9)	419±17 (9)
Blanket	352±7 (9)	425±10 (9)	509±13 (9)	466±17 (9)

Acute effect of MDMA microdialysed in the striatum on DA release

Pefusion of MDMA (400 µM) during 6 h produced a rapid and substantial increase in extracellular DA in the striatum (Fig. 6a). This change was accompanied by a sustained decrease in both HVA and DOPAC, the decrease in the latter starting earlier and being more pronounced than that of HVA. Two hours after the start of MDMA perfusion, the DOPAC and HVA levels were, respectively, 61±6% and 88±3% of control values (Fig. 6b, c).

Long-term effect of MDMA microdialysed in the striatum on DA and 5-HT content

Seven days after perfusion during 6 h with MDMA (400 µM) the concentrations of 5-HT and DA in the striatum were similar to those seen in rats perfused with aCSF (Table 3).

Long-term effect of 5,7-DHT microdialysed in the hippocampus on 5-HT content

Seven days after perfusion of rats with 5,7-DHT (400 µM) there was a pronounced decrease in the concentration of 5-HT and 5-HIAA in the right hippocampus (ipsilateral to the perfusion) compared with the left side (Fig. 7). This reduction did not occur in vehicle- or aCSF-treated rats (Fig. 7).

Table 3 Concentration of dopamine (DA) and 5-HT in the right and left striatum 7 days after perfusion with aCSF or MDMA (400 µM) through the probe for 6 h. Results shown as mean ± SEM (*n*)

Treatment	DA (ng/g tissue)		5-HT (ng/g tissue)	
	Right	Left	Right	Left
aCSF	8109±592 (10)	9491±459 (10)	441±22 (10)	518±28 (10)
MDMA 400 µM	8212±726 (8)	9331±667 (8)	443±25 (8)	521±40 (8)

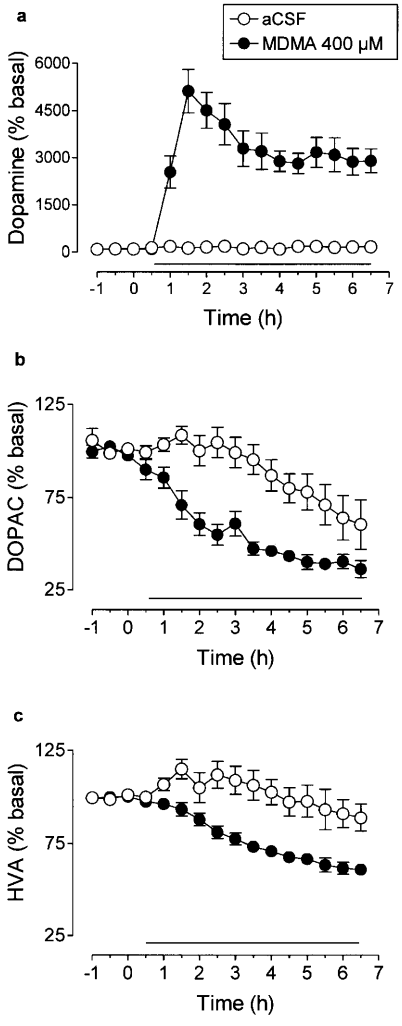

Fig. 6 Changes in the levels of (**a**) dopamine (DA), (**b**) DOPAC and (**c**) HVA in the striatal dialysate of rats perfused with aCSF or MDMA 400 µM for 6 h. The *horizontal bar* represents the period of perfusion. Values are expressed as percentage of the mean of three measurements before drug perfusion. Each value is the mean ± SEM of six or seven rats. There were no differences in the basal levels of DA, DOPAC and HVA in the dialysate. MDMA perfusion produced a significant increase in the levels of both DA [$F(1,11)=93.04$, $P<0.001$] and a pronounced decrease in the levels of DOPAC [$F(1,11)=21.70$, $P<0.001$] and HVA [$F(1,12)=15.21$, $P<0.01$]. Basal levels were for DA (pg/µl): aCSF (0.16±0.04, $n=7$), MDMA 400 µM (0.18±0.03, $n=6$); for DOPAC (pg/µl): aCSF (163±7, $n=6$), MDMA 400 µM (181±33, $n=7$); for HVA (pg/µl): aCSF (187±7, $n=7$), MDMA 400 µM (214±23, $n=7$)

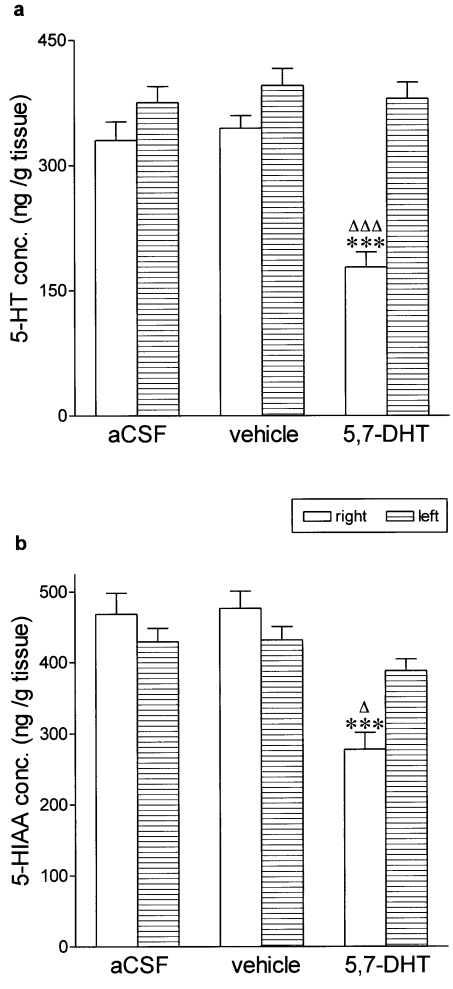

Fig. 7 Concentration of (**a**) 5-HT and (**b**) 5-HIAA in the right and left hippocampus 7 days after perfusion with aCSF, vehicle or 5,7-dihydroxytryptamine (5,7-DHT) 400 µM through the probe for 6 h. Results shown as mean ± SEM, $n=9$. Different from the right hippocampus of aCSF- and vehicle-perfused rats: ***$P<0.001$. Different from its corresponding left hippocampus: Δ$P<0.05$, ΔΔΔ$P<0.001$

Injection of desipramine 30 min before starting perfusion caused a prolonged hypothermia compared with saline-treated rats. This hypothermic effect was similar in aCSF- and 5,7-DHT-perfused rats (Fig. 8).

Discussion

This is the first study measuring the extracellular concentration of MDMA in the brain following systemic administration of neurotoxic doses which then examined the effect of a similar or higher concentration when it was injected directly into the brain. The hippocampal perfusion of MDMA over different periods of time in a range of concentrations similar to or greater than those reached after its intraperitoneal injection does not pro-

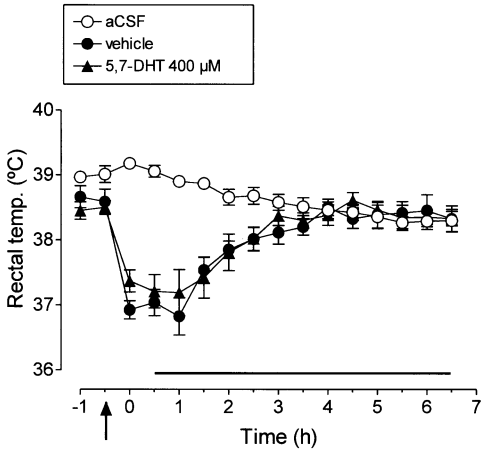

Fig. 8 Rectal temperature of rats perfused with aCSF, vehicle or 5,7-DHT 400 µM for 6 h. The *horizontal bar* represents the period of perfusion and the *arrow* the injection of desipramine (25 mg/kg, IP). Each value is the mean ± SEM of eight to ten rats. Desipramine significantly decreased temperature in both ascorbic acid [$F(1,15)=47.79$, $P<0.001$]- and 5,7-DHT [$F(1,17)=17.17$, $P<0.001$]-perfused rats

duce any long-lasting toxic effect on 5-HT nerve terminals.

After a single IP administration of MDMA at the doses of 10 and 15 mg/kg, the peak microdialysate level of the drug in the hippocampus of rats was estimated to be, respectively, 11 and 20 µM, between 30 and 60 min after injection. These same doses of MDMA (10 and 15 mg/kg) have previously been shown to induce, 7 days later, a marked loss (40–60%) in the concentration of 5-HT and its metabolite, 5-HIAA, and in the number of 5-HT uptake sites in the hippocampus of Dark Agouti rats (see, for example, Colado et al. 1995, 1997; O'Shea et al. 1998).

In our experimental design the measured MDMA concentrations of 2.5 and 4.35 µM in the dialysate correspond to an approximate extracellular level of 11 and 20 µM (calculated from the probe in vitro recovery efficiency) and 12.5 and 22 µM (calculated from the probe in vivo delivery efficiency). In order to facilitate drug delivery to the hippocampus at a concentration similar to or greater than the extracellular levels seen after systemic administration, MDMA was perfused through a microdialysis probe for 2.5 h at different concentrations (100, 200 and 400 µM). Considering the percentage of recovery in vitro as well as the percentage of delivery in vivo, the estimated concentration of MDMA diffusing to the extracellular space in the immediate area of the probe during perfusion time with 100, 200 and 400 µM should be between 20 and 80 µM. This concentration of MDMA diffusing into the hippocampus is either similar to or approximately 4 times higher than the calculated actual MDMA levels in the extracellular space after IP administration of neurotoxic doses of MDMA. Nevertheless neither concentration produced any change in the content of 5-HT 7 days later. When MDMA (400 µM) was perfused for a longer period (6 h) there was still no long-lasting effect on indole content.

Since MDMA is a very lipid soluble substance, it seemed possible that diffusion of the drug from the probe site, its rapid distribution to other brain areas and the removal from the brain due to plasma clearance, might lead to an MDMA concentration in the hippocampus below that required to produce neurotoxicity. In order to answer this point a second probe was implanted at the edge of the hippocampus to recover MDMA during the perfusion of the drug through the main probe. Results showed that the estimated actual extracellular concentration reached by MDMA (400 µM) during the 6 h perfusion is between 10.4 and 19.5 µM, i.e. in the range of concentrations obtained after IP administration of neurotoxic doses of MDMA. This drug concentration is high enough to produce an effect in the whole hippocampus since immediately after cessation of dialysis the content of 5-HT in the ipsilateral side was markedly decreased (around 50%). This effect was also evident in the contralateral hippocampus although to a lesser extent. During MDMA (400 µM) perfusion the concentration reached by the drug at the border of the hippocampus is therefore within the neurotoxic range and in the tissue surrounding the probe it would be expected to be higher. Consequently, the lack of neurotoxic effect of MDMA when applied directly through a microdialysis probe cannot be attributed to the analysis of amines in the whole hippocampus.

In contrast to MDMA, the local application of 5,7-DHT (400 µM) during 6 h induced a marked loss in the concentration of 5-HT and 5-HIAA in the ipsilateral hippocampus, which almost certainly reflects a degeneration of 5-HT nerve terminals. 5,7-DHT has very low liposolubility, so may have a different distribution pattern to MDMA when diffusing away from the immediate perfusion site. Nevertheless, the long-term depletion on 5-HT content is evident even using the whole hippocampus. Data from the 5,7-DHT experiment also confirmed the correct probe placement in our experiments.

It has been shown repeatedly that the hyperthermia subsequent to intraperitoneal MDMA injection plays a very important role in the neuronal damage and that drugs producing hypothermia or preventing the MDMA-induced hyperthermia prevent the long-term neurotoxicity (Farfel and Seiden 1995a, b; Malberg et al. 1996; Colado et al. 1998, 1999). During the perfusion time with MDMA there was no change in the rectal temperature of the rats. An experiment was therefore carried out where MDMA was perfused into the brains of rats whose body temperature was kept in a similar range to that observed after systemic administration of the drug. This aim was achieved by the use of a homeothermic blanket during the perfusion time. In this experimental condition MDMA did not, as before, produce any long-term effect on the brain concentration of 5-HT and 5-HIAA.

MDMA microdialysed in the brain caused the acute effects on 5-HT and DA function which occur after pe-

ripheral administration (Colado et al. 1999). Immediately after the start of perfusion, the extracellular concentration of 5-HT in the hippocampal dialysate increased rapidly in a dose-dependent way probably reflecting enhanced 5-HT release. Similarly, when MDMA was perfused in the striatum, it also produced an increase in the extracellular DA concentration and a decrease in the concentrations of DOPAC and HVA. In spite of producing these acute effects, MDMA perfusion in the striatum did not induce any effect on DA or 5-HT content 7 days later. The short-term effects induced by hippocampal administration of MDMA are in agreement with those reported by Schmidt and Taylor (1988). Although the amount of MDMA infused (0.6 mg/h, during 1 h) was greater than the maximum concentration used in our study (28 µg over 6 h), these authors found an acute depletion of 5-HT and a decrease in tryptophan hydroxylase activity in the cortex and hippocampus after constant intracerebroventricular infusion of MDMA.

A further explanation for the non-toxicity encountered could be the exposure time. However, it is unlikely that the perfusion period was not enough to maintain the required brain concentration to produce toxicity. When perfused through the probe, a high extracellular MDMA concentration was maintained in the hippocampus for up to 6 h, whereas after systemic administration of neurotoxic doses of MDMA, the extracellular concentration reached a maximum 30–60 min after injection, declining quite rapidly thereafter, with a calculated half life in the brain of 1.5 h (this paper).

The possibility that the MDMA-induced damage of 5-HT nerve terminals is a consequence of the action of the drug on raphe nuclei is unlikely given that in rats systemic administration of the drug did not induce changes in the 5-HT cell bodies (O'Hearn et al. 1988), and intraraphe infusion of MDMA (50 µg) did not result in a loss of 5-HT cell bodies in the dorsal or medium raphe nucleus (Paris and Cunningham 1991).

What seems to be very clear from the current study on the effect of MDMA given centrally is that the acute effects of MDMA on 5-HT and DA release do not result in long-term neurotoxicity. This conclusion has previously been made following studies using systemic administration of MDMA and is supported by several observations: (1) MDMA increases 5-HT release at doses which do not produce neurotoxicity, (2) neuronal damage only occurs after administration of the (+) enantiomer of MDMA, while acute 5-HT release is seen with either stereoisomer (Schmidt 1987) and (3) there are several compounds which protect against MDMA neurotoxicity but do not have any effect on acute 5-HT release (Colado and Green 1994).

The lack of neurotoxicity following local application of MDMA in the hippocampus suggests that MDMA-induced neurotoxicity is not due to the parent compound and that an active peripheral metabolite is responsible for the neuronal damage. Nevertheless, attempts to identify this compound have as yet, been unsuccessful. There is evidence that MDMA is metabolised to catechol and quinone metabolites (Hiramatsu et al. 1990; Lim and Foltz 1991; Lin et al. 1992; Tucker et al. 1994) and we have proposed that further metabolism of such compounds results in the formation of free radicals which induce oxidative stress and membrane damage (Colado and Green 1995; Colado et al. 1997). Another substituted amphetamine, *p*-chloroamphetamine (PCA), administered systemically also induces neurotoxic damage to 5-HT nerve terminals in the rat brain, but does not result in axonal degeneration when administered directly into the neocortex (Berger et al. 1990). This finding indicates that PCA itself is not responsible for the neurotoxicity occurring. PCA is predominantly metabolised to 3-chloro-4-hydroxyamphetamine (Parli and Schmidt 1975) producing reactive intermediates (Miller et al. 1986) which have been predicted to be metabolised to catechol metabolites (Jerina and Daly 1974) structurally similar to those produced during MDMA metabolism. In fact, recent data suggest that free radicals are involved in the neurotoxicity of PCA. Immediately after its administration an increase in hydroxyl radical generation is observed in the hippocampus (Colado et al. 1997) and the spin trap reagent, α-phenyl-*N*-tert-butyl nitrone, attenuates PCA-induced neurodegeneration (Murray et al. 1996).

These data demonstrate that the local application of relatively high concentrations of MDMA does not produce neurotoxicity of hippocampal 5-HT terminals in rats and suggest that the neuronal damage following systemic administration could be due to compounds initially formed peripherally.

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References

Baldwin HA, Williams JL, Snares M, Ferreira T, Cross AJ, Green AR (1994) Attenuation by chlormethiazole administration of the rise in extracellular amino acids following focal ischaemia in the cerebral cortex of the rat. Br J Pharmacol 112:188–194

Battaglia G, Sharkey J, Kuhar MJ, De Souza EB (1991) Neuroanatomic specificity and time course of alterations in rat brain serotonergic pathways induced by MDMA (3,4-methylenedioxymethamphetamine): assessment using quantitative autoradiography. Synapse 8:249–260

Berger UV, Molliver ME, Grzanna R (1990) Unlike systemic administration of *p*-chloroamphetamine, direct intracerebral injection does not produce degeneration of 5-HT axons. Exp Neurol 109:257–268

Colado MI, Green AR (1994) A study of the mechanism of MDMA ('ecstasy')-induced neurotoxicity of 5-HT neurones using chlormethiazole, dizocilpine and other protective compounds. Br J Pharmacol 111:131–136

Colado MI, Green AR (1995) The spin trap reagent α-phenyl-*N*-tert-butyl nitrone prevents 'ecstasy'-induced neurodegeneration of 5-hydroxytryptamine neurones. Eur J Pharmacol 280: 343–346

Colado MI, Murray TK, Green AR (1993) 5-HT loss in rat brain following 3,4-methylenedioxymethamphetamine (MDMA),

p-chloroamphetamine and fenfluramine administration and effects of chlormethiazole and dizocilpine. Br J Pharmacol 108:583–589

Colado MI, Williams JL, Green AR (1995) The hyperthermic and neurotoxic effects of 'ecstasy' (MDMA) and 3,4-methylenedioxyamphetamine (MDA) in the Dark Agouti (DA) rat, a model of the CYP2D6 poor metabolizer phenotype. Br J Pharmacol 115:1281–1289

Colado MI, O'Shea E, Granados R, Murray TK, Green AR (1997) In vivo evidence for free radical involvement in the degeneration of rat brain 5-HT following administration of MDMA ('ecstasy') and *p*-chloroamphetamine but not the degeneration following fenfluramine. Br J Pharmacol 121:889–900

Colado MI, Granados R, O'Shea E, Esteban B, Green AR (1998) Role of hyperthermia in the protective action of clomethiazole against MDMA ('ecstasy')-induced neurodegeneration, comparison with the novel NMDA channel blocker AR-R15896AR. Br J Pharmacol 124:479–484

Colado MI, O'Shea E, Granados R, Esteban B, Martín AB, Green AR (1999) Studies on the role of dopamine in the degeneration of 5-HT nerve endings in the brain of Dark Agouti rats following 3,4-methylenedioxymethamphetamine (MDMA or 'ecstasy') administration. Br J Pharmacol 126:911–924

Esteban B, O'Shea E, Martínez I, Green AR, Colado MI (1999) Similar brain concentrations of MDMA have different neurotoxic effects following central or peripheral drug administration. Br J Pharmacol 127:70P

Farfel GM, Seiden LS (1995a) Role of hypothermia in the mechanism of protection against serotonergic toxicity. I. Experiments with 3,4-methylenedioxymethamphetamine, dizocilpine, CGS 19755 and NBQX. J Pharmacol Exp Ther 272: 860–867

Farfel GM, Seiden LS (1995b) Role of hypothermia in the mechanism of protection against serotonergic toxicity. II. Effects with methamphetamine, *p*-chloramphetamine, fenfluramine, dizocilpine and dextromethorphan. J Pharmacol Exp Ther 272:868–875

Gudelsky GA, Nash JF (1996) Carrier-mediated release of serotonin by 3,4-methylenedioxymethamphetamine: implications for serotonin-dopamine interactions. J Neurochem 66:243–249

Hamberger A, Jacobson I, Larsson S, Lönnroth P, Nyström B, Sandberg M (1991) Microdialysis techniques for studying brain amino acids in the extracellular fluid: basic and clinical studies. In: Robinson TE, Justice JB Jr (eds) Microdialysis in the neurosciences. Elsevier, Amsterdam, pp 407–423

Hewitt KE, Green AR (1994) Chlormethiazole, dizocilpine and haloperidol prevent the degeneration of serotonergic nerve terminals induced by administration of MDMA ('ecstasy') to rats. Neuropharmacology 33:1589–1595

Hiramatsu M, Kumagai Y, Unger SE, Cho AK (1990) Metabolism of methylenedioxymethamphetamine: formation of dihydroxymethamphetamine and a quinone identified as its glutathione adduct. J Pharmacol Exp Ther 254:521–527

Jerina DM, Daly JW (1974) Arene oxides: a new aspect of drug metabolism. Science 185:573–582

König JFR, Klippel RA (1963) The rat brain. A stereotaxic atlas of the forebrain and lower parts of the brain stem. Krieger, New York

Lew R, Sabol KE, Chou C, Vosmer GL, Richards J, Seiden LS (1996) Methylenedioxymethamphetamine-induced serotonin deficits are followed by partial recovery over a 52-week period. Part II: radioligand binding and autoradiographic studies. J Pharmacol Exp Ther 276:855–865

Lim HK, Foltz RL (1991) Ion trap tandem mass spectrometric evidence for the metabolism of 3,4-(methylenedioxy)methamphetamine to the potent neurotoxins 2,4,5-trihydroxymethamphetamine and 2,4,5-trihydroxyamphetamine. Chem Res Toxicol 4:626–632

Lin LH, Kumagai Y, Cho AK (1992) Enzymatic and chemical demethylenation of (methylenedioxy)amphetamine and (methylenedioxy)methamphetamine by rat brain microsomes. Chem Res Toxicol 5:401–406

Malberg JE, Sabol KE, Seiden LS (1996) Co-administration of MDMA with drugs that protect against MDMA neurotoxicity produces different effects on body temperature in the rat. J Pharmacol Exp Ther 278:258–267

Miller KJ, Anderholm DC, Ames MM (1986) Metabolic activation of the serotonergic neurotoxin para-chloroamphetamine to chemically reactive intermediates by hepatic and brain microsomal preparations. Biochem Pharmacol 35:1737–1742

Molliver ME, O'Hearn E, Battaglia G, De Souza ER (1986) Direct intracerebral administration of MDMA and MDA does not produce serotonin neurotoxicity. Soc Neurosci Abstr 12:1234

Molliver ME, Berger UV, Mamounas LA, Molliver DC, O'Hearn E, Wilson MA (1990) Neurotoxicity of MDMA and related compounds: anatomic studies. Ann NY Acad Sci 600:649–661

Murray TK, Williams JL, Misra A, Colado MI Green AR (1996) The spin trap reagent PBN attenuates degeneration of 5-HT neurones in rat brain induced by *p*-chloroamphetamine but not fenfluramine. Neuropharmacology 35:1615–1620

O'Hearn E, Battaglia G, De Souza EB, Kuhar MJ, Molliver ME (1988) Methylenedioxyamphetamine (MDA) and methylenedioxymethamphetamine (MDMA) cause selective ablation of serotonergic axon terminals in forebrain: immunocytochemical evidence for neurotoxicity. J Neurosci 8:2788–2803

O'Shea E, Granados R, Esteban B, Colado MI, Green AR (1998) The relationship between the degree of neurodegeneration of rat brain 5-HT nerve terminals and the dose and frequency of administration of MDMA ('ecstasy'). Neuropharmacology 37:919–926

Paris JM, Cunningham KA (1991) Lack of serotonin neurotoxicity after intraraphe microinjection of (+)-3,4-methylenedioxymethamphetamine (MDMA). Brain Res Bull 28:115–119

Parli CJ, Schmidt B (1975) Metabolism of 4-chloroamphetamine to 3-chloro-4-hydroxyamphetamine in rat: evidence for an in vivo 'NIH shift' of chlorine. Res Commun Chem Pathol Pharmacol 10:601–604

Schmidt CJ (1987) Neurotoxicity of the psychedelic amphetamine, methylenedioxymethamphetamine. J Pharmacol Exp Ther 240: 1–7

Schmidt CJ, Taylor VL (1988) Direct central effects of acute methylenedioxymethamphetamine on serotonergic neurones. Eur J Pharmacol 156:121–131

Shankaran M, Yamamoto BK, Gudelsky GA (1999) Mazindol attenuates the 3,4-methylenedioxyamphetamine-induced formation of hydroxyl radicals and long term depletion of serotonin in the striatum. J Neurochem 72:2516–2522

Sharkey J, McBean DE, Kelly PA (1991) Alterations in hippocampal function following repeated exposure to the amphetamine derivative methylenedioxymethamphetamine ('ecstasy'). Psychopharmacology 105:113–118

Slikker W Jr, Holson RR, Ali SF, Kolta MG, Paule MG, Scallet AC, McMillan DE, Bailey JR, Hong JS, Scalzo FM (1989) Behavioral and neurochemical effects of orally administered MDMA in the rodent and nonhuman primate. Neurotoxicology 10:529–542

Tucker GT, Lennard MS, Ellis SW, Woods HF, Cho AK, Lin LY, Hiratsuka A, Schmitz DA, Chu TY (1994) The demethylenation of methylenedioxymethamphetamine ('ecstasy') by debrisoquine hydroxylase (CYP2D6). Biochem Pharmacol 47:1151–1156

Yeh SY (1999) *N*-tert-butyl-alpha-phenylnitrone protects against 3,4-methylenedioxymethamphetamine-induced depletion of serotonin in rats. Synapse 31:169–177