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ORIGINAL INVESTIGATION

The relationship between core body temperature and 3,4-methylenedioxymethamphetamine metabolism in rats: implications for neurotoxicity

Beatriz Goni-Allo · Brian Ó Mathúna · Mireia Segura · Elena Puerta · Berta Lasheras · Rafael de la Torre · Norberto Aguirre

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Abstract
Rationale A close relationship appears to exist between 3,4-methylenedioxymethamphetamine (MDMA)-induced changes in core body temperature and long-term serotonin (5-HT) loss.
Objective We investigated whether changes in core body temperature affect MDMA metabolism.
Materials and methods Male Wistar rats were treated with MDMA at ambient temperatures of 15, 21.5, or 30°C to prevent or exacerbate MDMA-induced hyperthermia. Plasma concentrations of MDMA and its main metabolites were determined for 6 h. Seven days later, animals were killed and brain indole content was measured.
Results The administration of MDMA at 15°C blocked the hyperthermic response and long-term 5-HT depletion found in rats treated at 21.5°C. At 15°C, plasma concentrations of MDMA were significantly increased, whereas those of three of its main metabolites were reduced when compared to rats treated at 21.5°C. By contrast, hyperthermia and indole deficits were exacerbated in rats treated at 30°C. Noteworthy, plasma concentrations of MDMA metabolites were greatly enhanced in these animals. Instrastriatal perfusion of MDMA (100 μM for 5 h at 21°C) did not potentiate the long-term depletion of 5-HT after systemic MDMA. Furthermore, interfering in MDMA metabolism using the catechol-*O*-methyltransferase inhibitor entacapone potentiated the neurotoxicity of MDMA, indicating that metabolites that are substrates for this enzyme may contribute to neurotoxicity.
Conclusions This is the first report showing a direct relationship between core body temperature and MDMA metabolism. This finding has implications on both the temperature dependence of the mechanism of MDMA neurotoxicity and human use, as hyperthermia is often associated with MDMA use in humans.

Keywords 3 · 4-Methylenedioxymethamphetamine (MDMA · "Ecstasy") · 5-Hydroxytryptamine (5-HT · serotonin) · Hyperthermia · Metabolism · Neurotoxicity

Beatriz Goni-Allo and Brian Ó Mathúna contributed equally to this work.

B. Goni-Allo · E. Puerta · B. Lasheras · N. Aguirre (✉)
Department of Pharmacology, School of Medicine,
University of Navarra,
c/ Irunlarrea 1,
31008 Pamplona, Spain
e-mail: popi@unav.es

B. Ó Mathúna · M. Segura · R. de la Torre
Pharmacology Research Unit,
Institut Municipal d'Investigació Mèdica (IMIM),
Barcelona, Spain

B. Ó Mathúna · M. Segura · R. de la Torre
Universitat Pompeu Fabra,
Barcelona, Spain

Introduction

The amphetamine derivative 3,4-methylenedioxymethamphetamine (MDMA, "Ecstasy") can produce long-term deficits of neurochemical and histological indices of the serotonergic function in the brain of rats and primates, depending on several factors such as the dose and its frequency, the route of administration, and the ambient temperature. Although it is difficult to relate these findings to the human condition, there is growing consensus that

MDMA is neurotoxic in the human (Green et al. 2003). Despite the efforts made during the last two decades, the mechanisms underlying MDMA toxicity remain unclear. Oxidative stress, excitotoxicity, and mitochondrial dysfunction appear to play a major role in the neurotoxicity produced by different amphetamine derivatives (Goni-Allo et al. 2006; Quinton and Yamamoto 2006). The focus of this investigation was on the involvement of hyperthermia and MDMA metabolic disposition in the aforementioned mechanisms of neurotoxicity. The close relationship between hyperthermia and neurotoxicity engendered by MDMA is well established (Broening et al. 1995; Malberg and Seiden 1998; Yuan et al. 2002; Johnson et al. 2004). Small changes in ambient temperature cause large changes in both core body temperature and MDMA-induced 5-HT neurotoxicity in the rat (Schmidt et al. 1990; Malberg and Seiden 1998). Additionally, a common feature of many drugs known to prevent MDMA toxicity relies on their ability to block the acute hyperthermia induced by MDMA, with such protection disappearing if the temperature of rats is kept elevated (Che et al. 1995; Farfel and Seiden 1995; Malberg et al. 1996; Taraska and Finnegan 1997; Colado et al. 1998; Colado et al. 1999; Hervias et al. 2000; O'Shea et al. 2002; Morley et al. 2004). Other nonpharmacological manipulations capable of preventing the acute hyperthermic response caused by MDMA also provide substantial protection against the neurotoxic effects of the drug (Sprague et al. 2003). However, some compounds have been shown to protect against MDMA-induced serotonergic changes without affecting hyperthermia, including fluoxetine, NOS inhibitors, and antioxidants (Sanchez et al. 2004; Zheng and Laverty 1998; Yeh 1999). This evidence suggests that the increase in temperature caused by MDMA is not a primary contributing factor to the serotonergic changes seen in the central nervous system.

There is a substantial body of evidence indicating that increased free radical formation is responsible for MDMA-induced neurotoxicity (e.g., Aguirre et al. 1999; Shankaran et al. 2001). It has been postulated that MDMA metabolic activation through the formation of thioether adducts capable of generating reactive oxygen species (ROS) contributes to neurotoxicity. Furthermore, direct injection of MDMA into the brain fails to reproduce the serotonergic neurotoxicity seen after systemic administration (Esteban et al. 2001). In rats, MDMA is cleared mainly by hepatic metabolism by *N*-demethylation to form methylenedioxyamphetamine (MDA). MDMA and MDA are further *O*-demethylenated to 3,4-dihydroxymethamphetamine (HHMA; *N*-methyl-α-methyldopamine; *N*-Me-α-MeDA) and 3,4-dihydroxyamphetamine (HHA; α-methyldopamine; α-MeDA), respectively. HHMA and HHA are highly redox-unstable catechols that conjugate with sulfate and glucuronic acid or can oxidize to their corresponding orthoquinones, forming adducts with glutathione (GSH) and other thiol-containing compounds (Hiramatsu et al. 1990; de la Torre and Farre 2004; de la Torre et al. 2004). It has been postulated that these thioether adducts cross the blood–brain barrier using GSH-specific transporters (Bai et al. 2001), and once inside the brain, they generate ROS in a 5-HT transporter-dependent manner (Jones et al. 2004; Monks et al. 2004). Interestingly, intracerebroventricular administration of GSH and *N*-acetylcysteine conjugates of MDMA metabolites resembles not only the acute neurobehavioral effects of this drug but also its neurotoxic pattern (Bai et al. 1999). Using in vivo microdialysis, it has been recently shown that GSH and *N*-acetylcysteine conjugates of 3,4-dihydroxymethamphetamine (Fig. 1) are present in the striatum of rats administered with MDMA systemically (Jones et al. 2005). This finding further strengthens the hypothesis that MDMA metabolic disposition contributes significantly to the induction of neurotoxicity.

According to these premises, in this study, we attempted to intervene in the hyperthermic response to MDMA and to evaluate changes in drug metabolism. MDMA-induced hyperthermia was prevented or exacerbated by altering ambient temperature. Furthermore, we investigated whether interfering with MDMA metabolism by means of inhibiting catechol-*o*-methyltransferase (COMT) activity (Fig. 1) would have the same effect as raising the ambient temperature in terms of long-term 5-HT depletions.

Materials and methods

Drugs and chemicals

MDMA–HCl was either purchased from Sigma (UK) or was provided by the "Servicio de Restricción de Estupefacientes" (Spanish Regulatory Body on Psychotropic Drugs); 5-HT creatinine sulfate, 5-HIAA, dopamine, dihydroxyphenylacetic acid and homovanillic acid were from Sigma (UK). MDMA, HHMA, 3-methoxy-4-hydroxymethamphetamine (HMMA), MDA, HHA, 3-methoxy-4-hydroxyamphetamine (HMA), and the internal standards (MDMA-d_5, MDA-d_5, and pholedrine-*p*-hydroxymethamphetamine) were purchased from Lipomed (Arlesheim, Switzerland). High-performance liquid chromatography (HPLC)-grade methanol, acetic acid (glacial), sodium acetate, ammonia solution, ethyl acetate, potassium hydrogen phosphate, and potassium dihydrogen phosphate were obtained from Merck (Darmstadt, Germany). Ultrapure water was obtained using a Milli-Q purification system (Milli-pore, Molsheim, France). β-glucuronidase from *Helix Pomatia* (HP-2) (145,000 and 370 U per ml for β-glucuronidase and sulfatase activities, respectively) was supplied by Sigma Chemicals (St. Louis, MO, USA).

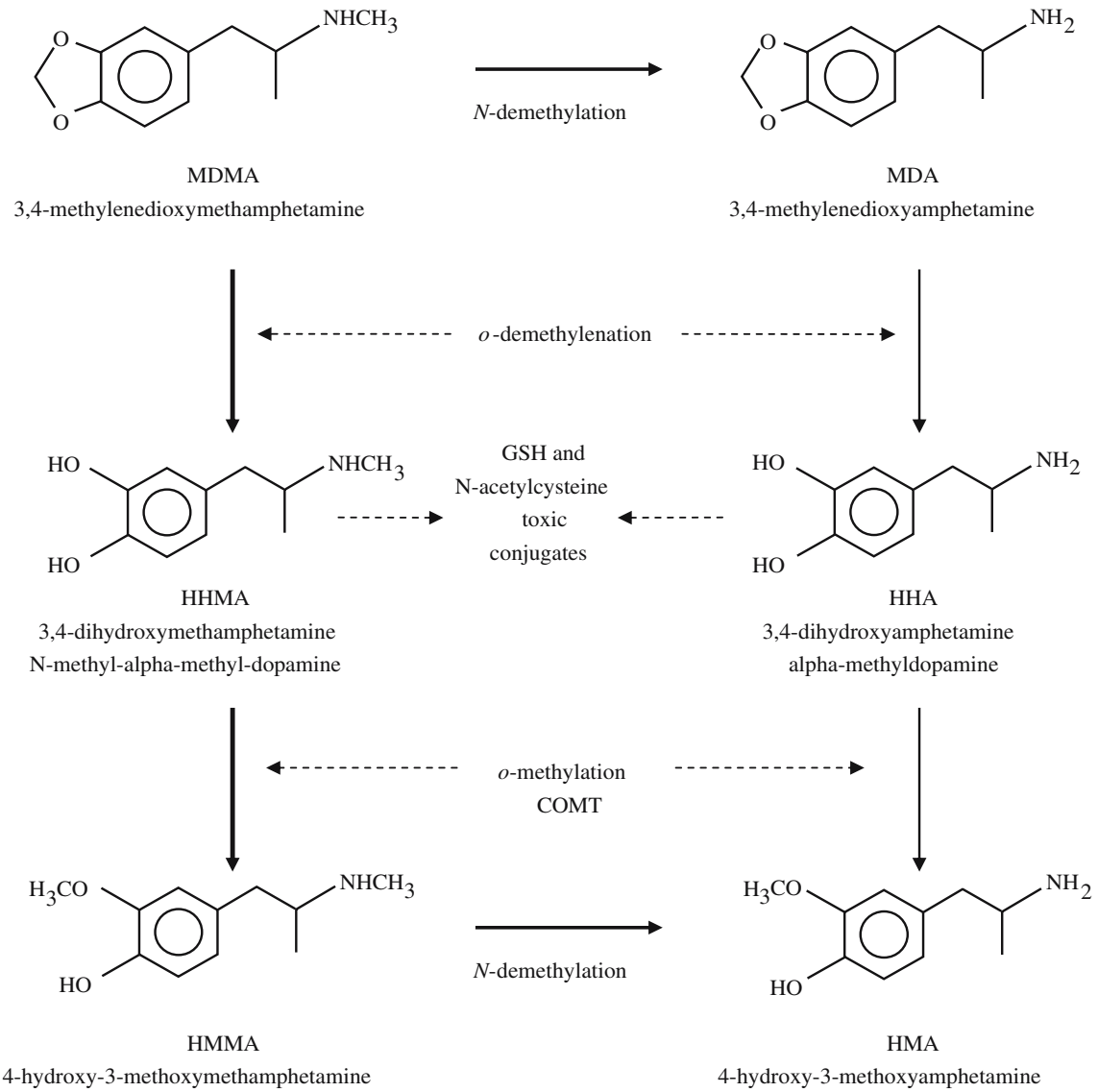

Fig. 1 Postulated pathways of MDMA metabolism (adapted from de la Torre and Farre 2004). Only structures considered in this paper are shown

N-Methyl-bis-trifluoracetamide (MBTFA) was purchased from Macherey-Nagel (Düren, Germany). Bond Elut Certify solid-phase extraction columns were purchased from Varian (Harbor City, CA, USA) and mounted on a Vac-Elut vacuum manifold (Supelco, Bellefonte, PA, USA).

Animals and MDMA treatments

Male Wistar rats (290–340 g) were housed in plastic cages in a temperature-controlled room (21.5±1° C) with free access to food and water, and maintained on a 12/12 h light/dark cycle (lights on at 0700 hours). Rats were treated intraperitoneally with either saline or MDMA (final dose equivalent to 15 mg/kg) with two different dosing regimens: 1×15 or 3×5 mg/kg every 2 h. Three sets of experiments were performed. Firstly, to ascertain the influence of different ambient temperatures on MDMA metabolism and long-term neurotoxicity, MDMA was administered at ambient temperatures of 15, 21.5, or 30± 1°C. Trunk blood was withdrawn by saphenous vein puncture to measure plasma concentrations of MDMA, and its main metabolites at different time points. Rats were sacrificed at 1 week and brain indole content was measured. In the second set, MDMA (100 μM) was reverse dialyzed into the striatum for 5 h (details are given below). The concentration of MDMA in the perfusate was chosen based upon a previous study showing that the extracellular concentration of MDMA under these experimental conditions is similar to that found after systemic neurotoxic doses of MDMA (Esteban et al. 2001). Finally, saline or the COMT inhibitor entacapone (30 mg/kg i.p.) was administered 30 min before MDMA (3×5 mg/kg i.p.) to prevent

the *O*-methylation of HHMA or HHA (Fig. 1). Rats were killed 1 h after MDMA administration for the measurement of MDMA, its metabolites, including HHMA and HHA, and serum concentrations of L-tyrosine. In parallel, a different batch of rats was sacrificed 1 week after MDMA to measure brain indole content. The dose of entacapone was chosen based on previous findings, indicating that rat liver COMT activity is completely inhibited 30 min after injection and remains reasonably inhibited (~75%) 6 h later (Learmonth et al. 2002). In all cases, the doses of MDMA used refer to the hydrochloride salt. All the procedures followed in the present work were in compliance with the European Community Council Directive (86/609/EEC) and were approved by the Ethical Committee of the University of Navarra.

Surgical procedures

Rats were anesthetized with a combination of ketamine (70 mg/kg i.p.) and xylazine (7 mg/kg i.p.), and placed in a Kopf stereotaxic frame, with the incisor bar set at 3.3 mm below the interaural line. The skull was exposed, and holes were drilled to allow implantation of a guide cannula (CMA/11, Sweden) into the right striatum (0.2 mm anterior, 3.0 mm lateral from bregma) or the right ventral hippocampus (5.6 mm posterior, 5.0 mm lateral from bregma; Paxinos and Watson 1997). The guide cannulae were secured to the skull with two bone screws, cyanoacrylate glue, and dental acrylic cement. After surgery, the animals were housed individually with free access to food and water. The rats were allowed to recover from surgery, and the reverse dialysis experiments were carried out 4–5 days later.

Reverse dialysis studies

The effect of direct application of MDMA into the striatum or entacapone into the hippocampus in combination with a toxic dosage regimen of MDMA (3×5 mg/kg i.p., given 2 h apart) was assessed. For this, 12 h before dialysis experiments, the obturators were removed from the guide cannulae, and a microdialysis probe (CMA/11) was inserted slowly through each cannula into the brain of the awakened rat such that the membrane protruded its full length (4 mm) from the end of the probe. The probes were connected via spring-covered fluorinated ethylene propylene tubing (CMA, Sweden) to a dual channel swivel (Instech, Plymouth Meeting, PA, USA) that allowed for relatively unrestrained movement of the animal. Probes were perfused overnight with artificial cerebro spinal fluid (aCSF; NaCl, 120 mM; KCl, 1.4 mM; $CaCl_2$, 1.2 mM; $MgCl_2$, 0.83 mM and D-glucose 10 mM, pH 7.4) at a flow rate of 1 μl/min. In the following morning, fresh aCSF was perfused at a flow rate of 2 μl/min for an additional 1.5 h equilibration period. The perfusion medium of the probe of each animal was then switched to one containing MDMA (100 μM) or entacapone (100 μM). At this time, rats also received the first of three injections of saline or MDMA (5 mg/kg i.p.) given every 2 h. The perfusion continued for an additional 5 h in the case of MDMA or 2 h in the case of entacapone. The concentration of MDMA in the perfusate was chosen based upon previous studies showing that the extracellular concentration of MDMA under these experimental conditions is similar to that found after systemic neurotoxic doses of MDMA (Esteban et al. 2001; Nixdorf et al. 2001; Breier et al. 2006). On the other hand, the concentration of entacapone in the perfusate was chosen based upon a previous study showing an effective inhibition of brain COMT activity (Forsberg et al. 2005).

Seven days after reverse dialysis experiments were completed, animals were killed by decapitation; their brains were rapidly removed, and one 1-mm thick tissue section was taken (approximately 0.5 mm to either side of the probe). Striatal tissue was dissected out from the side where the probe was implanted, and 5-HT, dopamine, and their respective metabolites were quantified using HPLC coupled with an electrochemical detector (ED; see below). In all cases, samples were frozen in dry ice and stored at −80°C until analysis.

Temperature measurements

Rectal temperature of the rats was measured at ambient temperatures of 15, 21.5, or 30±1°C with a lubricated digital thermometer probe (pb 0331, Panlab, Barcelona) inserted 3 cm into the rectum, the rat being lightly restrained by hand. Rats were exposed to low (15°C) or high (30°C) ambient temperatures from 60 min before the first MDMA administration up to 1 h after the last rectal temperature measurement. At this time, rats were put back in the colony room set at ambient temperature of 21.5±1°C. Temperature was recorded before any MDMA treatment and thereafter every 60 min up to 6 or 8 h. Probes were reinserted from time to time until the temperature stabilized.

Blood sampling

Rats were separated in two different groups. In one group, blood was withdrawn at 1, 3, and 5 h, whereas in the other group of animals, blood was withdrawn at 2, 4, and 6 h after MDMA. Saphenous vein puncture for blood sampling was used as previously described by Hem et al. (1998). Blood drops were collected into Microvette® CB 300LH tubes and centrifuged immediately. After centrifugation, plasma was transferred to sterile Eppendorf tubes contain-

ing metabisulfite (2 μl, 0.001% *w*/*v*). Samples were frozen and stored at −20°C until analysis.

Plasma concentrations of MDMA and its main metabolites

MDMA, MDA, HMMA, and HMA concentrations in plasma were determined after a previously described method (Pizarro et al. 2002). Briefly, aliquots of 100 μl of plasma were hydrolyzed enzymatically with β-glucuronidase at pH 5.2 (incubation for 16 h at 37°C). After hydrolysis, samples were adjusted to pH 6, and a solid-phase extraction with Bond Elut Certify® columns was carried out. The extracts were evaporated to dryness (40°C), and the dried extracts were derivatized with 50 μl of MBTFA for 45 min at 70°C. Once cooled, the samples were transferred to autosampler vials and analyzed using gas chromatography-mass spectrometry (GC–MS, MSD5973, Agilent, Palo Alto, CA, USA).

The presence of HHMA and HHA in plasma was determined by using a previously published method (Segura et al. 2001). An acidic hydrolysis was performed before a solid–liquid extraction using strong cation exchange (SCX) columns followed by HPLC with electrochemical detection.

Measurement of serum L-tyrosine concentrations

Serum L-tyrosine concentrations were measured by HPLC–ED as previously described by Bongiovanni et al. (2003). Data for L-tyrosine is presented in nanomoles per milliliter.

Biochemical measurements

Concentrations of 5-HT and 5-HIAA in the brain regions of the rats were determined by HPLC–ED as previously described (Goni-Allo et al. 2006).

Data analysis

Time course of temperature changes was analyzed by two-way analysis of variance (ANOVA) for repeated measures. Treatment was used as the between-subjects factor and time as the repeated measure. Temperature measures were also converted to a composite measure (temperature area under the curve, TAUC) as previously described by Miller and O'Callaghan (2003). The TAUC was calculated for each rat by the application of Simpson's rule to temperatures measured at times—30 min and every hour up 6 or 8 h after MDMA administration. This composite measure represents the area under the curve of a plot of temperature (°C) versus time (h) and has units of °C xh. Differences in TAUC and biochemical concentrations were analyzed either by unpaired Student's *t* test or one-way ANOVA. Multiple pairwise comparisons were performed using the Tukey's test. Treatment differences were considered statistically significant at $p<0.05$. Data analyses were performed using the Statistical Program for the Social Sciences (for Windows, 11.0)

Results

Effect of ambient temperature on the metabolism of a single high dose of MDMA

MDMA in rats is mainly *N*-demethylated to MDA (Fig. 1). Similar area under the curve (AUC) derived from plasma concentrations are observed for both compounds when MDMA is administered in a single dose (1×15 mg/kg) at 21°C. Both MDMA and MDA are *O*-demethylenated, giving rise after a further *O*-methylation to HMMA and HMA, respectively (the intermediate catechol metabolites were not measured because of insufficient biological sample). HMMA AUC is about one third of that of MDMA, whereas HMA AUC is about 15 times lower than MDA AUC when MDMA is administered in a single dose (1×15 mg/kg i.p.) at 21°C (Fig. 2).

As shown in Fig. 2, reducing ambient temperature from 21 to 15°C affects the principal metabolic pathways of MDMA. The rate of *O*-demethylenation of MDMA to HMMA and to HMA from MDA is significantly reduced at 15°C when compared to 21.5°C (HMMA, $t_{26}=3.584$, $p<0.001$; HMA, $t_{26}=5.929$, $p<0.001$). The *N*-demethylation pathway giving rise to MDA from MDMA was also affected by ambient temperature in a similar fashion ($t_{26}=4.197$, $p<0.001$). In contrast, statistical analysis of AUC derived from plasma MDMA concentrations revealed a significant increase at 15°C when compared to 21.5°C ($t_{26}=2.291$, $p<0.05$).

Lowering ambient temperature from 21.5 to 15°C protected against the neurotoxicity induced by a single high dose of MDMA (15 mg/kg i.p.) in different brain regions of the rat except for the hippocampus where the percentage decrease in 5-HT and its metabolite was only 30% compared to 65% at 21°C (Fig. 3a). Similarly, the hyperthermic effects of MDMA were absent in those rats treated at 15°C (Fig. 3b, c). It was not possible to obtain such data for rats treated at a high ambient temperature (30°C), as most of them died before the experiment terminated (Table 1).

Effect of ambient temperature on the metabolism of an MDMA "binge dosing" regimen

To avoid high mortality rates after single doses, we tried a different approach by administering a "binge-dosing" regimen of MDMA. When comparing dose regimens (1× 15 vs 3×5 mg/kg i.p.), lower recoveries (65%) of MDA or

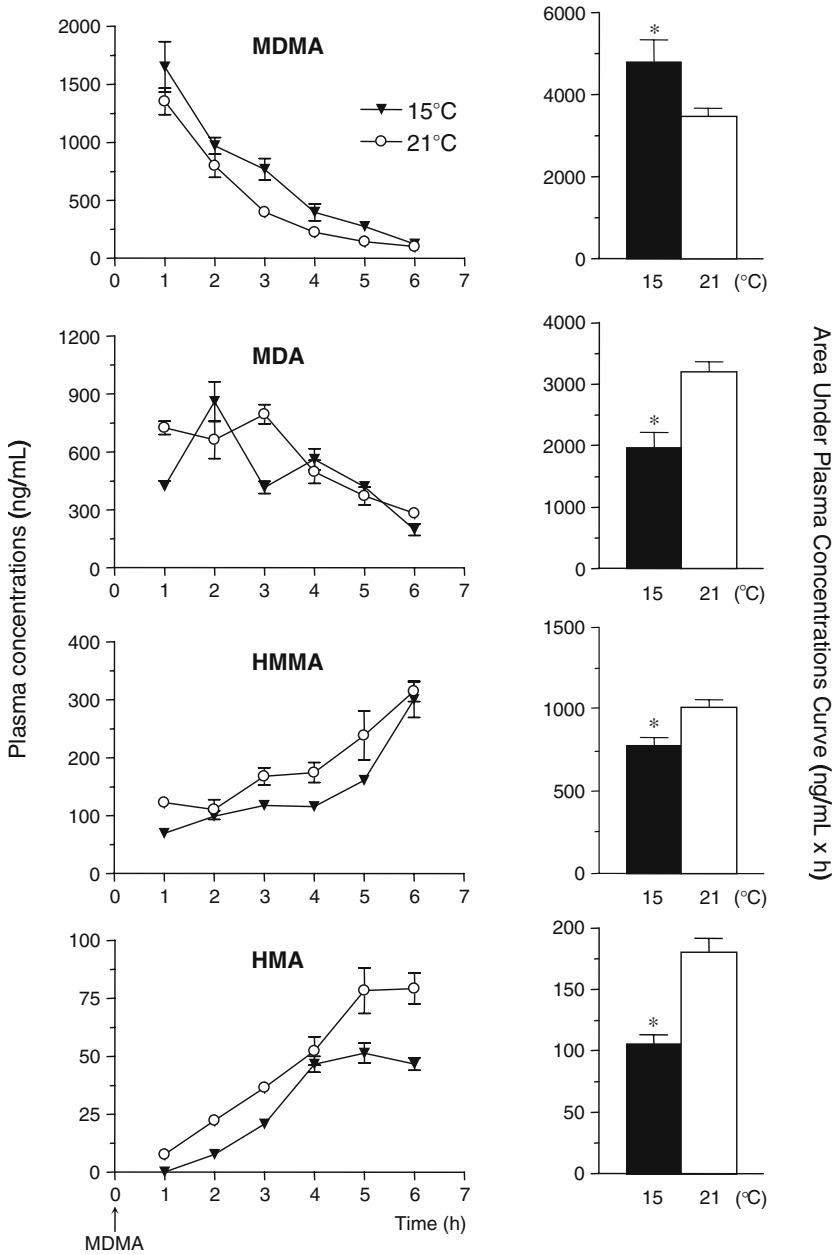

Fig. 2 Effect of ambient temperature on MDMA metabolic disposition. A single high dose of MDMA (15 mg/kg i.p.) was administered at ambient temperature (21.5±1°C) or at reduced ambient temperature (15±1°C). Plasma concentrations of MDMA and its main metabolites (MDA, HMMA, and HMA) were determined every hour from 1 to 6 h after MDMA administration. AUC were calculated by the trapezoidal rule. Data represent the mean ± SEM (n=13–15). AUC were analyzed using unpaired Student's t test *p<0.05 vs ambient temperature of 21.5±1°C

HMA are observed with the fractionated dose at a standard ambient temperature (21.5°C), whereas those of HMMA are unchanged (Fig. 4). Despite these differences, the overall influence of a low ambient temperature on MDMA metabolism was similar to that described earlier for a single high dose of MDMA. On the contrary, plasma concentrations of MDA, HMMA, or HMA were significantly higher in those rats administered at 30°C. Analysis of AUC revealed the following results: MDA increased 1.4-fold ($F_{2,44}$=21.595, p<0.001), HMMA increased 2.75-fold ($F_{2,44}$=101.141, p<0.001), and HMA concentrations were 2.4-fold higher ($F_{2,44}$=96.914, p<0.001). Although reducing ambient temperature produced a significant increase in MDMA concentrations ($F_{2,44}$=9.148, p<0.001), no statistical difference was found in rats treated at 21.5 vs 30°C.

As shown in Fig. 5a, lowering ambient temperature to 15°C afforded an almost complete protection against MDMA-induced serotonergic deficits produced at a standard ambient temperature of 21.5°C. Under these experimental conditions, hyperthermia was abolished (Fig. 5b and c). In

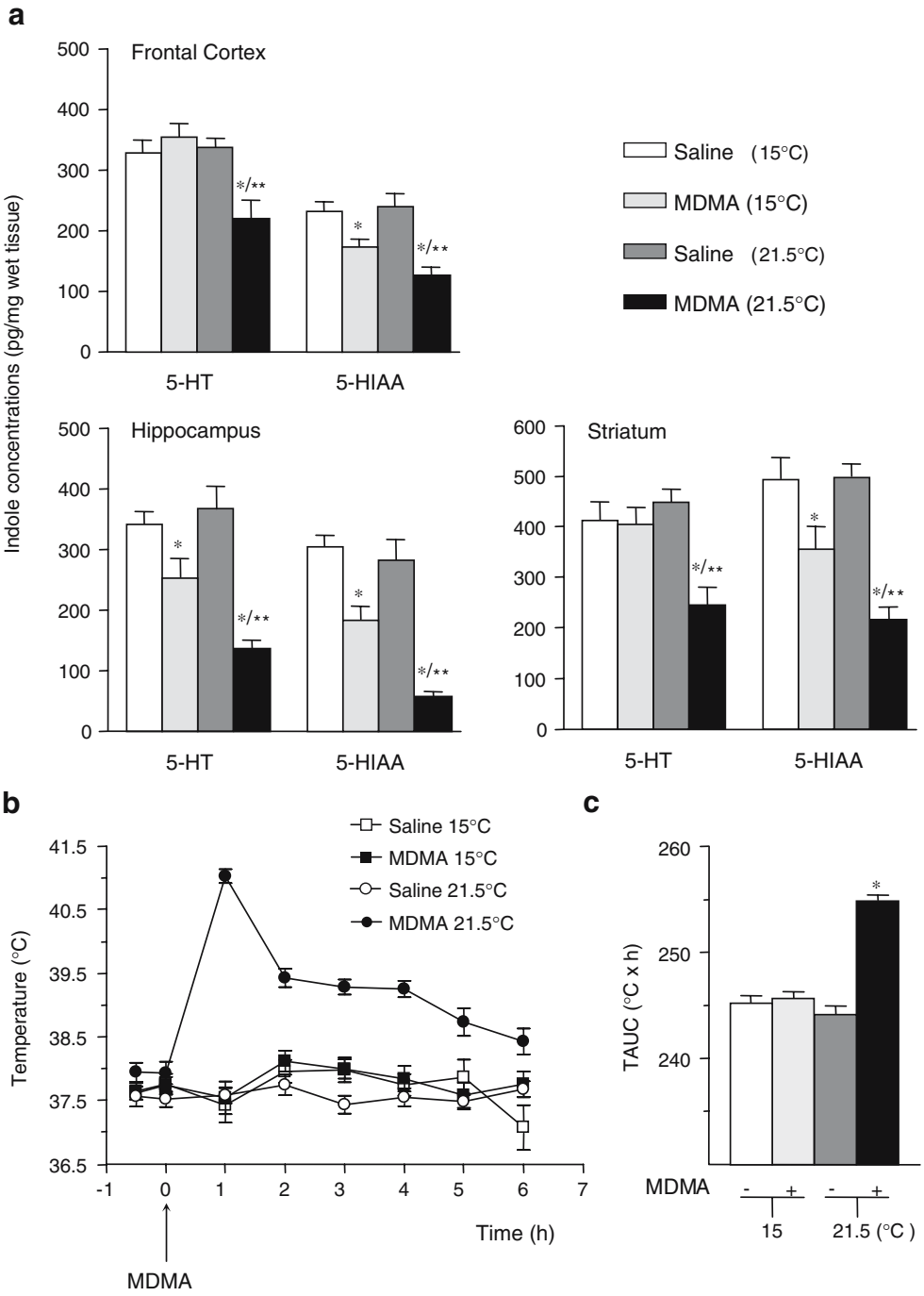

Fig. 3 Effect of ambient temperature on brain 5-HT and 5-HIAA content and hyperthermia induced by MDMA (15 mg/kg i.p.). MDMA was given at normal ambient temperature (21.5±1°C) or at reduced ambient temperature (15±1°C). **a** 5-HT and 5-HIAA content in different rat brain regions 7 days after MDMA. Values are means ± SEM in picogram per milligram wet tissue (n=13–15). **b** Rectal temperature of rats after saline given at 15°C (*open square*) or 21.5°C (*open circle*), or MDMA given at 15°C (*filled square*) or 21.5°C (*filled circle*). A two-way ANOVA for repeated measures revealed a significant interaction treatment × time ($F_{21,357}$=12.57, p<0.001). Statistical differences of single-time-point comparisons between groups are not shown for clarity. **c** The temperature measures shown in **b** were converted to a composite measure (TAUC). The TAUC was calculated for each rat by the application of Simpson's rule. Data were analyzed by one-way ANOVA followed by Tukey's test. *p< 0.05 vs saline and **p<0.05 vs MDMA given at 15°C

Table 1 Mortality rates of male Wistar rats after the two MDMA dosage regimens used in the present study given at low (15°C), standard (21.5°C), or high (30°C) ambient temperatures

Temperature (°C)	MDMA 3×5 mg/kg i.p., given every 2 h (%)	MDMA 1×15 mg/kg i.p. (%)
15	0	0
21	5	20
30	40	95–100

contrast, raising ambient temperature exacerbated both the acute hyperthermic effect and long-term loss of 5-HT and 5-HIAA content in different rat brain regions. Ambient temperature did not cause any significant change in 5-HT or 5-HIAA content in saline treated rats. For this reason, data from these animals were pooled together. As shown in Table 1, mortality rate also increased with ambient temperature. Thus, at 15°C, none of the animals died, whereas approximately 5 and 40% of the rats died at 21.5 and 30°C, respectively.

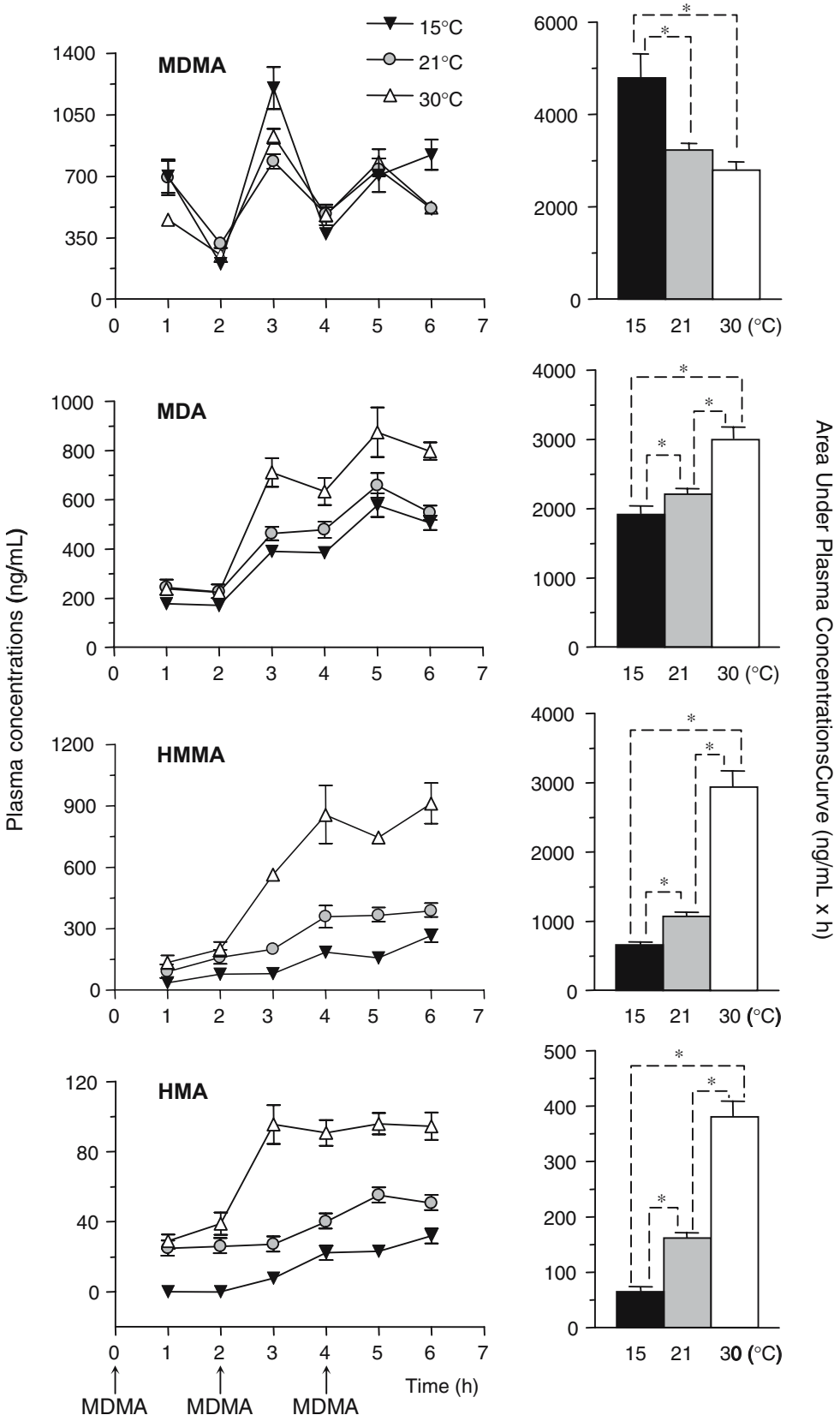

Fig. 4 Effect of ambient temperature on MDMA metabolic disposition. Repeated injections of MDMA (binge dosing; 3× 5 mg/kg i.p., given every 2 h) were administered at ambient temperature (21.5±1°C), at reduced ambient temperature (15± 1°C), or high ambient temperature (30°C). Plasma concentrations of MDMA and its main metabolites (MDA, HMMA, and HMA) were determined every hour from 1 to 6 h after the first injection of MDMA. AUC were calculated by the trapezoidal rule. Data represent the mean ± SEM (n=10–23). AUC were analyzed using one-way ANOVA followed by Tukey's test. *p<0.05

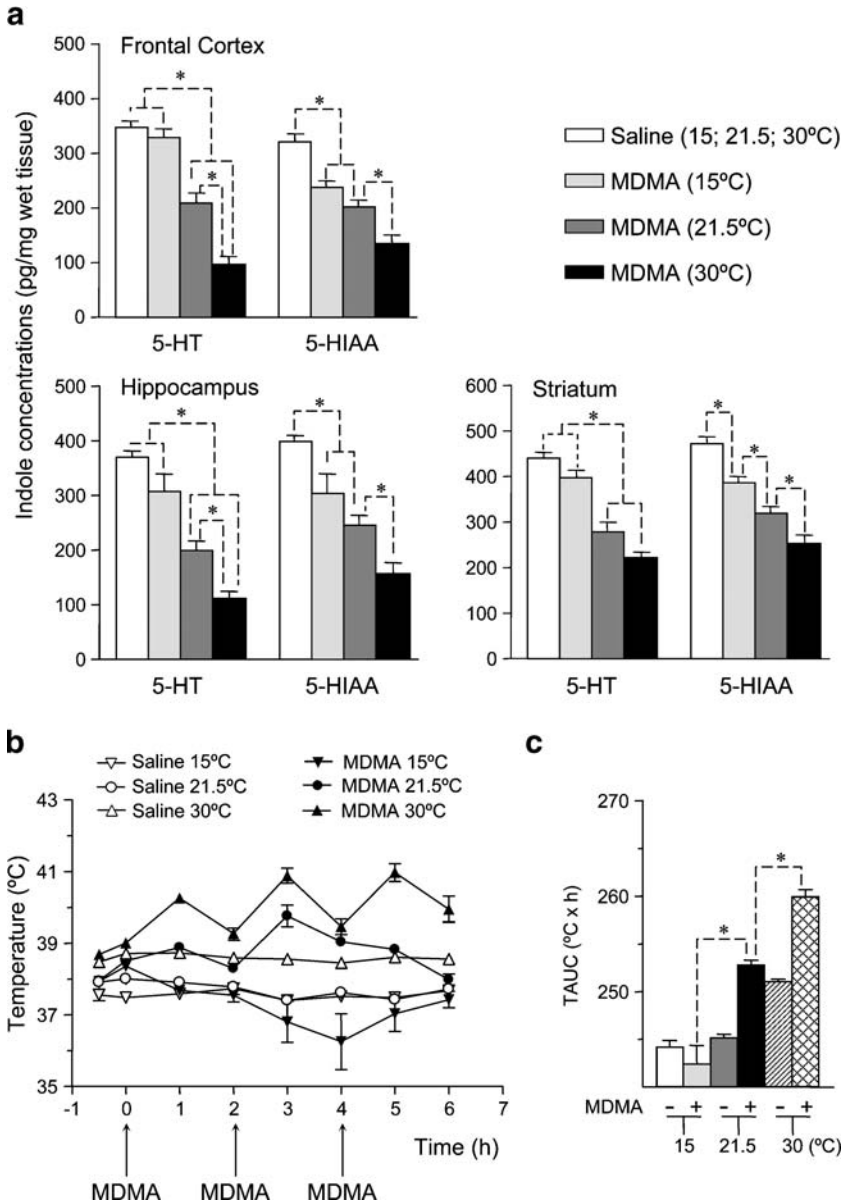

Fig. 5 Effect of ambient temperature on brain 5-HT and 5-HIAA content and hyperthermia induced by MDMA (3×5 mg/kg i.p., given every 2 h). MDMA was given at normal ambient temperature (21.5± 1°C) at reduced ambient temperature (15±1°C) or high ambient temperature (30°C). **a** 5-HT and 5-HIAA content in different rat brain regions 7 days after MDMA. Values are means ± SEM in picogram per milligram wet tissue (n=10–15). **b** Rectal temperature of rats after saline given at 15°C (*open inverted triangle*), 21.5°C (*open circle*), or 30°C (*open triangle*), or MDMA given at 15°C (*filled inverted triangle*), 21.5°C (*filled circle*), or 30°C (*filled triangle*). A two-way ANOVA for repeated measures revealed a significant interaction treatment × time ($F_{35,455}$=5.64, p<0.001). Statistical differences of single-time-point comparisons between groups are not shown for clarity. **c** TAUC of data shown in **b**. Data were analyzed by one-way ANOVA followed by Tukey's test. *p<0.05 vs saline given at 21.5°C

Long-term effect of striatal perfusion of MDMA in combination with systemic administration of MDMA on 5-HT content

No difference was observed in the concentration of 5-HT and 5-HIAA in the striatum between rats perfused during 5 h with MDMA (100 μM) or aCSF 7 days earlier (Fig. 6a). It should be noted that perfusion of MDMA into the striatum did not alter the core body temperature of the rats (Fig. 6b, c). Only when MDMA was given systemically, acute hyperthermia (AUC ANOVA, $F_{3,31}$=78.875, p<0.001) and long-term loss of 5-HT and 5-HIAA content in the striatum were evident. Such effects were independent of buffer composition reverse dialyzed 7 days earlier.

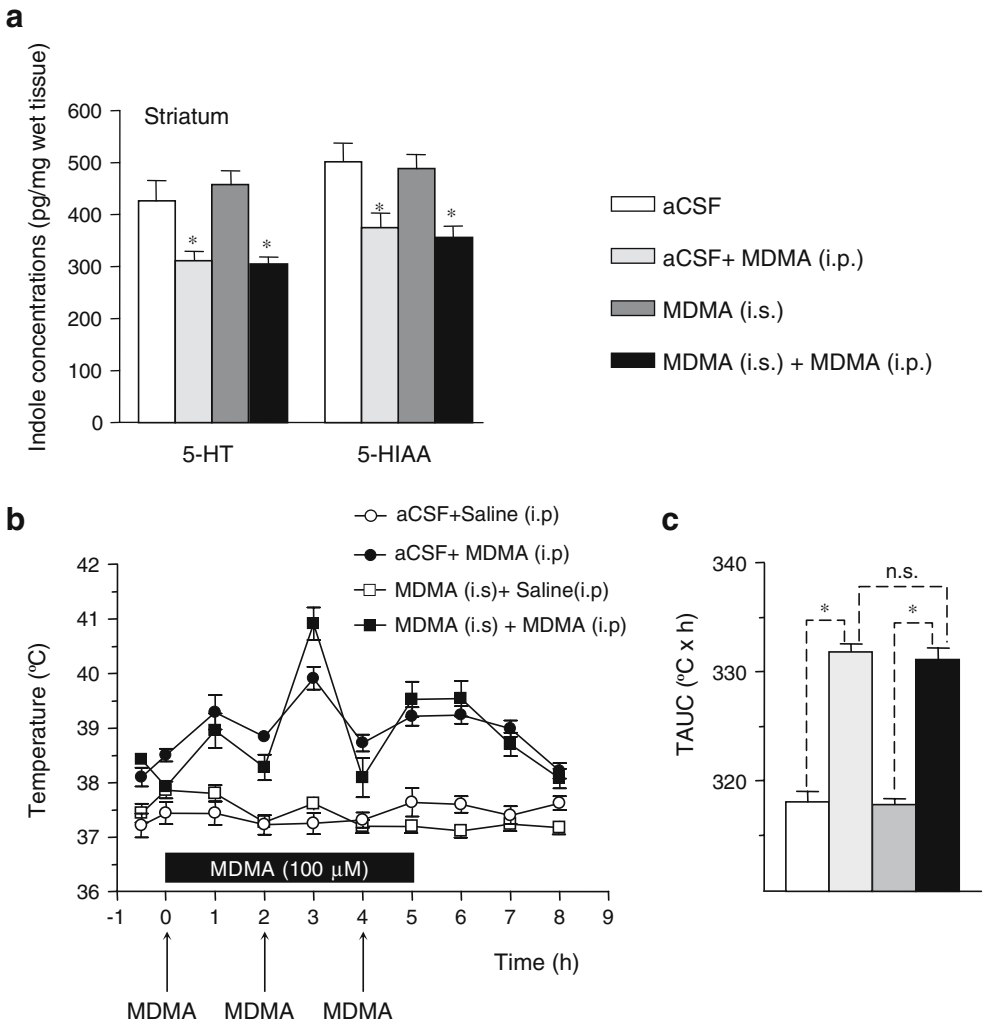

Fig. 6 The effect of reverse dialysis of MDMA (100 μM for 5 h in striatum) alone or in combination with MDMA (3×5 mg/kg i.p.) on striatal indole content and rat core temperature. Concentrations of 5-HT and 5-HIAA (**a**), temperature curves (**b**), and TAUC's (**c**) are shown. All drug treatments were given at ambient temperature (21.5± 1°C). Animals were killed 7 days later. **a** Data represent the means ± SEM of striatal 5-HT and 5-HIAA content in picogram per milligram of wet tissue (n=8 in all groups). **b** Rectal temperature of rats after the administration of aCSF + saline (*open circle*), aCSF + MDMA (i.p.; *filled circle*), MDMA (intrastriatal; i.s.) + saline (*open square*), MDMA (i.s.) + MDMA (i.p.; *filled square*). Temperatures were recorded at baseline right before MDMA and then every 60 min up to 8 h. Two-way ANOVA for repeated measures revealed a significant interaction (treatment × time, $F_{27,252}$=7.635, p<0.001). Statistical differences of single-time-point comparisons between groups are not shown for clarity. The *horizontal bar* represents the time of perfusion while the *arrow* represents the time at which MDMA was injected. **c** TAUC of data shown in **b**. Data were analyzed by one-way ANOVA followed by Tukey's test. *p<0.05 vs aCSF perfused group

Effect of COMT inhibition on MDMA metabolism, hyperthermia, and long-term 5-HT depletions

Inhibition of COMT activity by entacapone (30 mg/kg, i.p.) given 30 min before the first injection of MDMA (3×5 mg/kg i.p., given every 2 h at 21°C) resulted in a significant reduction of plasma concentrations of HMMA (t_{14}=7.286, p<0.001) and HMA (t_{14}=7.904, p<0.001) 1 h after the last dose of MDMA. Plasma concentrations of MDMA, the rest of the metabolites, or serum tyrosine levels were not affected by entacapone (Fig. 7).

As shown in Fig. 8a, entacapone exacerbated the long-term effects of MDMA on rat indole content measured in the frontal cortex and hippocampus 1 week after drug treatment. Rectal temperature analysis using two-way ANOVA for repeated measures over time revealed a significant interaction treatment × time ($F_{27,297}$=16.002, p<0.001). Entacapone caused a deep hypothermia when injected alone, although it did not affect the hyperthermic effect caused by MDMA (Fig. 8b). The statistical analysis of TAUC clearly shows that the effect of entacapone on 5-HT depletions caused by MDMA was independent of any

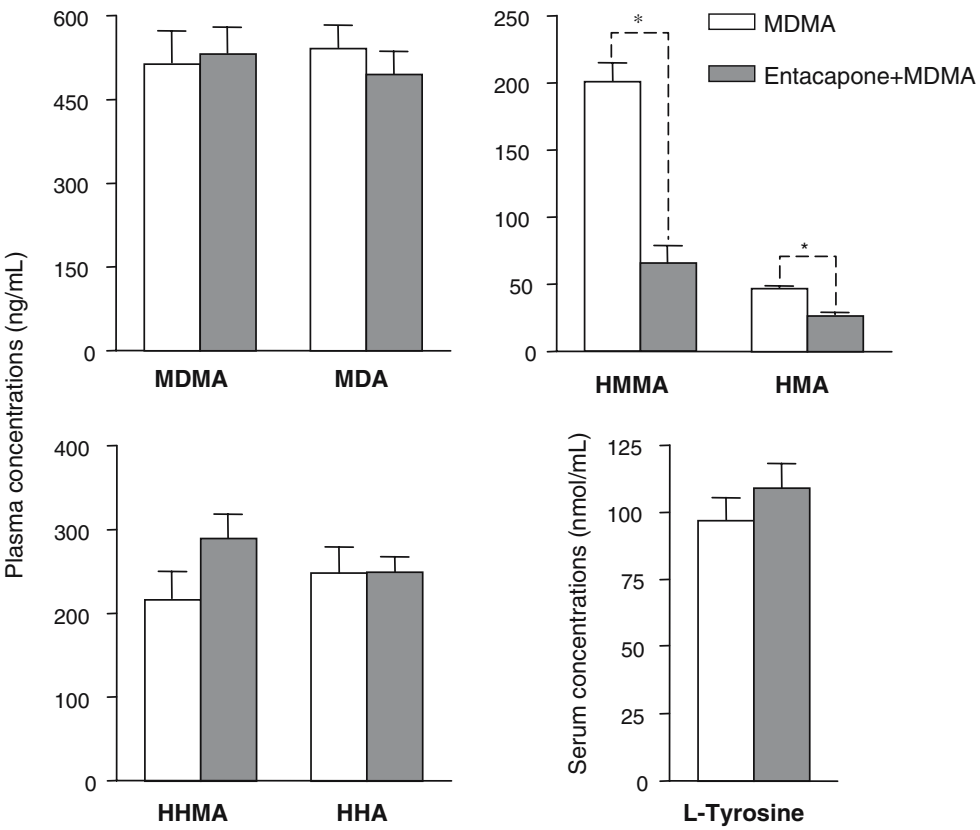

Fig. 7 Effect of entacapone on MDMA metabolic disposition and serum tyrosine levels. Entacapone (30 mg/kg i.p.) was administered 30 min before the first dose of MDMA (3×5 mg/kg i.p. given every 2 h). One hour later, plasma concentrations of MDMA, its main metabolites (MDA, HHMA, HHA, HMMA, and HMA), and serum tyrosine concentrations were determined. Data represent the mean ± SEM (n=8). *p< 0.05 vs MDMA (unpaired Student's t test)

effect of MDMA-induced hyperthermia ($F_{3,33}$=92.03, p< 0.001; Fig. 8c).

By contrast, as shown in Fig. 9, the perfusion of entacapone (100 μM) into the hippocampus for 2 h did not potentiate 5-HT or 5-HIAA depletions caused by MDMA (3×5 mg/kg i.p.).

Discussion

The acute hyperthermia induced by MDMA can strongly influence its long-term neurotoxic effects (Green et al. 2004). The blockade of the acute hyperthermic effect of MDMA is a feature common to many different pharmacological or nonpharmacological manipulations known to protect against MDMA-induced neurotoxicity, and such protection is abolished when the temperature of the animals is kept elevated (Farfel and Seiden 1995; Malberg et al. 1996). Conversely, the degree of long-term damage produced by MDMA appears to be closely related to the magnitude of the hyperthermic response (Malberg and Seiden 1998; Goni-Allo et al. 2007). Furthermore, Capela et al have demonstrated in vitro that neuronal cell death caused by MDMA and its metabolites is potentiated at higher temperatures. Indeed, the production of ROS is increased during hyperthermia (Halliwell 1992). The quinone thioether conjugates of MDMA metabolites by redox cycle interference are postulated to generate the ROS that leads to neurotoxicity (Monks et al. 2004). The hyperthermia caused by MDMA may increase ROS production even further and so lead to the serotonergic changes observed in animals. On the other hand, hyperthermia may not be a defining factor in MDMA-induced neurotoxicity. The administration of serotonin uptake inhibitor, fluoxetine, NOS inhibitors, and antioxidants protect against neurotoxicity without abolishing the hyperthermic response (Sanchez et al. 2004; Zheng and Laverty 1998; Yeh 1999). The mechanism underlying the relationship between MDMA-induced changes in core body temperature and long-term toxicity is as yet unclear. In this study, we report that MDMA metabolism is greatly affected by core body temperature, providing new clues to clarify the relationship between changes in core body temperature and 5-HT toxicity induced by MDMA.

In a first set of experiments, we administered a single high dose of MDMA (15 mg/kg i.p.) at two different ambient temperatures and analyzed the plasma concentrations of MDMA and its metabolites, MDA, HMMA, and HMA, every hour for a period of 6 h. The administration of MDMA at a low ambient temperature (15±1°C) prevented MDMA-induced hyperthermia and resulted in an almost complete protection. The only region where decreases in 5-HT and 5-HIAA were detected was in the hippocampus, and the percentage decrease from the saline control was

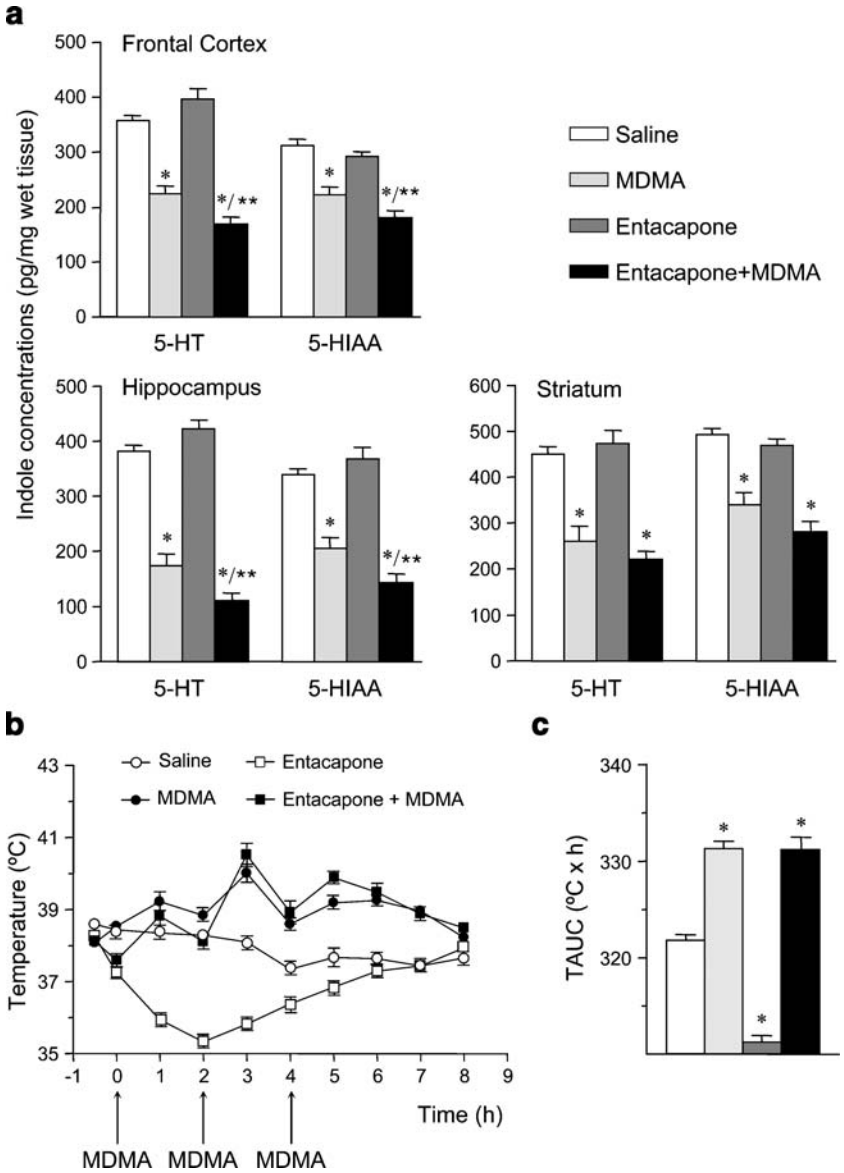

Fig. 8 Effect of entacapone on brain 5-HT and 5-HIAA content and hyperthermia induced by MDMA. Entacapone (30 mg/kg i.p.) was administered 30 min before saline or MDMA (3×5 mg/kg i.p., every 2 h), at normal ambient temperature (21.5±1°C). **a** 5-HT and 5-HIAA content in different rat brain regions 7 days after MDMA. Values are means ± SEM in picogram per milligram wet tissue (n=7–15). **b** Rectal temperature curves of rats treated with saline (*open circle*), MDMA (*filled circle*), entacapone (*open square*), or entacapone + MDMA (*filled square*). **c** TAUC of data shown in **b**. Data were analyzed by one-way ANOVA followed by Tukey's test. *p<0.05 vs saline and **p<0.05 vs MDMA

much less than the change at 21°C. Indeed, this result is not surprising, as the hippocampus appears to be more sensitive to MDMA-induced neurotoxicity than the striatum or the frontal cortex. Although consistently reported (Sprague et al. 2003; O'Shea et al. 2006), the reasons for such different sensitivity are still unknown. Interestingly, we also found that hepatic metabolism of MDMA was reduced. MDMA concentrations were higher than those obtained from rats treated at a standard ambient temperature (21.5± 1°C). Accordingly, plasma concentrations of MDA, HMMA, and HMA in rats treated at 15°C were significantly lower than those found in rats treated at 21.5°C. Because increases in ambient temperature exacerbate MDMA-induced 5-HT neurotoxicity and hyperthermia in the rat (Malberg and Seiden 1998), we also tried to verify whether increasing the ambient temperature up to 30°C would have consequences on the metabolism of MDMA. However, we could not successfully carry out these experiments because, under such experimental conditions, the mortality rate of the animals was above 90%.

One of the main objections often raised to MDMA studies is that the doses of MDMA used to cause 5-HT toxicity in laboratory animals are too high and do not resemble those taken by humans. A practice sometimes

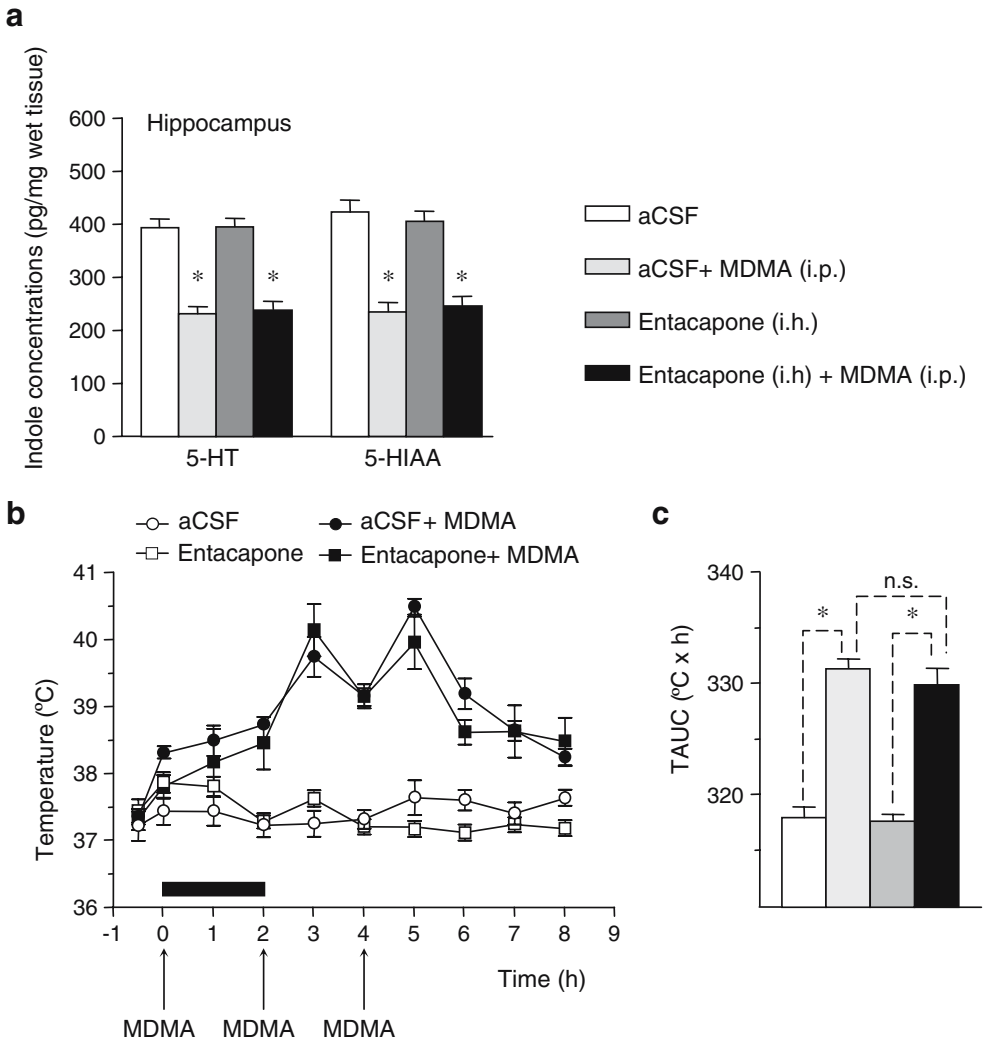

Fig. 9 The effect of reverse dialysis of entacapone (100 μM for 2 h) alone or in combination with MDMA (3×5 mg/kg i.p.) on hippocampal 5-HT and 5-HIAA content and rat core temperature. Concentrations of 5-HT and 5-HIAA (**a**), temperature curves (**b**), and TAUCs (**c**) are shown. All drug treatments were given at ambient temperature (21.5±1°C). Animals were killed 7 days later. **a** Data represent the means ± SEM of hippocampal 5-HT and 5-HIAA content in picogram per milligram of wet tissue (n=8 in all groups). **b** Rectal temperature of rats after the administration of aCSF+saline (*open circle*), aCSF + MDMA (i.p.; *filled circle*), entacapone (intrahippocampal; i.h.) + saline (*open square*), entacapone (i.h.) + MDMA (i.p.; *filled square*). Temperatures were recorded at baseline right before MDMA and then every 60 min up to 8 h. Two-way ANOVA for repeated measures revealed no significant interaction (treatment × time, $F_{27,252}$=0.993, p>0.05). The *horizontal bar* represents the time of perfusion while the *arrow* represents the time at which MDMA was injected. **c** TAUC of data shown in **b**. Data were analyzed by one-way ANOVA followed by Tukey's test. *p<0.05 vs aCSF perfused group

employed by recreational ecstasy users is "binge" dosing, comprising the ingestion of several doses on a single occasion (Weir 2000; Parrott 2004). We used this type of dosing regimen to examine how such a regimen influences the effect of MDMA on acute hyperthermia and long-term 5-HT toxicity. Our results indicate that, for the same dose exposure, "binge" dosing produces an identical acute hyperthermic response and modification of secondary biomarkers of 5-HT neurotoxicity than a similar nondivided dose. An interesting finding was that a single high dose of MDMA (15 mg/kg) resulted in an approximately 20% mortality, whereas the "binge" dosing regimen caused no deaths. Unpublished data from our laboratory and others (Bagdy et al., personal communication) show that the mortality rate after a single high dose of MDMA (15 mg/kg) increases up to 50–60% in male Dark Agouti rats, an effect that is almost completely prevented with a repeated dose regimen of MDMA (3×5 mg/kg i.p., every 2 h).

Hence, we tried to reproduce our first set of experiments by using the "binge-dosing" regimen given at low (15°C), standard (21.5°C), and high (30°C) ambient temperatures. As in the case of a single high dose of MDMA, low ambient temperature prevented MDMA-induced acute hyperthermia and long-term 5-HT toxicity. Although

somehow expected, MDMA metabolism was also reduced at 15°C, as plasma MDMA concentrations were significantly higher, whereas those of MDA, HMMA, and HMA were lower than those found in rats treated at 21.5°C. It is apparent from these results that, despite higher plasma concentrations of the serotonergic neurotoxin MDMA at low ambient temperature, hyperthermia and 5-HT depletions induced by MDMA are reduced. These observations would be in agreement with the hypothesis that a reduction in drug metabolism is correlated with reductions of biomarkers of neurotoxicity. The dose regimen used for an equal total dose exposure does not substantially modify these conclusions. Interestingly, the converse also occurred. The administration of MDMA (3×5 mg/kg) at 30°C potentiated hyperthermia and long-term toxicity, but what is most important is that the concentrations of all three metabolites of MDMA increased drastically over the 6 h after the first MDMA injection. As stated above, we could not measure the concentrations of the catechol-type metabolites of MDMA, HHMA, and HHA because of an insufficient amount of sample. However, because the only metabolic pathway leading to HMMA or HMA formation involves the methylation of HHMA and HHA (de la Torre and Farre 2004), it appears reasonable to suggest that, at some point, the plasma concentrations of HHMA and HHA (the proposed precursors of neurotoxic species) must have been higher in rats treated at 30 vs 21°C.

Furthermore, our data confirm and expand on those reported by others, indicating that direct perfusion of MDMA into the brain does not elicit any sign of 5-HT toxicity (Esteban et al. 2001). However, the absence of tissue depletions after the local infusion of MDMA has been suggested to be caused by the lack of effect on body temperature (Nixdorf et al. 2001). To bypass this inconvenience, intrastriatal perfusion of MDMA (100 μM, 5 h) was combined with MDMA (3×5 mg/kg i.p.) known to increase core body temperature. It should be noted that the concentration of MDMA used in these experiments produces an increase in extracellular DA concentrations similar to that seen after a toxic MDMA dosing regimen (Nash and Yamamoto 1992; Nixdorf et al. 2001). Furthermore, such concentration of MDMA in the perfusate gives rise to the range of extracellular concentrations of MDMA observed after peripheral administration of neurotoxic doses of MDMA (Esteban et al. 2001). Despite these considerations, the extent of 5-HT and 5-HIAA depletions was similar and independent of whatever was perfused 7 days earlier. Again, these data are consistent with the hypothesis that peripheral generation of neurotoxic metabolites contributes to MDMA-induced serotonergic neurotoxicity.

Finally, in an attempt to further investigate the role of the catechol type metabolites of MDMA in the neurotoxic process, we administered the COMT inhibitor entacapone 30 min before the binge-dosing regimen of MDMA. We speculated that inhibiting the *O*-methylation of HHMA and HHA would increase their plasma concentration, and this would, in turn, lead to larger 5-HT depletions. Entacapone exacerbated MDMA-induced long-term 5-HT depletions. The effect of entacapone was not related to an effect on core temperature, as pretreatment with entacapone caused a marked hypothermia when injected alone and did not significantly modify MDMA-induced hyperthermia. Noteworthy, plasma concentrations of HMMA and HMA were significantly lower in the entacapone-treated animals, indicating that the *O*-methylation pathway was successfully inhibited. By contrast, plasma concentrations of HHMA and HHA were unchanged, suggesting that these compounds must have been cleared someway. As stated above, HHMA and HHA are highly unstable catechols that cannot only conjugate with sulfate or glucuronic acid but can also be rapidly oxidized to their corresponding orthoquinones and form adducts with GSH and other thiol-containing compounds (Lim and Foltz 1988; Hiramatsu et al. 1990; Patel et al. 1991). Such compounds have been related to MDMA neurotoxicity (Miller et al. 1997; Bai et al. 1999, 2001; Jones et al. 2004, 2005). Therefore, what at first sight was an unexpected finding may offer an explanation as to why entacapone exacerbated MDMA-induced 5-HT depletions.

In a recent report by Breier et al. (2006), it has been suggested that increased tyrosine concentrations after MDMA and its eventual conversion to DA within 5-HT terminals could play an important role in MDMA neurotoxicity. Entacapone will necessarily interfere with DA turnover, and by reducing its metabolism by this pathway, it could enhance its accumulation or metabolization via other pathways, leading to ROS production that could enhance 5-HT neurotoxicity. According to our results, however, this possibility seems unlikely, as perfusion of entacapone into the hippocampus did not affect 5-HT or 5-HIAA depletions caused by MDMA. Furthermore, entacapone, when given systemically, caused no change in serum tyrosine concentrations. Taken together, these results suggest that entacapone exacerbates MDMA-induced 5-HT depletions by interfering with peripheral MDMA metabolism.

According to our data, COMT activity plays an important role in MDMA metabolism and toxicity in rats. It is known that the level of COMT enzyme activity is genetically polymorphic in human tissues with a trimodal distribution of low ($COMT_{LL}$), intermediate ($COMT_{LH}$), and high ($COMT_{HH}$) activities. This polymorphism, which, according to segregation analysis of family studies, is caused by autosomal codominant alleles, leads to three- to fourfold differences in COMT activity in human erythrocytes and liver (Mannisto and Kaakkola 1999), and could, therefore, be relevant for human users in terms of susceptibility to MDMA neurotoxicity.

In conclusion, our data suggest that peripheral metabolism of MDMA plays a key role in the whole neurotoxic process. Based on the literature and the results presented herein, it is tempting to speculate that the so far unclear relationship between the acute hyperthermic response and long-term damage produced by MDMA may involve the influence of core body temperature in the metabolic rate of this drug. We would also like to point out that these findings may be relevant not only for future animal investigations but also for recreational MDMA users in spite of the limitations of translating animal data to humans (de la Torre and Farre 2004; Easton and Marsden 2006). High doses (and or plasma concentrations) of MDMA are associated to acute toxicity and mortality, as it has also been observed in humans (Greene et al. 2003). The practice of taking low doses of MDMA repeatedly in a short period of time and for the same dose exposure (i.e., a high dose inducing acute toxicity and mortality) might reduce the risk of acute toxicity but does not modify the risk of neurotoxic effects, especially in crowded and hot dance club conditions where MDMA is often ingested.

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