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Original Investigation

Increased anxiety and "depressive" symptoms months after MDMA ("ecstasy") in rats: drug-induced hyperthermia does not predict long-term outcomes

Iain S. McGregor[1], Clint G. Gurtman[1], Kirsten C. Morley[1], Kelly J. Clemens[1], Arjan Blokland[2], Kong M. Li[3], Jennifer L. Cornish[1] and Glenn E. Hunt[4]

(1) School of Psychology, University of Sydney, Sydney, 2006 NSW, Australia
(2) Department of Psychology, University of Maastricht, Maastricht, The Netherlands
(3) Department of Pharmacology, University of Sydney, Sydney, 2006 NSW, Australia
(4) Department of Psychological Medicine, University of Sydney, Concord Hospital, Sydney, 2139 NSW, Australia

Iain S. McGregor
Email: iain@psych.usyd.edu.au
Phone: +61-2-93513571
Fax: +61-2-93518023

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Abstract

Rationale There is some uncertainty whether the acute hyperthermia caused by MDMA (ecstasy) plays a significant role in determining the long-term neurotoxic effects on brain 5-HT systems and associated changes in mood and behaviour.

Objective The present study assessed whether long-term behavioural and cognitive changes seen in MDMA-treated rats are affected by hyperthermia at the time of drug administration.

Method Male Wistar rats were treated with MDMA (4×5 mg/kg i.p. over 4 h on 2 consecutive days) or vehicle at either a high ambient temperature (28°C) or a low ambient temperature (16°C). Eight to 18 weeks later, rats were tested in behavioural measures of anxiety (social interaction and emergence tests), a test of cognition (object recognition test) and the forced swim test of depression. At the conclusion of behavioural testing the rats were killed and their brains analysed using HPLC.

Results MDMA treatment caused a clear and consistent hyperthermia at 28°C and hypothermia at 16°C. Months later, rats pre-treated with MDMA at either 16 or 28°C displayed increased anxiety in the social interaction and emergence tests and reduced escape attempts and increased immobility in the forced swim test. MDMA pre-treatment was also associated with poorer memory on the object recognition test, but only in rats given the drug at 28°C. Rats pre-treated with MDMA showed loss of 5-HT in the hippocampus, striatum, amygdala and cortex, regardless of body temperature at the time of dosing. However, 5-HIAA loss in the amygdala and hippocampus was greater in rats pre-treated at 28°C. Dopamine in the striatum was also depleted in rats given MDMA.

Conclusions These results indicate that hyperthermia at the time of dosing with MDMA is not necessary to produce subsequent 5-HT depletion and anxiety in rats. They also extend previous findings of long-term effects of brief exposure to MDMA in rats to include apparent "depressive" symptoms in the forced swim model.

Keywords MDMA - Ecstasy - Anxiety - Depression - Serotonin - 5-HT - Temperature

Introduction

MDMA (3,4 methylenedioxymethamphetamine; ecstasy) is a drug with unique prosocial and euphoric properties that

is currently among the most popular illicit recreational drugs in the world. For more than 15 years it has been known that MDMA and several related amphetamine derivatives produce long-term depletion of the neurotransmitter 5-HT in the brains of laboratory animals (Ricaurte et al. *1985*, *2000*). This loss of 5-HT reflects a distal axotomy of serotonergic neurons originating from midbrain raphe nuclei (Battaglia et al. *1987*; Scanzello et al. *1993*; Fischer et al. *1995*; Callahan et al. *2001*).

MDMA and other amphetamine derivatives can cause major changes in body temperature, with the environmental temperature at the time of dosing determining the direction of body temperature change. At cooler temperatures (e.g. 4–20°C), MDMA and methamphetamine induce a dose-dependent hypothermia (Schmidt et al. *1990*; Gordon et al. *1991*; Bowyer et al. *1992*; Dafters *1994*; Broening et al. *1995*; Marston et al. *1999*), while at high ambient temperatures a hyperthermia is evident (Dafters *1994*, *1995*; Broening et al. *1995*; Malberg and Seiden *1998*; Morley et al. *2001*; Gurtman et al. *2002*). These body temperature changes may influence the neurotoxic action of these drugs. With methamphetamine, the dopamine and 5-HT depleting effects of the drug were greatly attenuated when it was administered at 4°C, a temperature at which it induces hypothermia in rats (Bowyer et al. *1992*, *1994*).

Similarly, with MDMA, Malberg and Seiden (*1998*) reported that 5-HT depletion increased as the ambient temperature rose to levels at which hyperthermia was evident. At the lowest ambient temperatures, 20°, 22° and 24°C, MDMA caused hypothermia, with no loss of 5-HT evident 2 weeks later. At the highest ambient temperature of 30°C, a strong hyperthermic response was seen with clear subsequent 5-HT depletion. These results have been complemented by pharmacological experiments in which such MDMA and methamphetamine-induced neurotoxicity has been prevented or attenuated by co-administration of a range of drugs that prevent or attenuate hyperthermia (Schmidt et al. *1990*; Colado et al. *1993*, *1995*, *1998*, *1999*, *2001*; Farfel and Seiden *1995*; Malberg et al. *1996*). Such results may have implications for human MDMA users, given that the drug is frequently consumed in hot crowded night-clubs and causes hyperthermia in humans (Chadwick et al. *1991*; Henry et al. *1992*; Mallick and Bodenham *1997*; Liechti et al. *2000*; Parrott *2002*).

Nonetheless, careful scrutiny of the literature reveals that the relationship between ambient temperature hyperthermia, and MDMA-induced neurotoxicity is not always simple. In some studies, long-term 5-HT depletion has been evident despite clear hypothermia at the time of MDMA administration. (Farfel and Seiden *1995*; Marston et al. *1999*). Similar results have been evident with methamphetamine (Bowyer et al. *2001*) and fenfluramine (Malberg and Seiden *1997*). On the other hand, MDMA-induced hyperthermia has sometimes been evident without subsequent 5-HT depletion (O'Shea et al. *1998*). It appears then that the relationship between body temperature and neurotoxicity induced by substituted amphetamines is complex. It may be that hyperthermia exaggerates the 5-HT depletion produced by these drugs, without being necessary or sufficient for its occurrence.

In recent preclinical studies, we have reported that Wistar rats briefly exposed to MDMA in a hot environment showed long-term increases in anxiety-like behaviour in the social interaction, elevated plus maze and emergence tests 1–3 months post-drug (Morley et al. *2001*; Gurtman et al. *2002*). MDMA treated rats also displayed inferior memory relative to controls in an object recognition test of memory (Morley et al. *2001*). Post-mortem neurochemical analysis showed that these rats had approximately 40% loss of 5-HT in cortex, hippocampus, amygdala and striatum (Gurtman et al. *2002*).

In the present study, we further examined these long-term effects of MDMA in rats with a principle focus on body temperature at the time of dosing as a determinant of long-term behavioural and neurochemical changes. To this end, identical doses of MDMA were given to rats, with one group receiving this dose at a high ambient temperature (28°C) known to produce acute hyperthermia, long-term increases in anxiety-like behaviour and lasting 5-HT depletion (Morley et al. *2001*; Gurtman et al. *2002*). The other group received the MDMA at an ambient temperature of 16°C, a temperature at which MDMA induces hypothermia (Schmidt et al. *1990*; Gordon et al. *1991*; Dafters *1994*; Malberg and Seiden *1998*; Marston et al. *1999*). The question of interest was whether the two groups would differ in anxiety-like behaviour, memory, and 5-HT depletion measured several months later.

In addition to the social interaction, emergence and object recognition tests, rats were also tested on the forced swim test. This is a test of behavioural despair that is sensitive to the effects of antidepressant drugs (Cryan et al. *2002*). There is increasing concern that heavy MDMA use may lead to dysregulation of mood, perhaps associated with 5-HT depletion (Topp et al. *1999*; Morgan *2000*; Schifano *2000*; Parrott *2001*). If this were the case then it might be expected that rats previously exposed to MDMA would show a "depressive" profile in the forced swim test, with fewer escape attempts and greater immobility.

Materials and methods

Subjects

The subjects were 64 inbred male albino Wistar rats (Concord Hospital breeding facility) aged approximately 60–75 days old and weighing 334±6.88 g at the time of drug treatment. At the conclusion of testing 16–18 weeks after MDMA or vehicle administration the rats weighed 572±5.32 g.

The rats were housed in groups of eight per cage for the duration of the experiment with food and water freely available. Temperature in the colony room was controlled at 22°C and a 12-h reverse light cycle was in operation. All behavioural testing was conducted during the dark cycle. All experimentation was approved by the University of Sydney animal ethics committee.

Drug

(±)3,4-Methylenedioxymethamphetamine was supplied by the Australian Government Analytical Laboratories (Pymble, N.S.W., Australia). It was diluted in 0.9% saline and injected IP at a volume of 1 ml/kg.

Experimental techniques

Drug treatment, body temperature and locomotor activity measurement

Procedures during acute drug administration closely followed those described previously (Morley et al. *2001*; Gurtman et al. *2002*) with rats receiving MDMA or vehicle administration on 2 consecutive days during which locomotor activity and body temperature were measured. Drug treatment of all 64 rats in the study required 2 weeks to complete. This meant that there was up to a 2-week difference in the time period between acute drug treatment and subsequent behavioural and neurochemical tests across rats.

During acute drug administration, rats were placed in standard operant chambers (30×50×25.5 cm) with aluminium side and back walls and Perspex front wall and a metal grid floor. The chambers were placed inside wooden sound attenuation boxes that provided darkness and masking fan noise during testing. The ambient temperature in the room in which the test chambers were located was maintained at either 28±1.0°C or 16±1.0°C by means of a reverse-cycle room air conditioner. Temperature readings taken within the test chambers were always within±1°C of the ambient room temperature.

Rats given MDMA (n=32) received a 5 mg/kg dose of the drug every hour for 4 h on each of 2 consecutive days to give a cumulative total dose of 40 mg/kg (20 mg/kg per day). This dosing regime is intended to simulate a single weekend of heavy MDMA use in a human user (Boot et al. *2000*; Morley et al. *2001*; Gurtman et al. *2002*). Control rats (n=32) received an injection of 0.9% saline at 1 ml/kg every hour for 4 h on each of the 2 treatment days. Each hour, rats were briefly removed from the test chambers to administer their next injection and to measure body temperature.

Body temperatures were taken using a Braun Thermoscan Instant Thermometer (IRT 1020), which was inserted into the ear of the rat (Morley et al. *2001*; O'Loinsigh et al. *2001*; Gurtman et al. *2002*). This method provides a rapid and relatively stress-free reading of body temperature in rats that is highly correlated with rectal temperature.

The MDMA and vehicle conditions were split so that half of the rats received their treatment at an ambient temperature of 28°C while the other half received the drug at 16°C. This resulted in four groups (n=16 per group) that are referred to as MDMA (28°), MDMA (16°), Vehicle (28°) and Vehicle (16°). MDMA and vehicle treated rats were equally represented within each home cage which contained rats from the same temperature condition.

At the conclusion of the 4 h acute dosing sessions, rats were returned to their normal group housing in the main animal colony at 22°C. At this ambient temperature, MDMA produces little if any effect on body temperature (Gordon et al. *1991*; Malberg and Seiden *1998*). Rats pre-treated with MDMA with an identical regime to that used here (4×5 mg/kg over 4 h) show a return to normal body temperatures when placed in group housing at this ambient temperature (McGregor and Clemens, unpublished data).

Social interaction test

Approximately 8–10 weeks following acute drug administration, pairs of rats were assessed in the social interaction

test. Testing occurred in a square black Perspex box (52×52×40 cm) dimly lit with red light (40 W). A miniature video camera was mounted above the box and was connected to a video recorder and monitor in a neighbouring room where the interactions of the rats were recorded onto tape. The experimenter remained outside the test room during testing and the test arena was wiped down with 10% ethanol in between each test session.

Testing was performed across 2 consecutive days with each rat tested with a different partner on each of the 2 days. Partners were selected so as to be of approximately equal body weight and from the same treatment condition [MDMA (16°), MDMA (28°), Vehicle (16°) or Vehicle (28°)] but from a different home cage. Data for a total of 16 pairs from each condition were obtained.

Each social interaction session lasted for 10 min. The total duration of social interaction and number of interactions during this 10-min period was scored from video using ODLog software (www.macropodsoftware.com) by an observer who was blind to group assignment. Behaviours that were recorded as social interaction included sniffing, adjacent lying, following, crawling over/under and mutual grooming.

Emergence test

Two days after the social interaction test, rats were tested in the emergence test. The apparatus consists of a black Perspex walled rectangular arena (96×100×40 cm) with a black wooden hide box (24×40×15 cm) placed in the top left corner of the arena. The open part of the arena was illuminated with red light (40 W) and a video camera was mounted above the arena and connected to a video recorder. Note that the use of a black painted apparatus is different to the white apparatus used in our earlier reports (Morley et al. *2001*; Gurtman et al. *2002*), and resulted in substantially lower baseline anxiety in rats.

Rats were initially placed inside the wooden hide box (which had a hinged lid through which the rat could be placed inside the box). Testing continued for 5 min, during which time the experimenter remained outside the test room.

Subsequent video analysis by an experimenter blind to group assignment scored the latency of rats to emerge from the hide box, the duration of time spent in the open field and the amount of rearing. Analysis was accomplished using ODLog data logging software.

After each test session the apparatus was thoroughly wiped down with a damp cloth containing 10% ethanol.

Novel object recognition test

Approximately 10–12 weeks after MDMA treatment, 32 rats of the 64 rats in the study were tested on the object recognition task following methods described by Prickaerts and colleagues (Prickaerts et al. *2002*). The decision to proceed with only half of the treated rats for the remainder of the study was done on the basis of the labour intensive nature of scoring the object recognition and forced swim tests and the subsequent HPLC. Four out of the eight home cages (n=32 rats) were randomly selected for this further testing.

The object recognition testing took place in the same black Perspex box used for the social interaction test (52×52×40 cm) dimly lit with a red light (40 W) and equipped with a miniature black and white video camera.

Rats were first further familiarized in the test box without any objects for 3 min each. Testing comprised two separate 3 min trials. The first trial involved the rat exploring two identical objects for 3 min after which the rat was returned to its home cage. Following a delay of exactly 1 h, the rat was placed back in the arena for 3 min but this time with two dissimilar objects (one previously exposed, the other novel). The time spent exploring both objects in the first and second trial was recorded. Exploration of objects was defined as directing the nose towards the object with a distance of no more than 2 cm and/or touching the object with the nose.

The two sets of objects used in the task were (1) a solid steel bar attached to a lamp fitting with a large bolt, and (2) a sugar dispenser filled with charcoal balls. Both objects had been found in pilot studies to attract equal inspection times and were heavy enough that they did not need to be fixed with adhesives to prevent displacement by the rat. These objects were interchanged and counterbalanced across subjects as novel and familiar. The objects and arena were thoroughly cleaned with dilute ethanol solution in between trials.

Rats were given preliminary tests at a 1-h retention interval on each of 2 days to allow rats to become familiar with the testing procedure (Prickaerts et al. *2002*). The following day, a third test was given, and the data for that test used

in analysis of group differences. The percentage of time spent inspecting the novel versus the familiar object on the third day of testing was used as the index of recognition memory.

Forced swim test

At 16–18 weeks following MDMA treatment, the same 32 rats that had been tested on the object recognition test were assessed on the forced swim test across 3 consecutive days. The methods used were similar to those described by Blokland et al (*2002*) and Cryan et al (*2002*).

Rats were placed in one of three cylindrical clear Perspex tubes (40 cm high×17 cm diameter) located in a room illuminated with dim red light (40 W). The tubes were filled to a height of 25 cm with water maintained at a temperature of 23°C. The tubes were cleaned and refilled with fresh water in between each trial. Each trial lasted exactly 5 min.

A video camera was mounted near the apparatus which was connected to a VCR in a neighbouring room. Here a blind observer scored behaviours in real time using ODlog software. The primary behavioural variable scored was "immobility" which was defined as "making no movements except for those necessary to keep the nose above water" (Blokland et al *2002*; Cryan et al *2002*). The other behaviours analysed were the two active responses of "climbing" and "swimming", which are known to be sensitive to 5-HT acting compounds (Cryan et al *2002*).

Neurochemical analysis

Four days after the conclusion of forced swim testing (and 121–135 days after the start of the experiment) the 32 rats that had been used in the object recognition and forced swim tests were decapitated using a guillotine, and their brains rapidly removed for neurochemical analysis.

Four regions of interest were manually dissected out over dry ice using a method derived from that of Harkin and colleagues (Harkin et al. *2001*). Samples from the prefrontal cortex, striatum, hippocampus and amygdala were individually placed in centrifuge tubes and were stored in a freezer at –80°C until assayed.

Tissue samples were weighed and then homogenized with a 500 μl ice-cold solution of 0.2 M perchloric acid containing 0.1% cysteine and 200 nmol/l of internal standard 5-hydroxy-N-methyltryptamine (5-HMeT). The homogenate was centrifuged at 15,000 *g* for 10 min at 4°C and a 20 μl aliquot of the resulting supernatant fluid was then analysed by high performance liquid chromatography (HPLC) with electrochemical detection as described previously (Schworer et al. *1987*).

Briefly, the HPLC system consisted of a Shimadzu ADVP module (Kyoto, Japan) equipped with SIL-10 autoinjector with sample cooler and LC-10 on-line vacuum degassing solvent delivery unit. Chromatographic control, data collection and processing were carried out using Shimadzu Class VP data software. The mobile phase consisted of 0.1 mol/l phosphate buffer (pH 3.0), PIC B-8 octane sulphonic acid (Waters, Australia) 0.74 mmol/l, sodium EDTA (0.3 mmol/l) and methanol (12% v/v). The flow rate was maintained at 1 ml/min. Dopamine, 5-HIAA, 5-HT and 5-HMeT were separated by a Merck LiChrospher 100 RP-18 reversed phase column. Quantification was achieved via GBC LC-1210 electrochemical detector (Melbourne, Australia) equipped with a glassy carbon working electrode set at +0.75 V. The calibration curve of each standard was obtained by the concentration versus the area ratio of the standard and internal standard.

Data analysis

Data for body temperature and locomotor activity during acute drug administration, for behavioural indices in the social interaction, emergence, object recognition and forced swim tests, and for neurochemical data from the HPLC analysis were performed using analysis of variance (ANOVA). The two independent variables were drug (MDMA versus vehicle) and ambient temperature at the time of dosing (16°C versus 28°C).

When the overall ANOVA results suggested specific differences between (a) the MDMA (16°) and MDMA (28°) groups, or (b) the Vehicle (16°) and Vehicle (28°) groups, these specific groups were compared by means of a simple *t*-test.

The significance level for all statistical tests was set at $P<0.05$.

Results

Body temperature during drug treatment

The body temperatures in the four groups across the 2 days of acute drug treatment are shown in Fig. 1. The direction of MDMA effects on body temperature were entirely dependent on ambient temperature with hyperthermia evident in rats given MDMA at 28°C and hypothermia in rats given MDMA at 16°C.

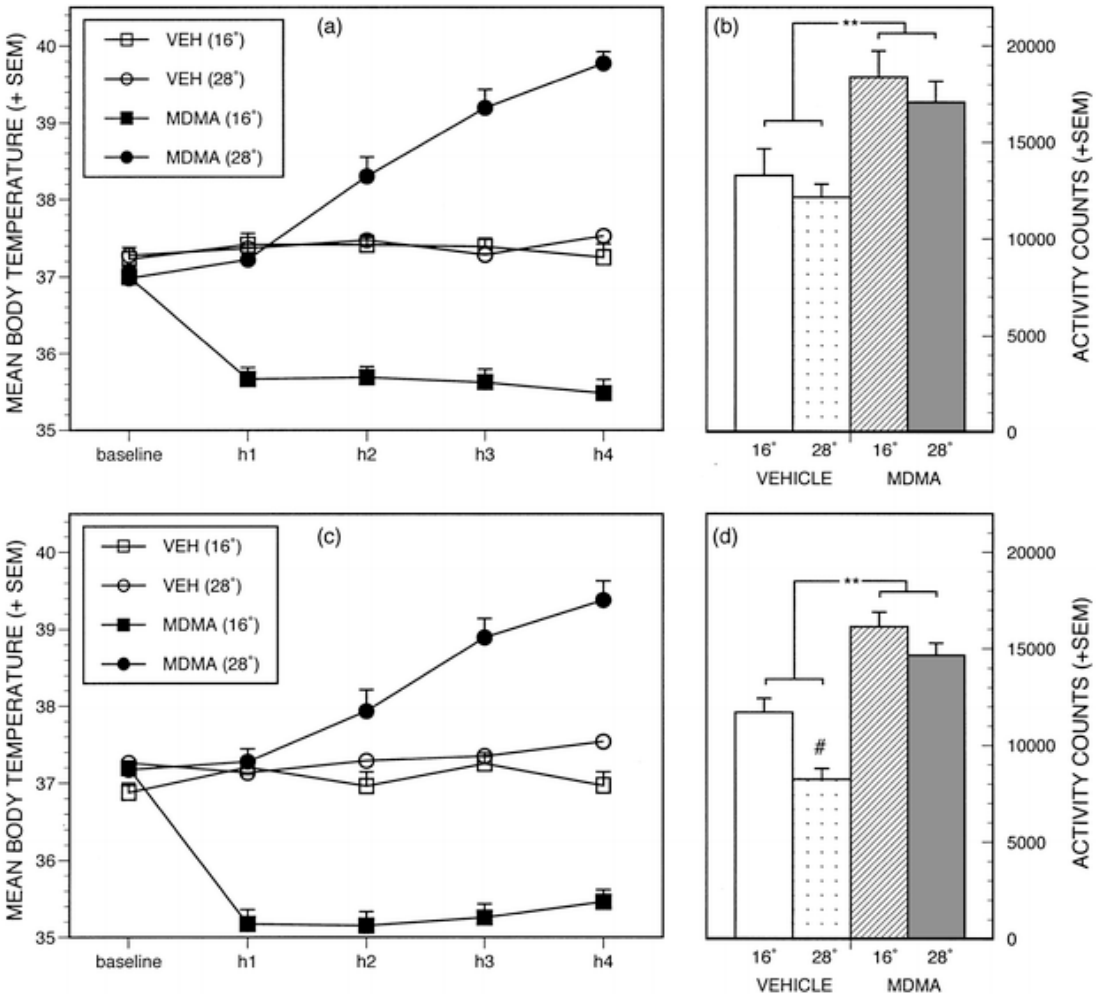

Fig. 1. Body temperature (*left*) and locomotor activity (*right*) on day 1 (**a**, **b**) and day 2 (**c**, **d**) of acute drug administration. Body temperature data are given for baseline (pre-test) and every hour for the four hours of testing. Locomotor activity data are given as a cumulative value for the entire 4-h test. Data represent mean±SEM for n=16 rats per condition. ** Significant overall difference between MDMA and vehicle groups, ANOVA, P<0.01; # significant difference between Vehicle 16°C and Vehicle 28°C groups, t-test, P<0.05

Repeated measures ANOVA for day 1 data showed a significant overall effect of drug [$F(1,60)=8.15$, $P<0.01$], of ambient temperature [$F(1,60)=170.96$, $P<0.0001$], and a significant drug by temperature interaction [$F(1,60)=156.61$, $P<0.0001$].

This pattern of results was similar on day 2 with a significant overall effect of drug [$F(1,60)=7.82$, $P<0.01$], of ambient temperature [$F(1,60)=170.84$, $P<0.0001$], and a significant drug by temperature interaction [$F(1,60)=112.01$, $P<0.0001$].

Locomotor activity during drug treatment

The data for locomotor activity during acute drug treatment are also shown in Fig. 1. ANOVA for total activity in the

4 h test session on day 1 revealed a significant effect of drug [$F(1,60)=23.74$, $P<0.0001$], but no significant effect of ambient temperature [$F(1,60)=2.11$, $P<0.15$] and no drug by temperature interaction ($F<1$). This pattern of results reflected an overall hyperactivity in the two groups given MDMA.

ANOVA for total activity on day 2 also revealed a significant effect of drug [$F(1,60)=66.28$, $P<0.0001$]. This again reflected greater overall activity in the animals given MDMA. There was also a significant effect of ambient temperature [$F(1,60)=13.84$, $P<0.001$] but no drug by temperature interaction [$F(1,60)=2.15$, $P<0.15$]. Further analysis showed significantly lower activity in the Vehicle (28°) group than Vehicle (16°) group [$t(1,31)=3.82$, $P<0.001$] but no significant difference between the MDMA (28°) and MDMA (16°) groups [$t(1,31)=1.52$, $P<0.14$].

Social Interaction test

The results of the social interaction tests conducted approximately 8–10 weeks post-MDMA are shown in Table 1. There was a significant effect of drug pre-treatment [$F(1,60)=11.76$, $P<0.01$] on duration of social interaction but no significant effect of temperature ($F<1$) and no significant drug by temperature interaction ($F<1$). As is shown in Table 1, both MDMA pre-treated groups showed less social interaction than vehicle treated groups.

Table 1. Results from the social interaction test conducted 10 weeks post-MDMA. Data are mean (SEM), and represent $n=16$ per condition. *A* significant overall effect of drug

	VEH (16°)	VEH (28°)	MDMA (16°)	MDMA (28°)	Statistics
Interaction time (s)	131.19 (9.16)	122.33 (5.28)	103.16 (7.16)	99.73 (7.39)	A
Interactions (number)	59.37 (3.07)	60.81 (1.60)	50.75 (2.90)	51.94 (3.08)	A

Rats in both MDMA pre-treated groups also showed significantly fewer social interaction bouts than controls as reflected in a significant overall drug effect [$F(1,29)=10.46$, $P<0.01$]. Again, there was no significant temperature effect or drug by temperature interaction effect ($F<1$).

Emergence test

The results of the emergence test conduced 8–10 weeks post MDMA are shown in Table 2. Rats in both MDMA pre-treated groups took longer to emerge in the open field than controls giving a significant overall effect of drug pre-treatment [$F(1,60)=7.37$, $P<0.01$]. There was no significant effect of temperature and no drug by temperature interaction ($F<1$).

Table 2. Results from the emergence test conducted 10 weeks post-MDMA. Data are mean (SEM), and represent $n=16$ per condition. *A* significant overall effect of drug

	VEH (16°)	VEH (28°)	MDMA (16°)	MDMA (28°)	Statistics
Emergence latency (s)	32.1 (8.62)	24.04 (3.55)	55.73 (17.20)	42.86 (10.86)	A
Open time (s)	160.49 (11.50)	166.09 (10.90)	131.44 (13.74)	122.15 (17.82)	A
Rearing (s)	22.40 (3.18)	18.26 (2.67)	12.31 (2.21)	10.27 (1.82)	A

There was also a significant effect of drug pre-treatment with respect to open field time [$F(1.60)=7.18$, $P<0.01$] with MDMA pre-treated rats spending less time in the open field. Again, there was no significant effect of temperature and no drug by temperature interaction ($F<1$).

Finally, there was also a significant effect of drug pre-treatment with respect to rearing [$F(1,60)=12.59$, $P<0.001$], reflecting lower levels of rearing in the two MDMA pre-treated groups. There was no significant effect of temperature or drug by temperature interaction on rearing ($F<1.5$).

Novel object recognition test

Data for the novel object recognition test, conducted 10–12 weeks after drug treatment, are shown in Fig. 2. Results indicated a significant overall effect of drug pre-treatment [$F(1,28)=7.84$, $P<0.01$], a significant effect of temperature [$F(1,28)=7.80$, $P<0.01$], but no significant drug by temperature interaction ($F<1$). The total time spent inspecting the objects did not vary significantly according to drug or temperature ($F<1.2$). It can be seen from Fig. 2 that the poorest memory was evident in the MDMA (28°) group. A *t*-test comparison between the MDMA (16° and MDMA (28°)

groups showed significantly poorer memory in the MDMA (28°) group [$t(1,14)=2.54$, $P<0.05$] but no difference between the two groups in overall inspection time ($t<1$).

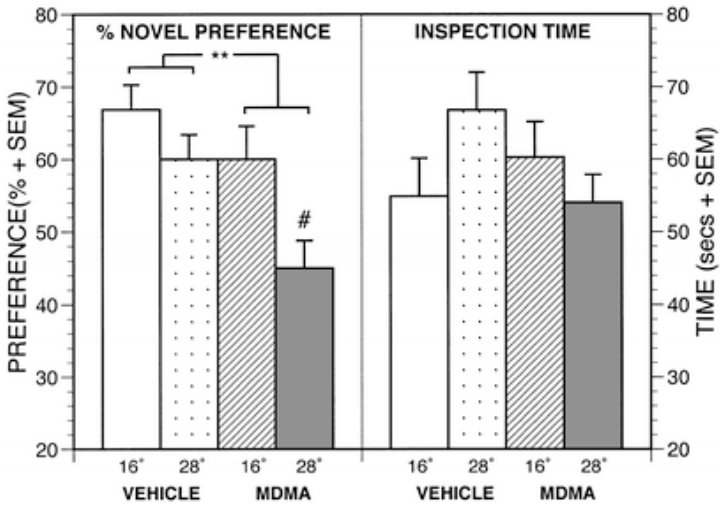

Fig. 2. Results from the novel object recognition test conducted 3 months after drug administration. The *left panel* shows percentage preference for the novel over the familiar object. The *right panel* shows the total amount of time spent inspecting the novel and familiar object combined. Data represent mean±SEM for $n=8$ rats per condition. ** Significant overall difference between MDMA and vehicle groups, ANOVA, $P<0.01$; # significant difference between MDMA 16°C and MDMA 28°C groups, *t*-test, $P<0.05$

Forced swim test

Data from the third and final day of the forced swim test are shown in Fig. 3. Day 3 was the only day on which significant effects were obtained.

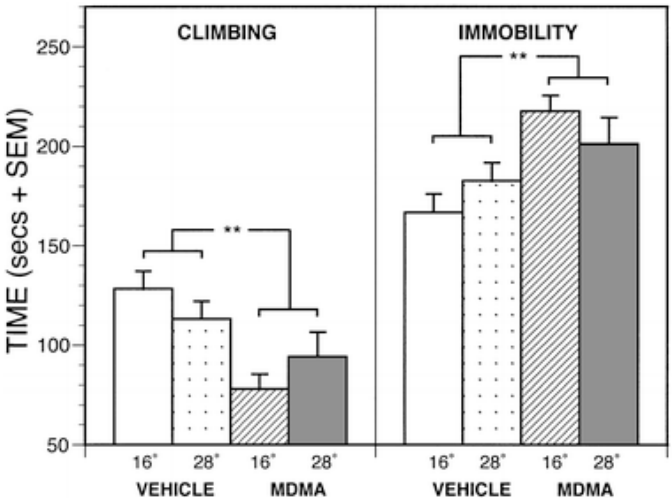

Fig. 3. Results from day 3 of the forced swim test conducted 16–18 weeks after drug administration. The *left hand panel* shows the time spent in active escape attempts (climbing). The *right hand panel* shows the time spent immobile (immobility). While swimming was also measured on day 3, the data are not shown as the values were very low and did not differ significantly between groups. Data represent mean±SEM for $n=8$ rats per condition. ** Significant overall difference between MDMA and vehicle groups, ANOVA, $P<0.01$

On day 1, there was no significant effect of drug pre-treatment on immobility [$F(1,28)=2.74$, $P<0.11$], climbing [$F(1,28)=2.17$, $P<0.16$] or swimming ($F<1.2$). There were no significant effects of temperature on any of these variables and no significant drug by temperature interactions ($F<1.2$).

On day 2, there was no significant effect of drug pre-treatment on immobility ($F<1$), climbing ($F<1$) or swimming

[$F(1,28)=2.40$, $P<0.13$]. There were no significant effects of temperature on any of these variables and no significant drug by temperature interactions ($F<1.5$).

On day 3, there was a significant effect of drug pre-treatment on immobility [$F(1,28)=9.35$, $P<0.01$], climbing [$F(1,28)=10.01$, $P<0.01$], but no effect on swimming ($F<1$). There were no significant effects of temperature on any of these variables and no significant drug by temperature interactions ($F<2.1$).

Neurotransmitter levels

The results of HPLC analysis of neurotransmitter content in key brain regions are shown in Table 3.

Table 3. Results of HPLC analysis. Data are mean (SEM) and represent $n=8$ per condition. All data are in ng/g whole tissue. *A* significant overall effect of drug, *B* significant overall effect of temperature, *C* significant difference between MDMA (16°) and MDMA (28°) groups

Region	Measure	Vehicle (16 °)	Vehicle (28 °)	MDMA (16 °)	MDMA (28 °)	Statistics
Prefrontal cortex	5-HT	366.4 (16.0)	381.8 (22.4)	298.9 (19.2)	280.4 (33.9)	A
	5-HIAA	145.0 (7.1)	154.0 (9.2)	122.0 (9.8)	108.2 (16.0)	A
Striatum	5-HT	399.6 (16.7)	380.2 (21.5)	332.7 (21.7)	315.5 (34.9)	A
	5-HIAA	350.2 (15.3)	313.4 (12.6)	281.7 (5.7)	259.7 (15.4)	A, B
	DA	9473.5 (320.9)	9518.9 (416.1)	8867.6 (296.5)	7852.9 (578.4)	A
Hippocampus	5-HT	331.0 (25.3)	337.3 (7.1)	213.9 (19.6)	213.5 (17.8)	A
	5-HIAA	311.3 (24.4)	274.0 (18.0)	196.9 (19.2)	163.4 (11.8)	A, C
Amygdala	5-HT	579.6 (45.8)	492.0 (49.5)	457.6 (34.3)	407.5 (23.2)	A
	5-HIAA	367.9 (30.9)	342.7 (30.2)	270.1 (15.2)	213.6 (19.4)	A, C

In the prefrontal cortex, there was significantly lower 5-HT [$F(1,28)=13.88$, $P<0.01$] and 5-HIAA [$F(1,28)=10.95$, $P<0.01$] in the MDMA pre-treated rats. There were no significant temperature effects and no drug by temperature interactions ($F<1$).

In the striatum, there was significantly lower 5-HT [$F(1,28)=43.69$, $P<0.0001$] and 5-HIAA [$F(1,28)=38.39$, $P<0.0001$] in the MDMA pre-treated rats. There was also a significant overall effect of temperature on 5-HIAA [$F(1,28)=5.01$, $P<0.05$]. However, further analysis failed to establish a significant difference in 5-HIAA between the MDMA (28°) and MDMA (16°) groups [$t(1,15)=1.56$, $P<0.15$] or between the Vehicle (28°) and Vehicle (16°) groups [$t(1,15)=1.86$, $P<0.09$]. There were no significant drug by temperature interaction effects.

There was also a significant overall effect of MDMA pre-treatment on dopamine in the striatum [$F(1,28)=7.38$, $P<0.05$] with lower dopamine in the MDMA pre-treated rats. There was no temperature effect or drug by temperature interaction for dopamine in the striatum ($F<1.6$).

In the hippocampus, there was significantly lower 5-HT [$F(1,28)=37.81$, $P<0.0001$] and 5-HIAA [$F(1,28)=27.56$, $P<0.0001$] in MDMA pre-treated rats but no significant temperature effect or drug by temperature interaction ($F<2.7$). There was also significantly lower 5-HIAA in the MDMA (28°) group than the MDMA (16°) group [$t(1,15)=2.49$, $P<0.05$].

Finally, in the amygdala, there was again lower 5-HT [$F(1,28)=7.13$, $P<0.05$] and 5-HIAA [$F(1,28)=22.46$, $P<0.001$] in MDMA pre-treated rats with no significant temperature effect or drug by temperature interaction ($F<2.7$). There was also significantly lower 5-HIAA in the MDMA (28°) group than the MDMA (16°) group [$t(1,15)=2.29$, $P<0.05$].

Discussion

The present study has tested the hypothesis that MDMA-induced hyperthermia influences the long-term 5-HT depletion and associated changes in emotionality and cognition produced by the drug. For the most part, the results have been rather surprising, with little apparent influence of MDMA-induced hyperthermia on long-term behavioural and neurochemical outcomes in rats.

The acute changes in body temperature reported here with MDMA are in line with the existing literature, with a clear and consistent hyperthermia at 28°C (Dafters *1994*, *1995*; Broening et al. *1995*; Malberg and Seiden *1998*; Morley et al. *2001*; Gurtman et al. *2002*) and hypothermia at 16°C (Schmidt et al. *1990*; Gordon et al. *1991*; Dafters *1994*; Broening et al. *1995*; Marston et al. *1999*). Acute hyperactivity was also evident in the MDMA-treated rats, in agreement with our previous reports (Morley et al. *2001*; Gurtman et al. *2002*). Interestingly, ambient temperature did not influence locomotor activity on the first day of drug administration when the test environment was novel and exploratory behaviour high. This agrees with earlier results noting no difference in the hyperkinetic response of rats to MDMA at 11°C and 24°C (Dafters *1994*, *1995*). On the second day of drug administration, when the testing environment was no longer novel, higher temperature was associated with decreased locomotor activity in vehicle-treated rats, but not in MDMA-treated animals.

The long-term effects of MDMA on anxiety-like behaviours were as previously reported (Morley et al. *2001*; Gurtman et al. *2002*), with MDMA pre-treated rats showing reduced social interaction relative to controls 8–10 weeks following drug administration. MDMA pre-treated rats also took longer to emerge into a novel open field, and showed less exploration and rearing in that open field. This further consolidates the evidence for a long-term anxiogenic effect of MDMA, albeit perhaps only in rat strains that have lower baseline levels of anxiety (Green and McGregor *2002*; Mechan et al. *2002*). The interesting aspect of the present findings is that hyperthermia at the time of dosing with MDMA does not influence these long-term anxiogenic effects.

The observation that MDMA pre-treated rats also showed poorer object recognition memory is also consistent with our previous report (Morley et al. *2001*). An absence of group differences in overall object inspection times between MDMA and vehicle pre-treated groups shows that the memory impairment was not simply due to a lack of object exploration in MDMA pre-treated rats. Object recognition memory was the only behavioural measure in the present study where rats showing hyperthermia at the time of MDMA administration had a worse prognosis than those showing hypothermia.

A novel and potentially important finding in the present study was the higher immobility and fewer active escape attempts in MDMA pre-treated rats tested in the forced swim model. This test is thought to be a useful animal model of depression, engendering a state of "behavioural despair" that is reversed by antidepressant drug treatment (Blokland et al. *2002*; Cryan et al. *2002*). There has been much speculation that heavy MDMA use in humans may lead to a depressed mood, both in the days immediately following MDMA administration (Parrott and Lasky *1998*) and perhaps more permanently (Schifano et al. *1998*; Topp et al. *1999*; Morgan *2000*; Parrott *2001*). The present study, to our knowledge, provides the first preclinical data that reflects on this issue, and suggests that rats given MDMA 16–18 weeks previously are more prone to depressive-like symptoms when confronted with repeated acute stress. One recent report indicates that MDMA pre-treated rats show a blunted 5-HT response in the hippocampus and prefrontal cortex to immobilization stress (Matuszewich et al. *2002*). Thus the present results might then reflect impairment in the neurochemical circuitry that underlies active coping responses.

The neurochemical data reported here agree with numerous previous studies showing long-term depletion of brain 5-HT and 5-HIAA after MDMA. There was some regional variation evident in this, with more than 40% 5-HT depletion in the hippocampus but less than 20% in the striatum. Some other reports have suggested that the hippocampus may be more vulnerable to the long-term neurotoxic effects of MDMA (Scanzello et al. *1993*; Fischer et al. *1995*). A significant depletion of dopamine was also evident in the striatum of rats given MDMA. Although we did not find significant dopamine depletion in a previous study (Gurtman et al. *2002*), there is a recent report of substantial dopaminergic neurotoxicity in monkeys given short-term exposure to MDMA (Ricaurte et al. *2002*). In general, the vulnerability to dopaminergic insult from MDMA seems to vary greatly across studies and may be highly dependent upon the dose regime and species employed.

The most surprising neurochemical result was that hypothermia at the time of MDMA administration did not protect much against long-term 5-HT depletion. The only neurochemical differences between the MDMA (16°) and MDMA (28°) groups were small and rather subtle: a greater loss of 5-HIAA in the MDMA (28°) group and a tendency towards greater dopamine depletion in this group. The lower 5-HIAA in the MDMA (28°) group may reflect decreased 5-HT uptake and metabolism in the amygdala and hippocampus of these rats, possibly indicative of a greater degree of neurotoxic damage in these regions. It is conceivable then that the poorer performance of MDMA (28°) rats in the novel object recognition task may in some way relate to such differences in the hippocampus and amygdala, or possibly the greater dopamine depletion in this group that is evident in the striatum.

The finding of long-term 5-HT depletion despite an acute hypothermia with MDMA agrees with the results of Marston et al. (*1999*) and also with observations of acute hypothermia followed by sizeable 5-HT loss with the other substituted amphetamine drug fenfluramine (Malberg and Seiden *1997*). However, the present results are inconsistent with previous indications that hypothermia might offer complete protection against MDMA-induced 5-HT depletion

(Malberg and Seiden *1998*). There are several factors that might potentially explain these major differences in outcome. One potentially important factor could be the age of the rats used. Broening and colleagues (*1995*) reported that MDMA-induced 5-HT depletion depends not only upon body temperature at the time of acute treatment but also the age of the rats. Thus 40-day-old male Sprague-Dawley rats were protected from MDMA induced 5-HT depletion when the drug was given at 10°C but not 25°C, while 70-day-old rats showed substantial 5-HT depletion even when MDMA was given at 10°C. Since the rats used in the present study were aged approximately 60–75 days old at the time of dosing, and were older than those used by Malberg and Seiden (*1998*), this could be a major factor in explaining our results.

Another important factor could be returning rats to group housing immediately after MDMA dosing, a procedure that might prolong hyperthermia and promote aggregation toxicity (see Green et al. *1995*). However at least one report suggests that individual housing and group housing are associated with equivalent long-term 5-HT depletion after MDMA given at ambient temperatures of 21°C (Broening et al. *1995*). We also note that returning hypothermic MDMA-treated rats to their home cages at 22°C does not produce hyperthermia in our rats (McGregor and Clemens, unpublished data).

Other factors of likely importance may include the dose regime and time-course of MDMA treatment used, and also the gap between acute treatment with MDMA and neurochemical assessment. Serotonergic parameters show a gradual recovery in the weeks and months after MDMA treatment (Scanzello et al. *1993*), It might then be the case that leaving 4 months between MDMA treatment and neurochemical assessment, as was done in the present study, will allow more time for recovery from 5-HT depletion and thereby minimise differences in 5-HT and 5-HIAA across groups dosed at different ambient temperatures.

Finally, it is interesting to speculate on the implications of the present results for human MDMA users. Animal models, such as those used here, allow the long-term effects of MDMA to be assessed in a way that avoids the confounds of polydrug use, drug purity and pre-drug psychopathology that compromise human MDMA research (Boot et al. *2000*). The present study, in conjunction with its predecessors (Morley et al. *2001*; Gurtman et al. *2002*), suggests a strong causal link between MDMA exposure and subsequent increases in anxiety, decreased social behaviour, impaired memory and loss of active coping in response to stress. This is consistent with increasing evidence from human studies that MDMA users are more vulnerable to mood disorders and memory dysfunction (Schifano et al. *1998*; Topp et al. *1999*; Morgan *2000*; Parrott *2001*). While the advice to human MDMA users to "chill out" while on the drug is undoubtedly of use in preventing acute hyperthermic reactions that may occasionally be life-threatening, the present study raises some doubts that cooler temperatures will prevent long-term neurotoxicity and related long-term adverse emotional effects of the drug.

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