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MDMA- and DPAT-induced hypothermia: Role and location of 5-HT_{1A} receptors

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Abstract

The popular drug of abuse 3,4 methylenedioxymethamphetamine (MDMA) has complex interactions with thermoregulatory systems resulting in either hyperthermia or hypothermia. MDMA induces hypothermia when given to animals housed at a low ambient temperature. In this study we report that MDMA (7.5 mg/kg i.p.) given at normal ambient temperatures of 24-25°C caused, in conscious freely moving rats, hypothermia (mean decrease from baseline of 1.1±0.06°C at 40 minutes). Pretreating animals with a 0.5 mg/kg intraperitoneal dose of the 5-HT1A antagonist N-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-N-2-pyridinylcyclohexanecarboxamide (WAY 100635 or WAY) not only prevented MDMA-induced hypothermia, but resulted in the development of hyperthermia (mean temperature increase from baseline of 0.74±0.2°C at 120 minutes). After treatment with WAY, MDMA also elicited an enhanced tachycardia (mean increases in heart rate from baseline of 110±16 beats per minute at 90 minutes). To identify the location of 5-HT1A receptors responsible for hypothermia induced by MDMA, we first investigated the role of 5-HT1A receptors in the rostral raphe pallidus (rRP) in decreases in temperature evoked by the known 5-HT1A agonist 8-Hydroxy-2-di-n-propylamino-tetralin (DPAT). Microinjections of 0.5 nmol of WAY into the rRP significantly attenuated DPAT (0.2 mg/kg, i.p.) elicited hypothermia. In parallel experiments, we found that microinjections of WAY into the rRP, while significantly augmenting MDMA-mediated tachycardia, did not alter body temperature. These results demonstrate that while hypothermia mediated by both MDMA and DPAT share a common dependence on the activation of 5-HT1A receptors, the location of these receptors is different for each drug.
Introduction

Persons using substituted amphetamines such as 3,4-methylenedioxymethamphetamine (MDMA) may, by mechanisms not completely understood, develop severe hyperthermia and cardiovascular collapse (Gowing et al., 2002). While hyperthermia remains the best studied physiologic effect related to MDMA usage, hypothermia can also occur, a phenomenon that is often ignored or when acknowledged attributed to low ambient temperatures (Malberg and Seiden, 1998). MDMA, however, can evoke decreases in body temperature at normal or even elevated ambient temperatures (Malberg et al., 1996; Bexis and Docherty, 2006). Even in studies in which MDMA causes hyperthermia, a transient initial period of hypothermia or a delay in the development of hyperthermia is often seen (Gordon et al., 1991; Malberg et al., 1996). To date few studies have addressed the mechanisms responsible for MDMA-induced hypothermia.

MDMA’s principal pharmacologic action is facilitation of transmission at monoaminergic nerve terminals causing the release of monoamines in the CNS and more specifically elevating extracellular levels of serotonin, dopamine and norepinephrine (Rothman et al., 2001; Gough et al., 2002; Sprague et al., 2007) all of which have been implicated in hyperthermia induced by MDMA. The mechanisms behind hypothermia induced by MDMA are however largely unknown. Serotonin can elicit opposite effects on body temperature depending on whether it binds to serotonergic 1A (5-HT₁A) or 2A (5-HT₂A) receptors. The divergent effects of serotonin on body temperature present an attractive hypothesis for changes in temperature induced by MDMA. To date, the role of serotonin in body temperature changes evoked by MDMA has focused on 5-HT₂A receptors with 5-HT₂A antagonists reducing hyperthermia and neurotoxicity elicited by MDMA (Schmidt et al., 1990). 5-HT₂A receptors are involved in hyperthermia evoked
by MDMA by impairing heat dissipation through the constriction of cutaneous vascular beds (Blessing et al., 2003; Ootsuka et al., 2004), and by increasing heat generation through interscapular brown adipose tissue (IBAT) (Blessing et al., 2006). While 5-HT$_{2A}$ receptors contribute to MDMA-mediated hyperthermia, the role of 5-HT$_{1A}$ receptors in temperature effects elicited by MDMA is largely unknown. As serotonin has a $>1000$-fold affinity for 5-HT$_{1A}$ receptors over that of 5-HT$_{2A}$ receptors (Peroutka et al., 1981), its release by MDMA likely activates 5-HT$_{1A}$ receptors. Activation of 5-HT$_{1A}$ receptors by agonists like 8-Hydroxy-2-di-n-propylamino-tetralin (DPAT) cause, similar to MDMA, the serotonin behavioral syndrome (Goodwin et al., 1987) and in a manner opposite to 5-HT$_{2A}$ receptor activation, hypothermia (Gudelsky et al., 1986; Ootsuka and Blessing, 2003). Along with their individual contributions, 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors have complex interactions affecting body temperature. Hyperthermia elicited by stimulating 5-HT$_{2A}$ receptors with (±)-1-(2,5-Dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI), is potentiated by the 5-HT$_{1A}$ antagonist N-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-N-2-pyrindinlcyclohexanecarboxamide (WAY 100635 or WAY), and prevented by the 5-HT$_{1A}$ agonist DPAT (Salmi and Ahlenius, 1998). Similarly WAY 100635 augments the hyperthermia induced by MDMA in a warm environment (Saadat et al., 2005). Together these data suggest that the overall effect of MDMA on body temperature may reflect the net sum of the combined activities of serotonin on 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors.

Muscle activity represents a key component of thermoregulation in the rat in the form of shivering and work related motor activity. A direct relationship exists between work intensity and core body temperature (Gordon, 1990). MDMA increases locomotion in rats, a factor likely
contributing to the development of hyperthermia (Spanos and Yamamoto, 1989). Locomotion by itself however, is insufficient to explain MDMA’s effects on body temperature as the degree of locomotion induced by a dose MDMA is similar regardless of whether the animal develops hypo or hyperthermia, depending on the ambient temperature in which it is given (Dafters, 1994). The role 5-HT1A receptors play in the locomotor effects induced by MDMA is unclear as 5-HT1A antagonists have been reported to both cause reductions and have no effect on MDMA-evoked locomotion (Callaway et al., 1992; McCreary et al., 1999).

The cardiovascular system plays a critical role in normal thermoregulation. Activation of the sympathetic nervous system in response to a cold environment results in the constriction of cutaneous vessels, along with an increase in heart rate and cardiac output (Blessing, 2004; Deussen, 2007). The net result of these effects is to conserve heat by shunting blood away from a cool skin surface while simultaneously supporting heat generation by diverting blood to heat producing central organs and brown adipose tissue. The reverse of these effects occurs in a warm environment facilitating heat release (Ootsuka and Blessing, 2006). While MDMA’s effects on cutaneous blood flow have been well studied (Pedersen and Blessing, 2001), it’s effects on other components of the cardiovascular system are less well known. While causing tachycardia in humans (Lester et al., 2000), the cardiovascular effects of MDMA in rats have not been clearly delineated as both an increase (Gordon et al., 1991) as well as no effect on heart rate has been reported (Irvine et al., 2001; Bexis and Docherty, 2006).

The rostral raphe pallidus (rRP) is a serotonin-rich region of the medulla containing sympathetic thermoregulatory premotor neurons that control the sympathetic innervation of cutaneous blood
vessels and IBAT in the rat (Dimicco et al., 2006). Neurons in the rRP are known to express 5-HT1A receptors (Helke et al., 1997) and studies to date have demonstrated that microinjections of DPAT into the rRP blocks thermogenesis induced by leptin and cutaneous vasoconstriction induced by cold (Morrison, 2004; Ootsuka and Blessing, 2006). Likewise microinjections of DPAT into various regions of the brainstem and rRP inhibit the cardioexcitatory responses to leptin and to hypothalamic stimulation (Morrison, 2004; Horiuchi et al., 2005).

As the cardiovascular and thermogenic responses to various stressors share similar hypothalamic and brainstem mechanisms (Dimicco et al., 2006), we sought to investigate the role of 5-HT1A receptors in the region of the rRP of conscious freely-moving rats in: (1) the thermoregulatory, cardiovascular, and locomotor effects induced by MDMA and (2) the hypothermia induced by systemic administration of DPAT.
Methods:
All procedures conformed to the guidelines set for by the NIH and were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee.

Drugs:
For systemic administration, WAY 100635 (WAY; Sigma, St. Louis, Mo, USA) was dissolved to concentration with normal saline and used the day of the experiment. For microinjections, WAY 100635 was dissolved in 0.1M phosphate buffered saline and used on the same day or frozen at -80°C and used within 2 weeks. 8-Hydroxy-2-di-n-propylamino-tetralin (DPAT; Sigma, St. Louis, Mo, USA) was dissolved in normal saline and used the day of the experiment. ± 3,4-methylenedioxymethamphetamine (MDMA) was generously provided by the NIH and prepared at the time of the experiments in normal saline.

Animals
Male Sprague-Dawley rats (325 ± 25 g; Harlan, Indianapolis, IN, USA) were maintained in a 12-h light: 12-h dark cycle beginning at 7am and fed ad libitum. Animals were singly housed to avoid interference from competing radiotelemetric signals and to avoid potential chewing or damage to CNS guide cannulas from other animals.

Surgical preparation
For studies employing MDMA and WAY 100635, rats were anesthetized (80 mg/kg ketamine and 11.5 mg/kg xylazine i.p., supplemented as required) and a telemetric transmitter (Model C50-PXT; Data Sciences, St. Paul, MN, USA) was implanted as previously described for the
measurement of mean arterial pressure (MAP), heart rate, locomotion and core body temperature (Zaretsky et al., 2003). For studies with DPAT and in determining the effects of different doses of MDMA on body temperature, a telemetric transmitter measuring only core temperature and locomotion (Model TA-F40; Data Sciences, St. Paul, MN, USA) was implanted in the abdominal cavity.

For experiments involving microinjections into the rRP, rats were anesthetized five days after transmitter implantation and placed in a stereotaxic apparatus with the incisor bar set 3.3 mm below the interaural line for placement of a guide cannula as previously described (Zaretsky et al., 2003). The target coordinates were: anterior-posterior -2.8 mm; left-right 0.0 mm; height-depth -1.1 mm; interaural line as reference point. Dummy wire cannulas were inserted in the guides, and rats were returned to their cages and left undisturbed until regaining their approximate pre-surgery weights (approximately 5-7 days).

**Experimental Procedures**

Experiments were conducted in the animal’s home cage in quiet and isolated rooms between 10:00 a.m. and 2:00 p.m. Animals acclimated to their new environment for a minimum of two hours before experiments were initiated. Throughout the experiment ambient temperature was maintained at 24–25 °C, a temperature typically associated with MDMA induced hyperthermia (Malberg and Seiden, 1998). In all the experiments, animals were randomized to study groups in blocks of 8 using a random number generator.

*Dose Response*
To determine the dose causing hypothermia and the time of maximum effect, rats received an intraperitoneal dose of either saline, or one of three doses of MDMA (1.5 mg/kg, 7.5 mg/kg, 15 mg/kg). We recorded temperature in degrees celsius (°C) and locomotion in units of activity (a.u.) every minute for 120 minutes after vehicle or MDMA.

Effect of systemic treatment with WAY 100635 on MDMA-induced hypothermia

Animals were assigned to one of four groups (Saline/Saline, WAY/Saline, Saline/MDMA, WAY/MDMA; n=8 rats/group). After an acclimation period, each animal received a 0.3 ml intraperitoneal injection of either saline vehicle (0.9%) or WAY 100635 (0.5 mg/kg) followed five minutes later by an equal volume of either saline or MDMA (7.5 mg/kg). Temperature (°C), locomotion (a.u.), heart rate in beats per minute (bpm) and mean arterial pressure in millimeters of mercury (mmHg) were recorded every minute for 120 minutes after MDMA.

Effect of microinjection of WAY 100635 into the rRP on DPAT-induced hypothermia

To confirm the dosing of WAY 100635 for microinjection experiments, we investigated the role of 5-HT1A receptors in the region of the rRP in body temperature decreases elicited by systemic administration of the 5-HT1A agonist DPAT. In these experiments, telemetric probes measured only temperature and locomotion. Animals were assigned to one of four groups (PBS/Saline, WAY/Saline, PBS/DPAT, WAY/DPAT). The dummy cannula was removed and a microinjector (33 gauge, Plastics One) connected to a 10-µl Hamilton syringe with Teflon tubing (ID 0.12 mm; OD 0.65 mm; Bioanalytic Systems, West Lafayette, IN) was placed through the guide cannula and positioned above the region of the rRP. The syringe was mounted in an infusion pump (KD Scientific, Holliston, MA) that was used to deliver 100 nL of a solution of either phosphate
buffered saline (PBS, 0.1M) vehicle or WAY 100635 (5 mM solution; 0.5 nmol) over 30 seconds. Five minutes after microinjection, animals received an intraperitoneal injection of a solution (0.3ml) of either 0.9% saline vehicle or DPAT (0.2 mg/kg). Preliminary studies demonstrated that 0.2 mg/kg of DPAT evoked a decrease in body temperature similar in magnitude to that produced by 7.5 mg/kg MDMA (data not shown). We recorded temperature (°C) and locomotion (a.u.) every minute for 120 minutes after DPAT injection. Two days later, animals receiving PBS/Saline crossed over to receive WAY/Saline and those animals originally receiving WAY/Saline crossed over to PBS/Saline. In a similar fashion, animals originally receiving PBS/DPAT crossed over to receive WAY/DPAT and vice versa. To assess the potential impact of damage caused by the placement of the guide cannula and microinjector into the region of the rRP on the response to DPAT, animals completing the above experiments, received an i.p. injection of DPAT (0.2 mg/kg). We defined a positive response to DPAT as a decrease of greater than 0.5°C within 30 minutes of injection. Data from four rats who failed to respond to DPAT were excluded from the final analysis. Fluorescent microspheres, 0.04 µm in diameter (Invitrogen, Carlsbad, California), were included in the microinjection solutions at a concentration of 0.25% in order to mark the exact site of injection. After the last session, rats were injected with pentobarbital (100 mg/kg), a 14 gauge feeding tube was placed in the left ventricle and the animals were perfused in situ with 30 ml of cold saline (4°C) followed by 30 ml of 4% paraformaldehyde. The brains were then removed and postfixed in 4% paraformaldehyde overnight, or longer, followed by placement in a solution saturated with 30% sucrose. Brains were cut into 40-µm coronal sections on a freezing microtome and injection sites were determined using a fluorescent microscope according to the atlas of Paxinos and Watson (Paxinos and Watson, 1998) by an observer blinded to group allocation.
Effect of microinjection of WAY 100635 into the rRP on MDMA-induced hypothermia

After determining microinjection of 0.5 nmol of WAY 100635 in the region of the rRP attenuated hypothermia elicited by systemic administration of DPAT, we sought to determine whether microinjections of WAY 100635 could similarly prevent hypothermia evoked by MDMA. Animals were assigned to one of four groups (PBS/saline, WAY/saline, PBS/MDMA, WAY/MDMA). As in the DPAT experiment described above, animals receiving i.p. injections of saline received microinjections of either PBS or WAY 100635 in a cross over fashion. In order to avoid the possibility of MDMA-induced serotonin neurotoxicity confounding the effect on body temperature, animals receiving MDMA were not crossed over. Animals were microinjected into the region of the rRP with 100nl of PBS (0.1 M) or WAY 100635 (5 mM solution; 0.5 nmol) over 30 seconds depending on group assignment. Five minutes later animals received an intraperitoneal injection of 0.3 milliliters of either saline vehicle or MDMA (7.5 mg/kg). We recorded temperature (°C), locomotion (a.u.), heart rate (bpm) and mean arterial pressure (mmHg) every minute for 120 minutes after MDMA injection. Injection sites were verified using fluorescent microspheres as described above.

Data Analysis

Data were captured using a receiver placed under the rat’s cage and transmitted to an IBM compatible computer using the Dataquest A.R.T data acquisition system (Data Sciences, St. Paul, MN, USA). Statistics were run using SPSS 14.0 for Windows (Chicago, Illinois). Graphs were made using Prism 4.0 (Graphpad Software, San Diego, Ca, USA) software. Grouped data are reported as 5 minute means ± one standard error measurement for the time points shown. To
compare changes in temperature and activity from baseline in the dose response experiment we performed a one way repeated measure ANOVA with pair wise comparisons done using the LSD procedure. For the remainder of the studies we performed a two way full factorial repeated measures analysis of variance. We included in the model main effects for study group, time, and their interaction. We performed post hoc comparisons among time points within a study group and between study groups within a time point. Pair wise comparisons were adjusted using a LSD procedure.
Results

Dose response of MDMA on temperature and locomotion (Figure 1)

Neither saline nor 1.5 mg/kg of MDMA significantly affected temperature or locomotion. At a dose of 7.5 mg/kg, MDMA evoked a purely hypothermic response with a maximum temperature decrease of 1.1±0.06°C from baseline (37.2±0.1°C) and returning to baseline by 80 minutes. Conversely, a 15 mg/kg dose of MDMA elicited a purely hyperthermic response with a maximum increase in temperature of 1.6±0.3 °C from baseline (37.0±0.08°C) occurring 120 minutes after injection. Although causing divergent effects on body temperature, doses of 7.5 mg/kg and 15 mg/kg of MDMA evoked similar increases in motor activity.

Effect of systemic treatment with WAY 100635 on MDMA-induced changes (Figure 2)

There were no differences in baseline values of any measured parameter among any of the treatment groups.

Temperature (Figure 2A)

Injections of saline had no effect on body temperature. Injections of WAY 100635 followed by saline however caused an increase in body temperature that by thirty minutes was significantly different from both its baseline (+0.8±0.2°C, p=0.002) and from the corresponding 30 min values of all other groups. The effects of WAY 100635 were transient however returning to baseline values by 60 minutes. The injection of saline followed by MDMA (Saline/MDMA) caused declines in body temperature at both 30 minutes (-0.9±0.2°C, p=0.001) and 60 minutes (-0.8±0.3°C, p=0.007) that were significant compared to both its own baseline and to the corresponding values of all other groups. Not only did pretreatment with WAY 100635 prevent
MDMA induced hypothermia, but animals treated with WAY followed by MDMA actually developed hyperthermia with temperatures significantly increasing 0.7±0.2°C from baseline at both 90 and 120 minutes (p<0.01 for both).

**Locomotion (Figure 2B)**

Outside of transient effects, secondary to the stress of the intraperitoneal injections, neither the injection of Saline/Saline nor WAY/Saline resulted in significant increases in locomotion. Regardless of pretreatment, the injection of MDMA resulted in significant increases in locomotor activity at every time point after its injection. Pretreatment with WAY attenuated the increases in locomotion induced by MDMA at 30 minutes (9.8±3 vs. 21.2±3 a.u., p=0.019) but by 60 minutes these effects were no longer evident (p=0.2).

**Heart Rate (Figure 2C)**

In all the groups, regardless of the pretreatment, the first injection (represented by 0 min) resulted in an increase in heart rate. In the Saline/Saline group no further increases were seen. In those animals injected with WAY/Saline however a significant increase in heart compared to baseline was still evident at 30 minutes (+51±18 bpm, p=0.009). The injection of saline followed by MDMA caused a steady increase in heart rate beginning at 30 minutes and becoming significantly increased compared to baseline by 90 minutes (+79±18 bpm, p<0.001). In those rats pretreated with WAY 100635, increases in heart rate after the injection of MDMA were immediate and sustained throughout the experiment. At both 30 and 60 minutes, values for heart rate in the WAY/MDMA group were greater then those of any of the other groups.
**Mean Arterial Pressure (Figure 2D)**

Similar to heart rate, mean arterial pressure transiently increased after pretreatment injections (represented by 0 min) in each group. In only those animals injected with WAY 100635 followed by MDMA however were increases in MAP significant compared to baseline at subsequent time points.

**Identification of microinjection sites: (Figure 3)**

Microinjection sites were determined using different colored fluorescent microspheres with the corresponding centers of the visualized microspheres plotted on representative drawings adapted from Paxinos and Watson (Paxinos and Watson, 1998) and shown in figure 3D. Shown in figures 3A-C are example fluorescent micrographs with the matching counter stained section. We considered positive injections as those in which the center of the fluorescence was contiguous with or within the area of the raphe pallidus.

**Microinjection of WAY 100635: DPAT (Figure 4)**

There were no differences in baseline values of any measured parameter among any of the treatment groups.

**DPAT: Core Body Temperature (Figure 4A)**

Microinjections of either 0.5 nmol of WAY 100635 (100nl, 5 mM) or PBS (100nl) into the region of the rostral raphe pallidus (rRP) followed by the intraperitoneal injection of saline caused a transient increase in core body temperature (Saline/Saline and WAY/Saline). As expected microinjection of PBS followed by intraperitoneal injection of 0.2 mg/kg of the 5-HT$_{1A}$
agonist DPAT (PBS/DPAT) caused a rapid decline in core body temperature. Temperatures in the PBS/DPAT group were significantly below baseline for the entire post injection period with the nadir occurring at 30 minutes (-1.4±0.2°C from baseline, p<0.001). The rRP was shown to be a key area involved in DPAT induced hypothermia as microinjections of WAY 100635 (0.5 nmol) in the rRP significantly attenuated the drop in temperature (-0.4±0.3°C, p=0.004) seen at 30 minutes in the PBS/DPAT group. If microinjections of WAY 100635 fell outside of the region of the rRP (WAY/DPAT (OR) reductions in DPAT evoked hypothermia were no longer evident at 30 minutes as decreases in body temperature (-1.4°C ±0.3°C) were identical to those seen in the PBS/DPAT (-1.4±0.2°C, p=0.6) group.

**DPAT: Locomotion (Figure 4B)**

As the locomotor effects elicited by DPAT are short lived, we added the 15 minute time point to the analysis of motor activity. Microinjections of either PBS or 0.5 nmol of WAY 100635 followed by the systemic injection of saline (PBS/DPAT and WAY/DPAT) had no effect on locomotor activity. All of the groups given intraperitoneal injections of DPAT (PBS/DPAT and WAY/DPAT) demonstrated significant and similar increases in locomotion 15 minutes post injection.

**Microinjection of WAY 100635: MDMA (Figure 5)**

There were no differences in baseline values of any measured parameter among any of the treatment groups.

**MDMA: Core Body Temperature (Figure 5A)**
Microinjections of PBS followed by the intraperitoneal injection of saline (PBS/Saline) increased core body temperature at both 30 and 60 minutes post injection in a manner comparable to that seen in the PBS/DPAT group (Figure 4A) in the previous experiment. Likewise, the microinjection of WAY 100635 followed by i.p. saline (WAY/Saline) increased body temperature, although differences from baseline did not achieve significance. Different from the systemic administration of saline or WAY 100635 followed by MDMA (7.5 mg/kg i.p.) (Figure 2A), microinjections of PBS or WAY 100635 followed by systemic MDMA (PBS/MDMA and WAY/MDMA) did not significantly reduce core body temperature compared to baseline values. While the core temperature for each MDMA group was significantly different at 30 minutes from their respective controls (37±0.2°C for PBS/MDMA vs 38±0.2°C for PBS/Saline, p=0.002 and 37.1±0.2°C for WAY/MDMA vs. 37.8±0.2°C for WAY/Saline, p=0.039) they were not different from one another (p=0.8).

**MDMA: Locomotion (Figure 5B)**

Systemic injections of MDMA significantly increased locomotion at all time points after its injection. Unlike the systemic administration of WAY 100635 (figure 2B) which attenuated MDMA mediated locomotion, microinjections of WAY 100635 had no affect on locomotion induced by MDMA.

**MDMA: Heart Rate (Figure 5C)**

As seen with Temperature (Figure 5A) the microinjection procedure caused a stress response in the animals manifested as transient increases in heart rate in both the PBS/Saline and WAY/Saline groups. Microinjections of PBS (100nl) followed by systemic administration of
MDMA (PBS/MDMA) caused a gradual increase in heart rate that was significantly different from baseline by 60 minutes (+42±17 bpm, p=0.02) and from then on. As with the systemic administration of WAY 100635 followed by MDMA (figure 2B), microinjections of WAY 100635 (0.5 nmol) into the rRP resulted in significant increases in MDMA mediated tachycardia at 30 minutes and from that point on compared to all other groups, except the WAY/MDMA (OR) group where numbers of animals were too small (n=4) for comparison. In animals with microinjection cannulae misplaced outside the rRP (OR), microinjections of WAY 100635 failed to alter significantly the effect of systemically administered MDMA on heart rate as compared to the PBS/MDMA.

**MDMA: Mean Arterial Pressure (Figure 5D)**

Except for the transient increases in mean arterial pressure seen after microinjections, consistent and sustained elevations in mean arterial pressure did not occur in any of the groups. This is different from that seen after the systemic administration of WAY 100635 followed by MDMA (Figure 2) where mean arterial pressure increased in a manner similar to heart rate.
Discussion

This study presents new findings regarding the physiologic changes induced by MDMA and DPAT. Our results indicate that the 5-HT$_{1A}$ antagonist WAY 100635 (WAY) not only prevents hypothermia mediated by MDMA at room temperature but also results in the development of hyperthermia. The effects of WAY 100635 on temperature changes elicited by MDMA are independent of locomotion, as WAY 100635 attenuates early increases in locomotion mediated by MDMA. In a similar manner to its effects on temperature, WAY 100635 unmasks significant MDMA-mediated tachycardia. Analogous to MDMA, systemic administration of DPAT causes hypothermia and for the first time we demonstrate that, the location of 5-HT$_{1A}$ receptors responsible for DPAT-induced hypothermia are located, at least in part, in the region of the rostral raphe pallidus (rRP). The location of 5-HT$_{1A}$ receptors involved in suppressing MDMA mediated tachycardia, but not those in mediating hypothermia, are likewise in the rRP.

A potential confounder in our results was that when administered systemically, WAY 100635 alone increased temperature and heart rate (Figure 2A and 2C). That WAY 100635 alone can increase temperature has been previously reported (Saadat et al., 2005). We do not however believe that the differences seen between animals treated with WAY/MDMA and Saline/MDMA is simply via a competitive physiologic mechanism. For one, the degree to which WAY 100635 alone increases temperature and heart rate cannot by itself account for the changes seen between WAY/MDMA and Saline/MDMA animals. Secondly, the effects WAY 100635 alone had on temperature and heart rate were transient while its effects on changes in temperature and heart rate induced by MDMA were more sustained.
Serotonin has long been implicated in the regulation of body temperature with initial studies showing that its microinjection into the anterior hypothalamus caused hypothermia (Cox et al., 1980). Contrary to this however, the systemic injection of drugs like MDMA that increase the central release of serotonin typically cause hyperthermia (Gordon et al., 1991). These seemingly conflicting findings may be due to the opposing effects of serotonin on 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors. Agonists of 5-HT$_{1A}$ receptors, such as DPAT, cause dose dependent hypothermia (Gudelsky et al., 1986) by increasing heat loss through the dilation of cutaneous vessels and by decreasing heat generation through inhibition of interscapular brown adipose tissue (IBAT) (Ootsuka and Blessing, 2006; Ootsuka and Blessing, 2006). Activation of 5-HT$_{2A}$ receptors on the other hand, causes hyperthermia through cutaneous vasoconstriction and by increasing heat production in brown adipose tissue (Ootsuka et al., 2004; Ootsuka and Blessing, 2006). Therefore drugs, such as MDMA, which cause a large release in serotonin may affect both 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors. Since serotonin has $>1000$ times the affinity for 5-HT$_{1A}$ receptors then 5-HT$_{2A}$ receptors (Peroutka et al., 1981), a low dose of MDMA might affect 5-HT$_{1A}$ receptors to a greater extent thereby causing hypothermia. Conversely, higher doses of MDMA could conceivably result in the activation of enough 5-HT$_{2A}$ or other non serotonin receptors to cause hyperthermia. We found evidence supporting this as a 7.5 mg/kg dose of MDMA caused pure hypothermia and 15 mg/kg of MDMA causes predominantly hyperthermia. On closer inspection of Figure 1, the initial increase in temperature after 15 mg/kg of MDMA is not evident until 30 minutes after injection suggesting that 5-HT$_{1A}$ receptor activation may have delayed the development of hyperthermia. We do not think this delay is secondary to drug absorption as decreases in temperature after 7.5 mg/kg of MDMA are evident within 15 minutes of injection. These results are similar to those of other studies in which doses of 40, 20, 15, and
10 mg/kg of MDMA produced either an initial hypothermia followed by hyperthermia or a delay in the development of hyperthermia (Gordon et al., 1991; Malberg et al., 1996). As doses of MDMA increase, the decrease in the initial hypothermia was likewise less (Malberg and Seiden, 1998). Data reported by Abdel-Fattah and colleagues supports the idea that lower concentrations of serotonin may preferentially activate 5-HT\textsubscript{1A} receptors causing hypothermia. In their study they demonstrated that rats systemically administered the serotonin precursor tryptophan and the monoamine oxidase inhibitor pargyline developed biphasic temperature responses, and that hypothalamic levels of 5HT were lower during the hypothermic phase and higher during the hyperthermic phase (Abdel-Fattah et al., 1997). Furthermore these authors showed that administering the 5-HT\textsubscript{1A} antagonist pindolol prevented the hypothermic phase caused by tryptophan and pargyline (Abdel-Fattah et al., 1995). In a similar fashion, the systemic administration of WAY 100635 prevented hypothermia evoked by the serotonin releasing agent dextenfluramine (Cryan et al., 2000). As hyperthermia correlates with neurotoxicity induced by MDMA, the finding in our study that 5-HT\textsubscript{1A} antagonists increase hyperthermia and the corollary that 5-HT\textsubscript{1A} agonists might prevent hyperthermia could lead to better selection of drugs to treat life threatening hyperthermia. An example is that the beta blocker propranolol has on occasion been suggested a treatment for toxicity from MDMA or in cases of serotonin syndrome (Jones and Story, 2005). As propranolol is a 5-HT\textsubscript{1A} antagonist, its usage could actually result in worsening hyperthermia.

The fact that other authors have not consistently reported MDMA-induced hypothermia may be due in part to the dose of MDMA used and the method of temperature measurement. Most of the studies to date reporting MDMA-induced hypothermia at room temperature use radiotelemetry to
measure body temperature (Berkey et al., 1990; Bexis and Docherty, 2006). As handling and rectal temperature measurements significantly increase body temperature and serotonin release (Berkey et al., 1990; Adell et al., 1997) animals given MDMA in which these methods of temperature measurement are employed may have increased amounts of serotonin release over animals in which biotelemetry is used to measure temperature. As we have previously discussed higher concentrations of serotonin may result in the activation of 5-HT$_{2A}$ or non-serotonergic receptors and hence less hypothermia. Although we saw very reproducible reductions in body temperature to 7.5 mg/kg of MDMA between the dose response experiment and the experiment in which WAY 100635 was systemically administered, the degree of hypothermia elicited by 7.5 mg/kg of MDMA when microinjections preceded systemic MDMA administration (Compare Figures 1 and 2A with Figure 5A) was less. One possible explanation to this may be that microinjection of PBS into the rRP causes local stimulation of neurons causing elevations in temperature and heart rate (See Figures 4 and 5). Another similar explanation may be the stress of the procedure itself. As the procedure requires the placement of a needle injector into a guide cannula followed several minutes later by its removal; it requires the investigator to be in proximity of the animal for a longer period of time then a simple i.p. injection. Lending support to either of these ideas, control animals (PBS/ Saline figure 5A) microinjected with PBS in rRP followed by i.p. saline injection had larger increases from baseline in core temperature temperatures at 30 minutes (+0.8±0.2°C) compared to the control animals receiving two i.p. doses of saline (Saline/Saline in Figure 2) and no microinjection (+0.2°C ±0.2). Another possible explanation for the reduced MDMA-mediated hypothermia when drugs were microinjected is that the placement of the microinjection cannula may have damaged neurons in the rRP reducing hypothermia induced by MDMA. We could account for this possibility in the experiments with
DPAT by making sure that animals with microinjection cannula in the rRP were capable of developing hypothermia to DPAT and eliminating those that did not from the final analysis. We did not do a similar screening procedure with MDMA as we were concerned that the neurotoxicity of MDMA might potentially affect the responses to further doses of MDMA.

Similar to Callaway and Kehne we showed that blocking 5-HT1A receptors significantly reduced MDMA mediated locomotion (Callaway et al., 1992). That decreases in MDMA mediated locomotion in animal pretreated with WAY 100635 occurred at 30 but not 60 minutes is consistent with findings reported by Kehne and colleagues (Kehne et al., 1996). In their study, they suggest that early (0 – 30 min) locomotion induced by MDMA may be dependent on activation of 5-HT1A and dopamine D2 receptors, while later increases (30-60 min) depend on activation of 5-HT2A receptors (Kehne et al., 1996). Our results conflict however with those of McCreary and colleagues who reported that Way 100635 had no effect on MDMA (3 mg/kg s.c.) mediated locomotion (McCreary et al., 1999). Differences between our studies may be explained by the lower dose of MDMA used or in the method of analysis; McCreary et al. analyzed summed data over a 90 minute period.

While administration of MDMA increases heart rate and systolic blood pressure in human volunteers (Lester et al., 2000) few studies have studied the MDMA’s cardiovascular effects in animals. Administering 20 mg/kg of MDMA subcutaneously to five Long Evan rats, Gordon et al. reported a steady rise in the heart rate of three animals and dramatic increases in both heart rate and temperature in two rats who subsequently died (Gordon et al., 1991). Irvine and colleagues gave MDMA in a range of doses from 5 to 20 mg/kg subcutaneously to female
Sprague Dawleys at a temperature of 20°C and did not report increases in heart rate and blood pressure (Irvine et al., 2001). At a temperature of 30°C however, all doses of MDMA tested increased heart rate and blood pressure (Irvine et al., 2001). Bexis and colleagues recently reported that a dose of MDMA (20 mg/kg s.c.) that caused a purely hypothermic response did not significantly effect heart rate, but did increase mean arterial pressure (Bexis and Docherty, 2006). In our study, intraperitoneal injections of saline followed by MDMA (7.5 mg/kg) at an ambient temperature of 24-25°C increased heart rate but not mean arterial pressure. Differences between the strains of rats used in our study (Sprague-Dawley) and that of Bexis et al (Wistar) may explain these contrary findings (Gaudreault et al., 2001; van den Buuse and Wegener, 2005). In our study when WAY 100635 was administered, a subsequent dose of MDMA evoked a greater initial increase in heart rate and mean arterial pressure then MDMA alone. This suggests that activation of 5-HT1A receptors initially suppress tachycardia and hypertension mediated by MDMA.

The results of our study reveal for the first time that activation of 5-HT1A receptors in the region of the rRP mediate, at least in part, the hypothermic response from the systemic administration of the 5HT1-a receptor agonist DPAT. DPAT lowers body temperature in rats (Gudelsky et al., 1986) by inhibiting IBAT thermogenesis and decreasing tail blood flow (Ootsuka and Blessing, 2006), effects which are blocked by pre-treatment with WAY 100635 (Ootsuka and Blessing, 2006). The rRP is the principal location of sympathetic premotor neurons regulating activity of tail and pinna cutaneous vasoconstrictor nerves in rats and rabbits, respectively, and sympathetic innervation for IBAT in rats (Morrison et al., 1999). Considerable evidence suggests that the major excitatory neurotransmitter released by terminals of these sympathetic premotor neurons
in the rRP onto thermoregulatory sympathetic preganglionic neurons in the spinal cord is glutamate (Nakamura et al., 2005; Stornetta et al., 2005). However, serotonergic neurons in the rRP also project to the same sympathetic regions of the spinal cord, and markers for both glutamate and serotonin are found in some regions (Helke et al., 1997). Likewise microinjections of serotonin potentiate increases in brown adipose sympathetic nerve activity elicited by subsequent spinal microinjection of n-methyl-d-aspartate (Madden and Morrison, 2006).

Serotonergic neurons in the rRP are transsynaptically labeled after injection of pseudorabies virus into the rat tail (Toth et al., 2006), and are at least in part responsible for cutaneous vasoconstrictor responses in the pinna of the rabbit that are evoked from the rRP (Ootsuka and Blessing, 2006). Spinally-projecting serotonergic and non-serotonergic neurons in the rRP possess 5-HT$_{1A}$ receptors (Helke et al., 1997). Microinjection of DPAT into the region of the rRP blocks leptin-induced thermogenesis in rats (Morrison, 2004) and cold-induced cutaneous vasoconstriction in rabbits (Ootsuka and Blessing, 2006). The location of neurons containing 5-HT$_{1A}$ receptors involved in the cardiovascular responses to leptin and chemical disinhibition of the dorsomedial hypothalamus has likewise been reported to be in the region of the rRP and medullary brainstem (Morrison, 2004; Horiuchi et al., 2005).

The results of our study demonstrate for the first time that activation of 5-HT$_{1A}$ receptors in the region of the rRP after a systemic dose of MDMA suppresses tachycardia but not hyperthermia. Blessing and colleagues likewise report that inhibition of the rRP with the 1 nmol of the GABA agonist muscimol did not prevent subsequent increases in sympathetic nerve activity to ear pinna of rabbits given MDMA (6 mg/kg i.v.) (Ootsuka et al., 2004). The systemic administration of the 5-HT$_{2A}$ antagonist SR46349B (0.1 mg/kg i.v.) however completely reversed both the

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sympathetic nerve activity and vasoconstriction of the rabbit ear pinna mediated by MDMA (Ootsuka et al., 2004).

These data suggest that vasoconstriction mediated by MDMA occurs downstream of the rRP likely by neurons expressing 5-HT_{2A} receptors in the spinal cord. Whether 5-HT_{1A} receptors involved in MDMA-mediated hypothermia are likewise in the spinal cord is unknown. In their paper Madden and Morrison demonstrated that microinjection of 5HT into the intermediolateral column of the spinal cord, a region containing pre-ganglionic sympathetic neurons, increased sympathetic nerve activity (SNA) to IBAT after a delay of approximately 20 minutes (Madden and Morrison, 2006). The authors present several plausible explanations for this delay, but one area not explored was whether activation of 5-HT_{1A} receptors may have initially suppressed SNA to IBAT with later activation occurring after 5-HT_{1A} receptor desensitization (Araneda and Andrade, 1991).

In conclusion, we demonstrate that MDMA can cause hypothermia as well as hyperthermia and that unlike DPAT; hypothermia mediated by MDMA does not involved 5-HT_{1A} receptors in the rostral raphe pallidus. We also demonstrate that activation of 5-HT_{1A} receptors in rRP suppress tachycardia mediated by MDMA. These results suggest that the thermogenic and cardiovascular effects caused by MDMA involve activation of both 5-HT_{1A} and 5-HT_{2A} receptors with their interactions having potential consequences on the development of hyperthermia and toxicity in humans.
References


Blessing WW, Zilm A and Ootsuka Y (2006) Clozapine reverses increased brown adipose tissue thermogenesis induced by 3,4-methylenedioxymethamphetamine and by cold exposure in conscious rats. *Neuroscience*** **141**:2067-2073.


Dafters RI (1994) Effect of ambient temperature on hyperthermia and hyperkinesis induced by 3,4-methylenedioxymethamphetamine (MDMA or "ecstasy") in rats. *Psychopharmacology (Berl)** **114**:505-508.
Deussen A (2007) [Hyperthermia and hypothermia: Effects on the cardiovascular system.].

*Anaesthesia*.


Helke CJ, Capuano S, Tran N and Zhuo H (1997) Immunocytochemical studies of the 5-HT(1A) receptor in ventral medullary neurons that project to the intermediolateral cell column and contain serotonin or tyrosine hydroxylase immunoreactivity. *J Comp Neurol* 379:261-270.


McCreary AC, Bankson MG and Cunningham KA (1999) Pharmacological studies of the acute and chronic effects of (+)-3, 4-methylenedioxymethamphetamine on locomotor activity: role of 5-hydroxytryptamine(1A) and 5-hydroxytryptamine(1B/1D) receptors. *J Pharmacol Exp Ther* **290**:965-973.


Saadat KS, O'Shea E, Colado MI, Elliott JM and Green AR (2005) The role of 5-HT in the impairment of thermoregulation observed in rats administered MDMA (‘ecstasy’) when housed at high ambient temperature. **Psychopharmacology (Berl)** **179**:884-890.


Footnotes

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Legends for Figures

Figure 1: Changes in core temperature (TOP) and locomotion (BOTTOM) after the intraperitoneal injection (t=0) of saline and three different doses of MDMA in conscious freely moving rats. Solid horizontal bars indicate times in which there were significant differences from baseline (by repeated measures ANOVA and LSD analysis, p<0.05) in rats treated with 7.5 mg/kg. Dashed horizontal lines indicate times in which significant differences were evident with 15 mg/kg of MDMA. n=6 for each group.

Figure 2: Changes in core temperature (A), locomotion (B), heart Rate (C) and mean arterial pressure (D) after systemic injections (t=-5 min) of WAY 100635 (0.5 mg/kg, i.p) or saline followed by injections (t=0 min) of MDMA (7.5 mg/kg, i.p.) or saline. Asterisk (*) = significantly different from its own baseline. Double plus (‡)= significantly different compared to all other groups at the specified time point. ANOVA and LSD, p<0.05. n=6 for each group.

Figure 3: Photomicrographs of coronal rat brain sections from a representative microinjection experiment. The distribution of red (A) and green (B) fluorescent microspheres marks the sites of injections of 0.5 nmol of WAY 100635 (100 nl, 5 mM) and PBS (100 nl, 0.1M) respectively. The corresponding neutral red counter stained section (C) shows anatomic landmarks used for marking injection sites. (D) Schematic diagrams of coronal sections through rat medulla adapted from the atlas of Paxinos and Watson (Paxinos and Watson, 1998) illustrating approximate sites of injection for all of the described microinjection experiments. Numbers indicate distance from bregma in millimeter. Open squares represent injections of WAY 100635 or PBS in followed by systemic injection of DPAT. Closed squares represent injections of WAY 100635 or PBS outside
the region of the rRP followed by the systemic injection of DPAT. Black triangles represent injections of PBS and open triangles that of WAY 100635 followed by the systemic injections of MDMA. Open Diamonds represent injections of WAY 100635 outside the region of the rRP followed by systemic injections of MDMA. Abbreviations are as follows: py, pyramids; rRP, rostral raphe pallidus; bas, basilar artery.

Figure 4: Changes in core temperature (A) and locomotion (B) after microinjections (t=-5 minutes) of WAY 100635 (100 nl, 5 mM) or PBS (100 nl) into the rRP followed by (t=0min) by intraperitoneal injections of DPAT (0.2 mg/kg) or saline. Animals in which microinjections of WAY 100635 were outside the rRP are designated by the abbreviation (OR). Asterisk (*) = significantly different from its own baseline. Double plus (‡)= significantly different compared to all other groups at the specified time point. ANOVA and LSD, p<0.05. (PBS/Saline n=6, WAY/Saline n=6, PBS/DPAT n=8, WAY/DPAT n=8, WAY/DPAT (OR) n=7)

Figure 5: Changes in core temperature (A), locomotion (B), heart rate (C) and mean arterial pressure (D) after microinjecting (t=-5 minutes) 0.5 nmol of WAY 100635 (100 nl, 5 mM) or PBS (100 nl) into the rRP followed by (t=0 min) the intraperitoneal administration of MDMA (7.5 mg/kg) or saline. Animals in which microinjections of WAY 100635 were outside the rRP are designated by the abbreviation (OR). Asterisk (*) = significantly different from its own baseline. Double plus (‡)= significantly different compared to all other groups at the specified time point except WAY/MDMA (OR). ANOVA and LSD, p<0.05. (PBS/Saline n=7, WAY/Saline n=7, PBS/MDMA n=8, WAY/MDMA n=5, WAY/MDMA (OR) n=4)
Figure 4

A

Temperature (°C) vs. Minutes

B

Locomotion (au) vs. Minutes

- PBS/Saline
- WAY/Saline
- PBS/DPAT
- Way/DPAT
- WAY/DPAT (OR)

* indicates significant difference compared to control.