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Studies on the mechanisms underlying amiloride enhancement of 3,4-methylenedioxymethamphetamine-induced serotonin depletion in rats

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Abstract

Amiloride and several of its congeners known to block the Na^+/Ca^{2+} and/or Na^+/H^+ antiporters potentiate methamphetamine-induced neurotoxicity without altering methamphetamine-induced hyperthermia. We now examine whether amiloride also exacerbates 3,4-methylenedioxymethamphetamine (MDMA)-induced long-term serotonin (5-HT) loss in rats. Amiloride (2.5 mg/kg, every 2 h×3, i.p.) given at ambient temperature 30 min before MDMA (5 mg/kg, every 2 h×3, i.p.), markedly exacerbated long-term 5-HT loss. However, in contrast to methamphetamine, amiloride also potentiated MDMA-induced hyperthermia. Fluoxetine (10 mg/kg i.p.) completely protected against 5-HT depletion caused by the MDMA/amiloride combination without significantly altering the hyperthermic response. By contrast, the calcium channel antagonists flunarizine or diltiazem did not afford any protection. Findings with MDMA and amiloride were extended to the highly selective Na^+/H^+ exchange inhibitor dimethylamiloride, suggesting that the potentiating effects of amiloride are probably mediated by the blockade of Na^+/H^+ exchange. When the MDMA/amiloride combination was administered at 15 °C hyperthermia did not develop and brain 5-HT concentrations remained unchanged 7 days later. Intrastriatal perfusion of MDMA (100 μM for 8 h) in combination with systemic amiloride caused a small depletion of striatal 5-HT content in animals made hyperthermic but not in the striatum of normothermic rats. These data suggest that enhancement of MDMA-induced 5-HT loss caused by amiloride or dimethylamiloride depends on their ability to enhance MDMA-induced hyperthermia. We hypothesise that blockade of Na^+/H^+ exchange could synergize with hyperthermia to render 5-HT terminals more vulnerable to the toxic effects of MDMA.
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1. Introduction

3,4-Methylenedioxymethamphetamine (MDMA, "ecstasy") is an amphetamine derivative which has become a very popular drug among young adults despite its potential neurotoxic effects and psychiatric complications reported in recreational MDMA users (see Green et al., 2003 for review). It is well known that single or repeated injections of MDMA cause several changes in neurochemical and histological markers of the serotonergic function in the brain of rodents (Ricaurte et al., 2000), primates (Hatzdimitrou et al., 1999) and, possibly, in humans (Kish et al., 2000; McCann et al., 1998). Such neurotoxicity is evident by the decline in the activity of tryptophan hydroxylase (Stone et al., 1998), a decrease in the content of 5-hydroxytryptamine (5-HT) and the number of [^{3}H]paroxetine-labelled 5-HT transporters in several regions of the brain (Aguirre et al., 1995, 1999; Hervias et al., 2000), the reduction in the density of 5-HTergic terminals (O'Hearn et al., 1988) and impairments of central 5-HT function (Aguirre et al., 1998; Hatzdimitrou et al., 2002). Oxidative stress is a major feature in MDMA toxicity, however, to date, despite the interest shown, the exact mechanisms underlying MDMA neurotoxicity remain unclear.

It has been recently proposed by Callahan et al. (2001), that ionic dysregulation is an important feature of methamphetamine-induced neurotoxicity. This hypothesis was based on two considerations. First, the prolonged activation of the dopamine transporter induced by methamphetamine would

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largely increase intracellular Na^+ concentrations. Second, direct and/or indirect inactivation of the Na^+/K^+ ATPase would gradually compromise the Na^+ gradient, and thereby impair the function of Na^+/Ca^{2+} and/or Na^+/H^+ exchangers leading to an eventual toxic build-up of H^+ and Ca^{2+} within the dopaminergic and/or serotonergic nerve terminals. Consistent with this hypothesis, Callahan et al. (2001) showed that amiloride and several of its congeners, known to block the Na^+/Ca^{2+} and/or Na^+/H^+ ion-exchangers (Kleyman and Cragoe, 1988), exacerbate methamphetamine-induced neurotoxicity without producing any significant effect on methamphetamine-induced hyperthermia.

MDMA, in contrast to methamphetamine, mainly targets the 5-HT transporter (Rudnick and Wall, 1992). The 5-HT transporter, akin to the dopamine transporter, belongs to the Na^+/Cl^- dependent neurotransporter subfamily. Therefore, 5-HT uptake into the presynaptic terminal is an Na^+-driven process (Lesch et al., 1996; Mann and Hrdina, 1992; Rothman and Bauman, 2002). Accordingly, we speculated that a similar chain of biochemical events to those proposed for methamphetamine (see above) could be occurring within the serotonergic terminal after MDMA administration. If this hypothesis is correct, drugs that block the Na^+/Ca^{2+} and/or Na^+/H^+ ion-exchange should also exacerbate MDMA-induced 5-HT loss without altering MDMA-induced hyperthermia. For this purpose we used amiloride as a non-selective Na^+/Ca^{2+} and Na^+/H^+ exchange inhibitor or its analogue, dimethylamiloride, a more selective inhibitor for the Na^+/H^+ exchanger. Amiloride is a potassium-sparing diuretic used in conjunction with other drugs to lower blood pressure, to treat edema or to treat or prevent hypokalemia.

Hyperthermia induced by MDMA can strongly influence its long-term neurotoxic effects (see Green et al., 2004 for review). It has been shown that prevention of MDMA-induced hyperthermia prevents neurotoxicity, and that many drugs that protect against MDMA-induced neurotoxicity lower the core temperature of the animals (Farfel and Seiden, 1995; Malberg et al., 1996). The converse also occurs; 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). Due to the important role of hyperthermia in the mechanisms underlying MDMA-induced neurotoxicity, core temperature measurements were taken after all the treatments used in the present study.

2. Materials and methods

2.1. Drugs and chemicals

MDMA–HCI was a gift from the "Servicio de Restricción de Estupefacientes" (Spanish regulatory body on psychotropic drugs). Amiloride, dimethylamiloride, diltiazem, flunarizine, 5-HT creatinine sulfate, dopamine hydrochloride and their major metabolites, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindole acetic acid (5-HIAA) were from Sigma (Madrid, Spain); Fluoxetine–HCI was generously donated by Eli-Lilly and Co. (Indianapolis, IN, U.S.A.). All other chemicals were from Merck (Darmstadt, Germany).

2.2. Animals

Experiments were carried out in male Wistar rats (Harlan, Barcelona), weighing 290–340 g at the beginning of drug treatment. Animals were housed four per cage in constant conditions of humidity and temperature (21.5±1 °C) with a 12-h/12-h light–dark cycle (lights on at 07.00). Food and water were available *ad libitum*. All the procedures followed in the present work were in compliance with the European Community Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Ethical Committee of the University of Navarra. Animals received drug injections in their home cages (40×55×20 cm) except in those experiments in which MDMA was perfused into the striatum (see below). In this case, animals were placed in the microdialysis tank (36×40 cm) right after recovering from anaesthesia, approximately 12 h before MDMA perfusion.

2.3. Drug treatments

Saline or MDMA (5 mg/kg, referred to the free base) was administered intraperitoneally (i.p.) three times, at 2 h intervals. Amiloride (2.5 mg/kg i.p.) or dimethylamiloride (2.5 mg/kg i.p.) were given 30 min before each dose of MDMA. Doses and times of administration of amiloride and dimethylamiloride were chosen based upon literature reports on their ion-exchange inhibiting effects in intact animals (Callahan et al., 2001 and references therein). In a different set of experiments, flunarizine (20 mg/kg i.p.) or diltiazem (50 mg/kg i.p.) were also administered 30 min before each administration of MDMA alone or in combination with amiloride. The doses of flunarizine and diltiazem were chosen based upon studies showing their capacity to cross the blood brain barrier and inhibit some of the effects caused by methamphetamine or MDMA (Finnegan et al., 1993; Johnson et al., 1992). A single injection of fluoxetine (10 mg/kg i.p.) was given 30 min before the first dose of MDMA given alone or combination with amiloride or dimethylamiloride. This dose of fluoxetine has been repeatedly shown to block MDMA neurotoxicity (e.g. Aguirre et al., 1998). When appropriate, saline (2 ml/kg i.p.) was injected to the rats so that all the animals used in each experiment received the same number of injections. In a different set of experiments, MDMA with or without amiloride was administered in a cold room set at 15 °C in order to block the acute MDMA-induced hyperthermia. In this case animals were allowed to accommodate to the new environment for 2 h before injections. All drugs were dissolved in 0.9% saline. Seven days after drug administration rats were killed by decapitation, their brains being rapidly removed thereafter and placed on ice. The hippocampus, striatum and frontal cortex were dissected free, frozen on dry ice and stored at −80 °C until chromatographic studies were performed. Brain areas were chosen based upon their sensitivity to MDMA-induced 5-HT depleting effects (Green et al., 2003; O'Hearn et al., 1988).

2.4. Reverse dialysis studies

Rats were anaesthetised 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 one hole was drilled to allow implantation of a concentric-style microdialysis probe into the right striatum at coordinates: AP+0.2 mm, ML 3 mm from bregma and 7 mm below the surface of the brain, according to the atlas of Paxinos and Watson (1997). The length of the dialysis membrane was 4 mm (Cuprophan™, Applied Neuroscience; 10,000 MW cutoff, 220 μm outside diameter). After surgery, the animals were placed individually in the microdialysis tank with free access to food and water. Probes were perfused overnight with artificial cerebrospinal fluid (aCSF) (NaCl 0.148 M, KCl 3×10^{-3} M, $CaCl_2{\cdot}2H_2O$ 1.4×10^{-3} M, $MgCl_2{\cdot}6H_2O$ 8×10^{-4} M, $NaHPO_4{\cdot}7H_2O$ 1.2×10^{-3} M and $NaH_2PO_4{\cdot}H_2O$ 1.9×10^{-4}M) at a flow rate of 1 μl/min using a CMA 100 perfusion pump.

Reverse dialysis experiments were carried out the following day. Flow rate was increased to 2 μl/min and after 90 min of stabilisation, rats received the first of 3 injections of amiloride (2.5 mg/kg i.p.) separated by 2 h. Thirty minutes after the first amiloride injection MDMA (100 μM, dissolved in aCSF) or aCSF was perfused for an additional 8 h. The concentration of MDMA in the perfusate was chosen based upon a previous study showing that the extracellular concentration of MDMA is similar to that seen with systemic neurotoxic doses of MDMA (Esteban et al., 2001). Because intrastriatal perfusion of MDMA did not elicit a hyperthermic response, we performed a different set of experiments in which rats perfused with MDMA had their rectal temperature kept elevated to near that seen in rats given MDMA peripherally. This was achieved by covering the base of the microdialysis tank with a homeothermic blanket. Blankets were turned on right after the first amiloride or saline injection and were turned off 4 h later. In these experiments, temperature of animals was measured only at 1, 3 and 5 h time points after beginning MDMA perfusion.

2.5. Biochemical measurements

Concentrations of 5-HT, 5-HIAA, dopamine, DOPAC and HVA in the brain regions of the rats were determined by high performance liquid chromatography with electrochemical detection as previously described (Perez-Otano et al., 1991). Briefly, samples were injected using an automatic sample injector (Waters 717 plus) onto a Spherisorb ODS-2 reverse phase C18 column (5 μm, 150×4.6 mm; Teknokroma, San Cugat del Valles, Spain) connected to a DECADE amperometric detector (Antec Leyden, Zoeterwoude, The Netherlands), with a glassy carbon electrode maintained at 0.7 V with respect to a Ag/AgCl reference electrode. The mobile phase consisted of citric acid 0.1 M, Na_2HPO_4 0.1 M, octanesulphonic acid 0.74 mM, EDTA 1 mM and methanol 16% (pH 3.4), pumped at a flow rate of 1 ml/min.

2.6. Temperature measurements

Rectal temperature of the rats was measured at an ambient temperature of 21.5±1 °C or at low ambient temperature of 15 °C with a lubricated digital thermometer probe (pb 0331, Panlab, Barcelona) inserted approximately 3 cm into the rectum, the rat being lightly restrained by holding in the hand. Temperature was recorded before any drug treatment and thereafter every 60 min up 6 or 8 h. Probes were re-inserted from time to time until the temperature stabilized.

2.7. Data analysis

Temperature curves were analysed by two-way ANOVA for repeated measures. In this case, treatment was used as the between-subjects factor and time as the repeated measure. Single-time-point comparisons between groups were made using one-way ANOVA and Tukey's test. Temperature measures were also converted to a composite measure (temperature area under the curve, TAUC) as previously described (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 8 h following 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×h. For the analysis of the TAUC and the neurochemical data, differences were analysed by one-way ANOVA. Multiple pair wise comparisons were performed using the Tukey's test. Treatment differences for all the data were considered statistically significant at $P<0.05$. Data analyses were performed using the Statistical Program for the Social Sciences (SPSS for Windows, 11.0).

3. Results

3.1. Effect of amiloride on MDMA-induced hyperthermia and long-term 5-HT loss

Amiloride (2.5 mg/kg, i.p., given 30 min before each MDMA injection) exacerbated the long-term effects of MDMA (3×5 mg/kg i.p., given every 2 h) on rat indole content measured in the frontal cortex, hippocampus and striatum 1 week after drug treatment (Fig. 1A). Rectal temperature analysis using two-way ANOVA for repeated measures over time revealed a significant interaction treatment×time [$F(27,441)=10.234$, $P<0.001$]. Single time point comparisons using one-way ANOVA showed significant differences at 3 h [$F(3,52)=79.808$, $P<0.001$], 7 h [$F(3,52)=11.031$, $P<0.001$] and 8 h time points [$F(3,52)=12.797$, $P<0.001$] between MDMA-treated rats versus rats treated with the amiloride/MDMA combination, indicating that amiloride prolonged MDMA-induced hyperthermia (Fig. 1B). Statistical analysis of temperature data expressed as a composite measure of temperature response (TAUC, Fig. 1C) clearly shows that amiloride potentiates MDMA-induced hyperthermia [$F(3,52)=84.206$, $P<0.001$].

3.2. Flunarizine or diltiazem do not block the potentiating effects of amiloride on MDMA-induced hyperthermia or long-term 5-HT loss

In an attempt to ascertain the involvement of Ca^{2+} accumulation in the exacerbating effect of amiloride, we carried out one experiment in which the non-selective voltage-dependent Ca^{2+} and Na^{+} channel blockers flunarizine or

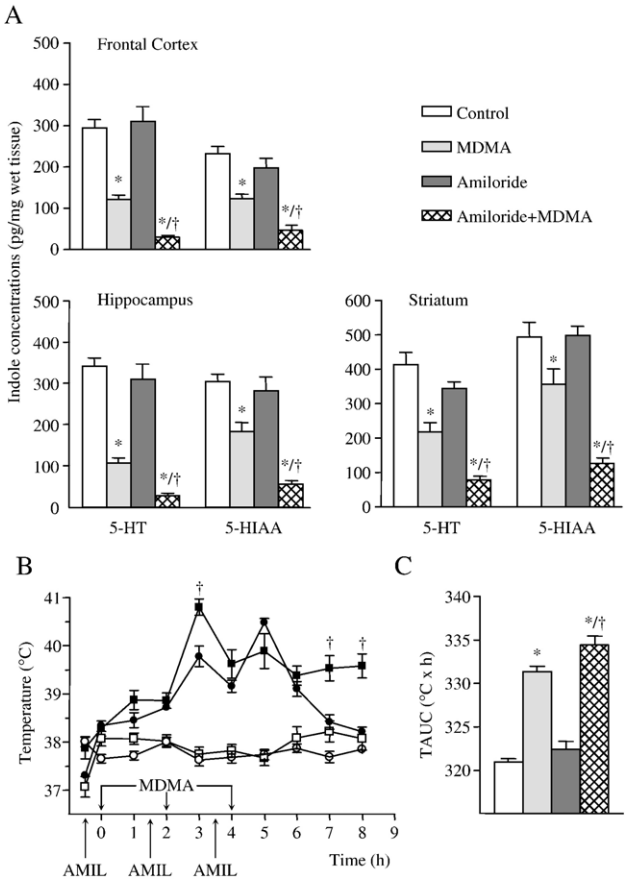

Fig. 1. Amiloride potentiates MDMA-induced 5-HT depletions and acute hyperthermia. The effect of a toxic regimen of MDMA (3×5 mg/kg i.p., every 2 h) alone, or in combination with amiloride (3×2.5 mg/kg i.p.) on rat 5-HT and 5-HIAA levels in the frontal cortex, hippocampus and striatum (A), core temperatures (B) and TAUC (C). Amiloride was given 30 min prior to each MDMA administration at ambient temperature (21.5±1 °C). Rat temperatures were recorded at baseline, right before the first injection of MDMA (t=0 h) and then every hour up to 8 h. Rats were killed 1 week after injections for determination of 5-HT and 5-HIAA levels by HPLC. Results are the means±S.E.M. (n=6–16 rats/group). *Designates significant difference from control; †Designates significant difference from MDMA. Significance level was set at $P<0.05$. In panel B: ○, control; ●, MDMA; □, amiloride; ■, amiloride/MDMA. Only differences between MDMA vs. amiloride/MDMA are shown. All other differences of single-time-point comparisons between groups are omitted for clarity. Panel C presents the means±S.E.M. of TAUC, which is an integration of the temperature versus time curves shown in panel B.

diltiazem were co-injected with MDMA alone or in combination with amiloride. Flunarizine (3×20 mg/kg i.p.) given 30 min before each MDMA injection, partially prevented MDMA-induced 5-HT loss (Fig. 2A), but did not block the toxic effects of the amiloride/MDMA combination in the frontal cortex [$F(5,68)=47.545$, $P<0.001$], hippocampus [$F(5,68)=84.173$, $P<0.001$] or striatum [$F(5,68)=65.677$, $P<0.001$]. Analysis of temperature curves using two-way ANOVA for repeated measures revealed a significant treatment×time interaction [$F(45,630)=9.670$, $P<0.001$] (Fig. 2B). Single time point comparisons using one-way ANOVA revealed that flunarizine did not produce any significant effect on MDMA-induced acute hyperthermia or prevent the potentiation of the hyperthermic effect caused by amiloride. Although flunarizine alone caused a significant reduction in body temperature compared to saline treated animals, analysis of TAUCs revealed that flunarizine was not able to prevent MDMA-induced hyperthermia or the potentiation of MDMA-induced thermal response by amiloride [$F(5,75)=115.943$, $P<0.001$] (Fig. 2C).

Administration of diltiazem (3×50 mg/kg i.p.) instead of flunarizine yielded similar results with the only difference that, in this case, diltiazem did not prevent MDMA-induced 5-HT depletions in any of the brain regions examined (Fig. 3A). Analysis of temperature curves (Fig. 3B) using two-way ANOVA for repeated measures revealed a significant treatment×time

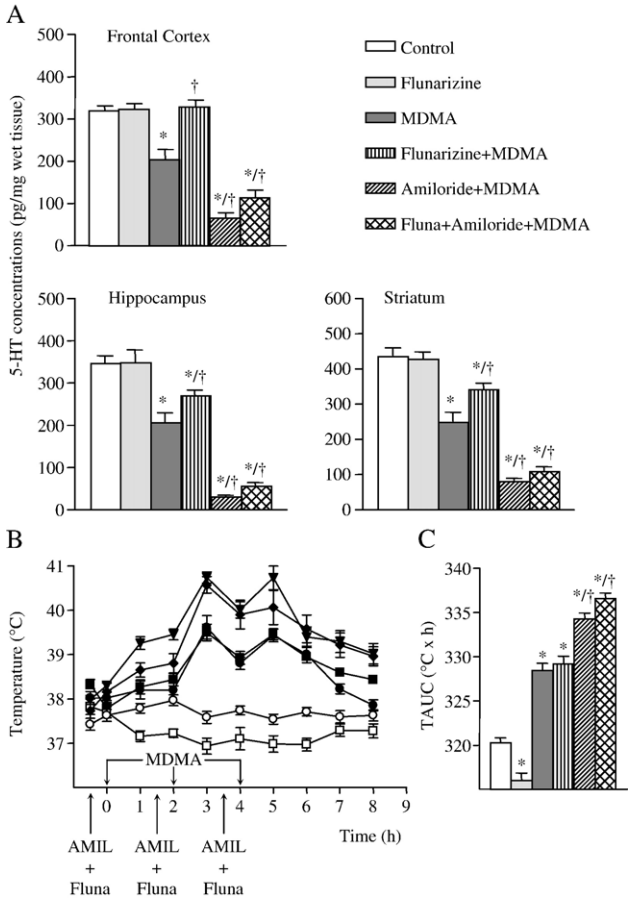

Fig. 2. Flunarizine does not prevent the effects of amiloride on MDMA-induced 5-HT depletions or hyperthermia. The effect of the voltage-dependent Ca^{2+} channel blocker flunarizine (20 mg/kg i.p., every 2 h×3) on 5-HT content changes induced by MDMA (3×5 mg/kg i.p., every 2 h) alone, or in combination with amiloride (3×2.5 mg/kg i.p.) (panel A). Flunarizine and/or amiloride were given at the times shown in the figure. All drug treatments were given at ambient temperature (21.5×1°C). Rats were killed one week after injections for determination of 5-HT levels by HPLC. Results are the means±S.E.M. (n=9–16 rats/group). Panel B: Lack of effect of flunarizine on temperature changes induced by MDMA either given alone or in combination with amiloride. Rat temperatures were recorded at baseline, right before MDMA (t=0) and then every hour up to 8 h. Statistical significant differences between groups are omitted for clarity. ○, Control; ●, MDMA; □, flunarizine; ■, flunarizine/MDMA; ◆, amiloride/MDMA; ▼, flunarizine/amiloride/MDMA. Panel C presents the means±S.E.M. of TAUC, which is an integration of the temperature versus time curves shown in panel B. *Designates significant difference from control; †Designates significant difference from MDMA (one-way ANOVA and Tukey's test).

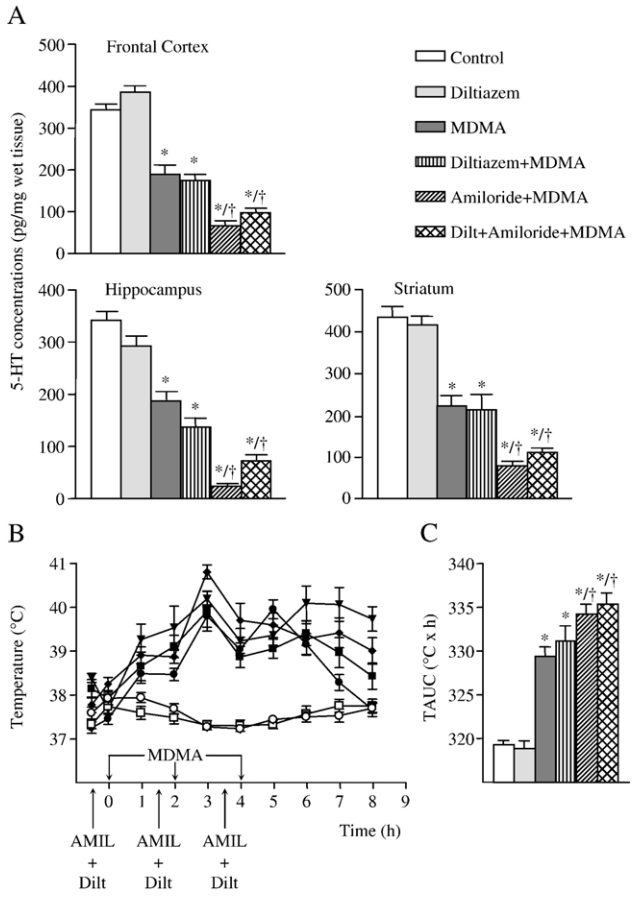

Fig. 3. Diltiazem does not prevent the effects of amiloride on MDMA-induced 5-HT depletions or hyperthermia. The effect of the voltage-dependent Ca^{2+} channel blocker diltiazem (50 mg/kg i.p., every 2 h × 3) on 5-HT content changes induced by MDMA (3 × 5 mg/kg i.p., every 2 h) alone, or in combination with amiloride (3 × 2.5 mg/kg i.p.) (panel A). Diltiazem and/or amiloride were given at the times shown in the figure. All drug treatments were given at ambient temperature (21.5 ± 1 °C). Rats were killed one week after injections for determination of 5-HT levels by HPLC. Results are the means ± S.E.M. (n = 7–16 rats/group). Panel B: Lack of effect of diltiazem on temperature changes induced by MDMA either given alone or in combination with amiloride. Rat temperatures were recorded at baseline, right before MDMA (t = 0) and then every hour up to 8 h. Statistical significant differences between groups are omitted for clarity. ○, Control; ●, MDMA; □, diltiazem; ■, diltiazem/MDMA; ♦, amiloride/MDMA; ▼, diltiazem/amiloride/MDMA. Panel C presents the means ± S.E.M. of TAUC, which is an integration of the temperature versus time curves shown in panel B. *Designates significant difference from control; †Designates significant difference from MDMA (one-way ANOVA and Tukey's test).

interaction [$F(45,648) = 9.862$, $P < 0.001$]. Analysis of TAUCs revealed a significant effect of treatments [$F(5,77) = 47.594$, $P < 0.001$]. Post-hoc analysis showed that the hyperthermic response induced by MDMA was potentiated by amiloride and this effect was not prevented by diltiazem (Fig. 3C).

3.3. Fluoxetine prevents the long-term 5-HT deficits caused by the amiloride/MDMA combination without altering the hyperthermic response

Given the importance of the 5-HT transporter in MDMA neurotoxicity (Green et al., 2003), we next determined if the 5-HT transporter inhibitor fluoxetine (10 mg/kg i.p.), protected against amiloride potentiation of MDMA-induced 5-HT depletions. As shown in Fig. 4, fluoxetine afforded complete neuroprotection against the toxic effects of the amiloride/MDMA combination (Fig. 4A), without significantly altering the hyperthermic response (Fig. 4B and C).

3.4. Effect of dimethylamiloride on MDMA-induced hyperthermia and long-term 5-HT loss and its reversal by fluoxetine

Studies with one amiloride analogue more selective for the Na^+/H^+ exchanger than the Na^+/Ca^{2+} exchanger (Vigne et al., 1984), dimethylamiloride, yielded similar results to those

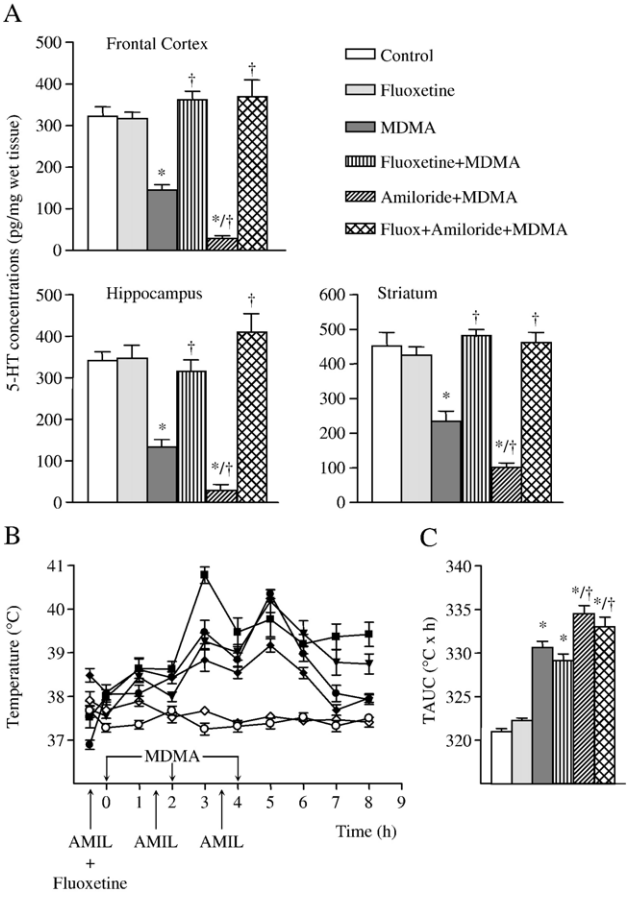

Fig. 4. Fluoxetine prevents 5-HT depletion caused by the amiloride/MDMA combination. The effect of the 5-HT uptake inhibitor fluoxetine (10 mg/kg i.p.) on 5-HT content changes induced by MDMA (3 × 5 mg/kg i.p., every 2 h) alone, or in combination with amiloride (3 × 2.5 mg/kg i.p.) (panel A). Fluoxetine and/or amiloride were given at the times shown in the figure. All drug treatments were given at ambient temperature (21.5 ± 1 °C). Rats were killed one week after injections for determination of 5-HT levels by HPLC. Results are the means ± S.E.M. (n = 6–16 rats/group). Panel B: Lack of effect of fluoxetine on temperature changes induced by MDMA either given alone or in combination with amiloride. Rat temperatures were recorded at baseline, right before MDMA (t = 0) and then every hour up to 8 h. Statistical significant differences between groups are omitted for clarity. ○, Control; ●, MDMA; ■, amiloride/MDMA; ◇, fluoxetine; ♦, fluoxetine/MDMA; ▼, Fluoxetine/amiloride/MDMA. Panel C presents the means ± S.E.M. of TAUC, which is an integration of the temperature versus time curves shown in panel B. *Designates significant difference from control; †Designates significant difference from MDMA (one-way ANOVA and Tukey's test).

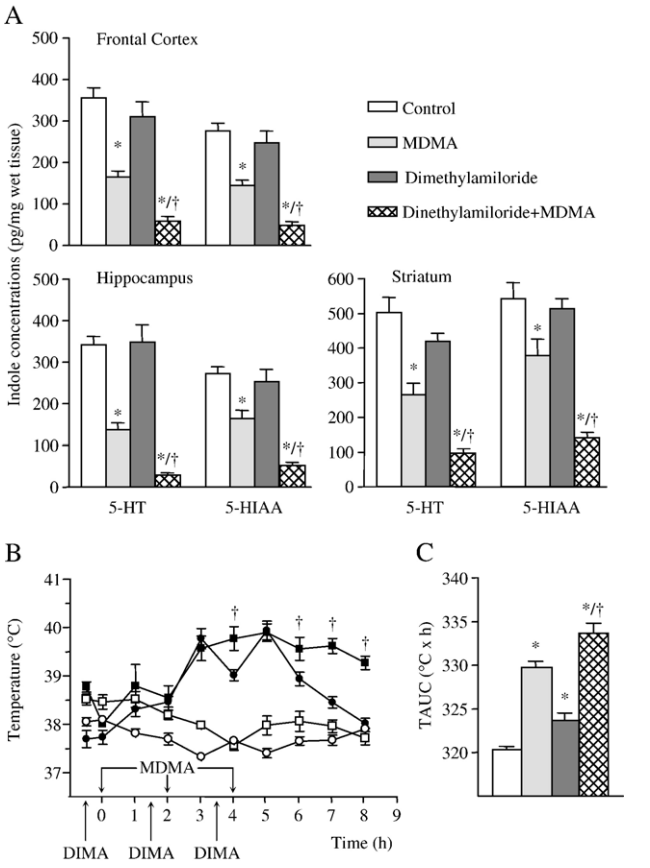

Fig. 5. Dimethylamiloride potentiates MDMA-induced 5-HT depletions and acute hyperthermia. The effect of a toxic regimen of MDMA (3×5 mg/kg i.p., every 2 h) alone, or in combination with dimethylamiloride (3×2.5 mg/kg i.p.) on rat 5-HT and 5-HIAA levels in the frontal cortex, hippocampus and striatum (A), core temperatures (B) and TAUC (C). Dimethylamiloride was given 30 min prior to each MDMA administration at ambient temperature (21.5±1 °C). Rat temperatures were recorded at baseline, right before the first injection of MDMA (t=0 h) and then every hour up to 8 h. Rats were killed 1 week after injections for determination of 5-HT and 5-HIAA levels by HPLC. Results are the means±S.E.M. (n=6–16 rats/group). *Designates significant difference from control; †Designates significant difference from MDMA. Significance level was set at $P<0.05$. In panel B: ○, control; ●, MDMA; □, dimethylamiloride; ■, dimethylamiloride/MDMA. Only differences between MDMA vs. dimethylamiloride/MDMA are shown. All other differences of single-time-point comparisons between groups are omitted for clarity. Panel C presents the means±S.E.M. of TAUC, which is an integration of the temperature versus time curves shown in panel B.

obtained with amiloride. In particular, dimethylamiloride potentiated MDMA-induced depletions of 5-HT and 5-HIAA in the three brain regions examined (Fig. 5A). Statistical analysis of 5-HT content yielded the following results: striatum [$F(3,38)=33.802$, $P<0.001$], frontal cortex [$F(3,38)=10.101$, $P<0.001$] and hippocampus [$F(3,38)=16.782$, $P<0.001$]. Rectal temperature analysis using two-way ANOVA for repeated measures over time revealed a significant interaction treatment×time [F (27,315)=14.108, $P<0.001$]. Single time point comparisons using one-way ANOVA showed significant differences at 4 h [$F(3,38)=46.122$, $P<0.001$], 6 h [$F(3,38)=22.748$, $P<0.001$], 7 h [$F(3,38)=34.846$, $P<0.001$] and 8 h time points [$F(3,38)=29.364$, $P<0.001$] between MDMA-treated rats versus rats treated with the dimethylamiloride/MDMA combination (Fig. 5B). Statistical analysis of TAUCs (Fig. 5C) clearly shows that dimethylamiloride causes a slight hyperthermia when injected alone and potentiates MDMA-induced hyperthermia [$F(3,38)=46.621$, $P<0.001$].

On the other hand, the administration of the 5-HT transporter inhibitor, fluoxetine (10 mg/kg i.p., given at the time of injecting the first dose of dimethylamiloride) afforded complete protection, not only against MDMA-induced neurotoxicity, but it also reversed the toxic effects of the MDMA/dimethylamiloride combination (Fig. 6A). Protection afforded by fluoxetine was independent of effects on core body temperature (Fig. 6B and C).

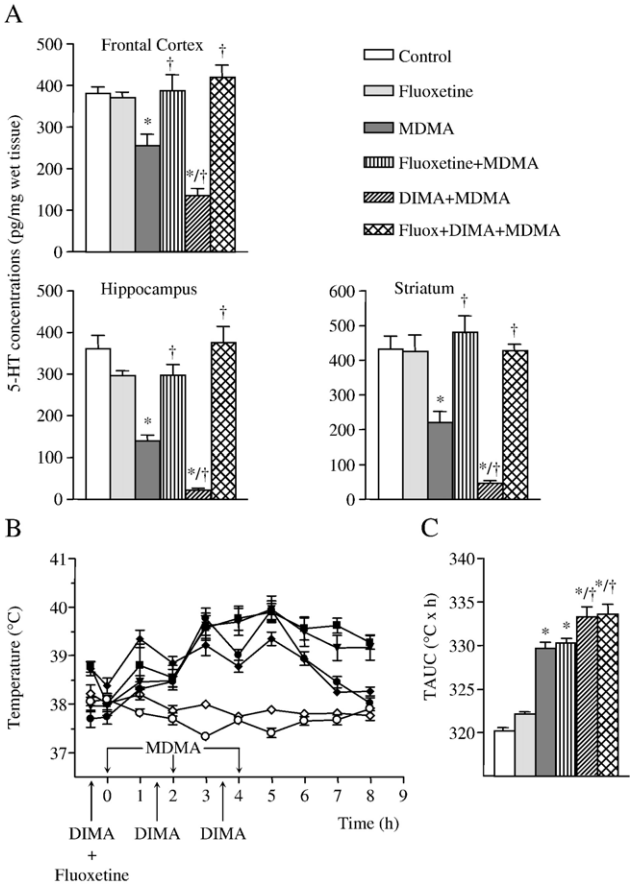

Fig. 6. Fluoxetine prevents 5-HT depletion caused by the dimethylamiloride/MDMA combination. The effect of the 5-HT uptake inhibitor fluoxetine (10 mg/kg i.p.) on 5-HT content changes induced by MDMA (3×5 mg/kg i.p., every 2 h) alone, or in combination with dimethylamiloride (3×2.5 mg/kg i.p.) (panel A). Fluoxetine and/or dimethylamiloride were given at the times shown in the figure. All drug treatments were given at ambient temperature (21.5±1 °C). Rats were killed one week after injections for determination of 5-HT levels by HPLC. Results are the means±S.E.M. (n=6–16 rats/group). Panel B: Lack of effect of fluoxetine on temperature changes induced by MDMA either given alone or in combination with dimethylamiloride. Rat temperatures were recorded at baseline, right before MDMA (t=0) and then every hour up to 8 h. Statistical significant differences between groups are omitted for clarity. ○, Control; ●, MDMA; ■, dimethylamiloride/MDMA; ◇, fluoxetine; ◆, Fluoxetine/MDMA; ▼, Fluoxetine/dimethylamiloride/MDMA. Panel C presents the means±S.E.M. of TAUC, which is an integration of the temperature versus time curves shown in panel B. *Designates significant difference from control; †Designates significant difference from MDMA (one-way ANOVA and Tukey's test).

3.5. Effect of cool ambient temperature (15 °C) on amiloride potentiation of MDMA-induced hyperthermia and long-term 5-HT loss

Given the importance of core body temperature in MDMA neurotoxicity (Green et al., 2004), we examined if blocking MDMA-induced hyperthermia would protect against amiloride potentiation of MDMA-induced 5-HT depletions. As shown in Fig. 7, lowering ambient temperature to 15 °C completely prevented not only MDMA-induced 5-HT loss but also that caused by the amiloride/MDMA combination in the hippocampus [$F(3,31)=1.656$, $P>0.05$], striatum [$F(3,31)=1.715$, $P>0.05$] and partially in the frontal cortex [$F(3,31)=5.679$, $P<0.05$]. In the case of 5-HIAA concentrations, Tukey post-hoc analysis revealed no significant difference between MDMA- versus amiloride/MDMA-treated rats (Fig. 7A). As shown in Fig. 7B and C, the hyperthermic effects of MDMA were absent in those rats treated at 15 °C.

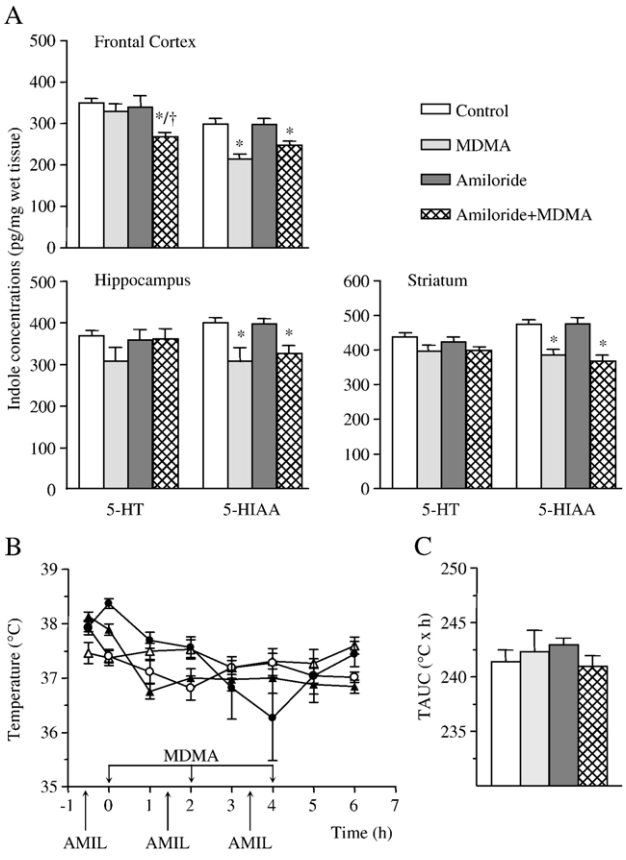

Fig. 7. Effect of ambient temperature on brain 5-HT and 5-HIAA content and hyperthermia induced by MDMA (3×5 mg/kg i.p., given every 2 h) alone or in combination with amiloride (3×2.5 mg/kg i.p., given every 2 h) 30 min before MDMA. Drugs were injected at reduced ambient temperature (15±1 °C). Panel A: 5-HT and 5-HIAA content in different rat brain regions 7 days after drug administrations. Values are means±S.E.M. ($n=8$ in all groups). *Designates significant difference from control; †Designates significant difference from MDMA. Panel B: Rectal temperature of rats after saline (○), MDMA (●), amiloride (△) or amiloride+MDMA (▲). A two-way ANOVA for repeated measures revealed a significant interaction treatment×time [$F(21,196)=4.154$, $P<0.001$]. Statistical differences of single-time-point comparisons between groups are not shown for clarity. Panel C: TAUCs of data shown in panel B. Data were analysed by one-way ANOVA followed by Tukey's test. No significant difference was found among groups [$F(3,31)=0.343$, $P>0.05$].

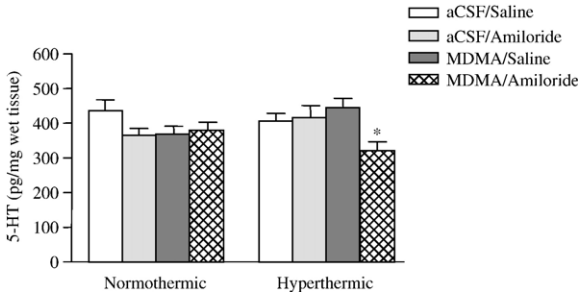

Fig. 8. Concentration of 5-HT in the striatum 7 days after perfusion with aCSF or MDMA (100 μM) through the probe for 8 h in normothermic and hyperthermic rats. The first injection of amiloride (3×2.5 mg/kg i.p., every 2 h) or saline was given 30 min before MDMA perfusion. Data represent the mean±S.E.M. in pg/mg wet tissue weight. ($n=7$–11). No statistical significant difference was found among non-heated rats. *, $P<0.05$ vs. aCSF/Saline/Heat (one-way ANOVA and Tukey test).

3.6. Effect of systemic administration of amiloride in combination MDMA (100 μM) reverse dialysed into the striatum

We performed one last experiment, in which MDMA (100 μM) was reverse dialysed into the striatum for 8 h with and without amiloride (3×2.5 mg/kg i.p., every 2 h). MDMA alone or in combination with amiloride did not cause any significant reduction in striatal 5-HT concentrations 7 days later. During the perfusion time with MDMA there was no change in the rectal temperature of the rats (not shown). Thus, the experiment was also carried out in rats whose temperature was kept elevated for 5 h (mean average 39.45±0.063 °C). Under these experimental conditions the amiloride/MDMA combination caused a small but significant depletion of striatal 5-HT content [$F(3,33)=3.079$, $P=0.042$] (Fig. 8).

Finally, none of the treatment regimens presented in this work produced any significant change in striatal dopamine, DOPAC or HVA content (data not shown), indicating that the exacerbating effects of amiloride or dimethylamiloride were restricted to 5-HT containing neurones.

4. Discussion

Amiloride and several of its congeners known to block the Na^+/Ca^{2+} and/or Na^+/H^+ exchangers potentiate methamphetamine-induced dopamine toxicity in mice as well as dopamine and 5-HT toxicity in rats independent of effects on core temperature (Callahan et al., 2001). We hypothesised that MDMA by prolonged activation of the 5-HT transporter (Rothman and Bauman, 2002; Rudnick and Wall, 1992) and a possible inhibition of the Na^+/K^+ ATPase (Darvesh and Gudelsky, 2005) could initiate a similar chain of biochemical events to those proposed for methamphetamine (Callahan et al., 2001).

To test this hypothesis, in a first set of experiments we administered amiloride in combination with MDMA. Amiloride markedly exacerbated MDMA-induced 5-HT loss. Therefore,

akin to methamphetamine, it would appear reasonable to suggest that ionic dysregulation is a feature of MDMA toxicity. However, these data should be interpreted with caution because, in contrast to methamphetamine (Callahan et al., 2001), amiloride exacerbated the hyperthermic effect of MDMA and, as stated above, increased body temperature can potentiate MDMA neurotoxicity (Broening et al., 1995; Malberg and Seiden, 1998; Sanchez et al., 2004).

Nevertheless, according to our initial hypothesis, accumulation of Na^+ due to prolonged 5-HT transporter activity in concert with Na^+/K^+ ATPase inhibition would lead to membrane depolarisation, which would then trigger the opening of voltage-gated Ca^{2+} channels leading to Ca^{2+} accumulation inside the serotonergic terminals. Accordingly, we injected the non-selective voltage-dependent Ca^{2+} channel blockers, flunarizine or diltiazem in combination with amiloride and/or MDMA. Flunarizine but not diltiazem prevented the decline of 5-HT content caused by MDMA alone. Such findings are in agreement with previous reports showing that flunarizine but not other L-type calcium channel antagonists such as diltiazem, nimodipine, verapamil or nifedipine prevent MDMA-induced 5-HT toxicity (Finnegan et al., 1993; Johnson et al., 1992). It is known that flunarizine has dopaminergic (Finnegan et al., 1993) and histaminic (Holmes et al., 1984) antagonist properties. Because some antihistaminic drugs or the blockade of dopaminergic transmission prevents MDMA-induced depletion of serotonin in rats (Sprague et al., 1998; Yeh et al., 1999), it is possible that flunarizine exerted its protective effect at a site other than a calcium channel. Flunarizine but not diltiazem also caused a slight hypothermia when injected alone, however, it is important to note that neither flunarizine nor diltiazem attenuated the hyperthermic effect or the long-term 5-HT depletion caused by the amiloride/MDMA combination. Such data suggests that Ca^{2+} entrance through voltage-gated Ca^{2+} channels is not involved in the toxic mechanisms induced by the amiloride/MDMA combination.

Because amiloride not only blocks the Na^+/Ca^{2+} but also the Na^+/H^+ exchanger, we tested whether the highly selective Na^+/H^+ exchanger inhibitor dimethylamiloride (Kleyman and Cragoe, 1988), would result in a similar exacerbation of 5-HT depletion when combined with MDMA. Despite their different pharmacological profile, our results show that dimethylamiloride exacerbates MDMA-induced 5-HT depletion to a similar extent as that caused by amiloride. There is a substantial body of evidence indicating that increased free radical formation is responsible for MDMA-induced 5-HT neurotoxicity in rats (e.g. Aguirre et al., 1999; Guldesky, 1996; Shankaran et al., 1999, 2001). Because intracellular acidosis impairs antioxidant capacity and promotes oxidative stress (LaVoie and Hastings, 1999; Ying et al., 1999), it appears reasonable to suggest that the blockade of Na^+/H^+ exchange by amiloride or dimethylamiloride could be potentiating MDMA-induced 5-HT depletion by promoting free radical formation inside the serotonergic terminal.

Nevertheless, it is worth noting that dimethylamiloride not only caused a slight hyperthermia when injected alone but also potentiated the hyperthermic response produced by MDMA which raises the question of whether amiloride and dimethylamiloride exacerbate 5-HT loss by blocking Na^+/H^+ exchange or by their effect on core temperature. In an attempt to solve such question we administered the amiloride/MDMA combination at a low ambient temperature (15 °C), hoping that such approach would not only prevent MDMA-induced hyperthermia (Malberg and Seiden, 1998; Schmidt et al., 1990), but also that caused by the amiloride/MDMA combination. Our findings indicate that lowering ambient temperature prevented not only the acute hyperthermia caused by the amiloride/MDMA combination but also the loss of brain 5-HT content. To further analyse the role of hyperthermia we administered amiloride intraperitoneally in combination with MDMA perfused directly into the striatum. It is known that perfusion of MDMA into the brain does not elicit the hyperthermic response achieved when the drug is given systemically (Esteban et al., 2001; Nixdorf et al., 2001). Under these experimental conditions, the amiloride/MDMA combination did not cause any significant effect on striatal 5-HT content. Taken together, these two latter experiments suggest that hyperthermia is necessary for amiloride to exacerbate MDMA-induced 5-HT loss. We therefore carried out a parallel experiment in which MDMA was perfused into the striatum of rats whose body temperature was kept in a similar range to that observed after systemic administration of the drug. In accordance with a previous study (Esteban et al., 2001), 7 days after perfusion of MDMA the striatal concentration of 5-HT was unchanged despite hyperthermia. By contrast, the amiloride/MDMA combination produced a significant long-term loss of striatal 5-HT concentration, although, to a much smaller extent than that produced in rats treated systemically with the amiloride/MDMA combination. This, together with the fact that fluoxetine completely prevented 5-HT depletions caused by the amiloride/MDMA or dimethylamiloride/MDMA combinations without producing any significant effect on hyperthermia suggests that hyperthermia and the blockade of Na^+/H^+ exchange are not sufficient for amiloride to exacerbate MDMA-induced 5-HT loss.

Hepatic metabolism of MDMA results in the formation of several glutathione and other thiol adducts in rats (Hiramatsu et al., 1990; Jones et al., 2005). Such compounds are known to generate reactive oxygen species in a 5-HT transporter-dependent manner (Jones et al., 2004; Monks et al., 2004), and decrease brain 5-HT concentrations (Bai et al., 1999). Interestingly, preliminary data from our laboratory indicate that the potentiation of MDMA-induced hyperthermia facilitates MDMA metabolism into such putative toxic compounds. This, together with the fact that hyperthermia increases free radical formation (Globus et al., 1995; Kil et al., 1996), and that intracellular acidosis promotes oxidative stress (LaVoie and Hastings, 1999; Ying et al., 1999), could somehow explain why amiloride or dimethylamiloride potentiate MDMA-induced 5-HT depletion.

In summary, this study demonstrates that amiloride or dimethylamiloride potentiate MDMA-induced 5-HT depletion and points to an effect related to changes in body temperature as a possible underlying mechanism. The blockade of the Na^+/H^+ exchanger in combination with a more sustained hyperthermia could render 5-HT terminals more vulnerable to free radical

damage. This contention is supported by many studies in the field of oncology showing that the sensitivity of tumour cells to hyperthermia is markedly increased when intracellular pH is lowered by Na^+/H^+ inhibition (e.g. Kitai et al., 1998; Li and Liu, 1997; Lyons et al., 1992; Song et al., 1994). Additional studies, however, are needed to clarify the mechanisms underlying MDMA toxicity.

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