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ORIGINAL ARTICLE

Inhibition of the dorsomedial hypothalamus, but not the medullary raphe pallidus, decreases hyperthermia and mortality from MDMA given in a warm environment

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Abstract

The central mechanisms through which 3,4-methylenedioxymethamphetamine (MDMA) mediates life-threatening hyperthermia when taken in a warm environment are not well described. It is assumed that MDMA alters normal thermoregulatory circuits resulting in increased heat production through interscapular brown adipose tissue (iBAT) and decreased heat dissipation through cutaneous vasoconstriction. We studied the role of the dorsomedial hypothalamus (DMH) and medullary raphe pallidus (mRPa) in mediating iBAT, tail blood flow, and locomotor effects produced by MDMA. Rats were instrumented with guide cannulas targeting either the DMH or the mRPa brain regions involved in regulating iBAT and cutaneous vascular beds. In all animals, core temperature and locomotion were recorded with surgically implanted telemetric transmitters; additionally, either iBAT temperature (via telemetric transmitter) or tail artery blood flow (via tail artery Doppler cuff) were also recorded. Animals were placed in an environmental chamber at 32°C and microinjected with either control or the gamma-aminobutyric acid (GABA) agonist muscimol (80 pmol) followed by an intravenous injection of saline or MDMA (7.5 mg kg^{-1}). To prevent undue suffering, a core temperature of 41°C was chosen as the surrogate marker of mortality. Inhibition of the DMH, but not the mRPa, prevented mortality and attenuated hyperthermia and locomotion. Inhibition of either the DMH or the mRPa did not affect iBAT temperature increases or tail blood flow decreases. While MDMA increases iBAT thermogenesis and decreases heat dissipation through cutaneous vasoconstriction, thermoregulatory brain regions known to mediate these effects are not involved. Rather, the finding that inhibiting the DMH decreases both locomotion and body temperature suggests that locomotion may be a key central contributor to MDMA-evoked hyperthermia.

Abbreviations

MDMA, 3,4-methylenedioxymethamphetamine; DMH, dorsomedial hypothalamus; mRPa, medullary raphe pallidus; iBAT, interscapular brown adipose tissue; aCSF, artificial cerebrospinal fluid; ANOVA, analysis of variance.

Introduction

Amphetamine-type stimulants are among the most abused drugs in the world. Ranked second only to cannabis, they are currently used more than cocaine and heroin combined (UNODC 2012). Of the many complications related to amphetamine use, one of the most acute and life-threatening is severe hyperthermia. A large number of hyperthermia and heatstroke cases have occurred with use of the substituted amphetamines like 3,4-methylenedioxymethamphet-

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amine (MDMA) (Henry 1992; Milroy et al. 1996; Liechti et al. 2005). The severity of hyperthermia is directly linked with adverse clinical outcomes, with one series showing that death occurred in two-thirds of the cases when core temperatures exceed 41.5°C (Gowing et al. 2002).

The mechanisms by which MDMA causes hyperthermia are complex, but are thought to involve increased heat generation through interscapular brown adipose tissue (iBAT) combined with decreased heat dissipation through the constriction of cutaneous blood vessels (Pedersen and Blessing 2001; Blessing et al. 2006). MDMA also causes spontaneous activity in rodents (Spanos and Yamamoto 1989) that may also contribute to hyperthermia and toxicity. Ambient temperature has a profound effect on temperature responses to MDMA. When given in a cold environment MDMA causes hypothermia, in a thermoneutral environment it can cause either hypo- or hyperthermia, and in a hot environment it causes hyperthermia (Dafters 1994; Malberg and Seiden 1998). This is of particular importance to humans, as MDMA is often used to facilitate dancing at both nightclubs and outdoor summer events where ambient temperatures can be elevated (Irvine et al. 2006). Ambient temperature may also affect the treatment of hyperthermia from MDMA. Many drugs that prevent hyperthermia and neurotoxicity from MDMA at room temperature fail to do so when environmental temperatures are elevated (Malberg et al. 1996; Colado et al. 1998, 1999). Therefore, understanding the mechanisms by which MDMA causes hyperthermia in a warm environment is an important step in designing effective treatment strategies.

The mechanisms through which MDMA increases sympathetic outflow to iBAT and cutaneous vessels, and increases spontaneous locomotion, involve a number of neurotransmitters (e.g., serotonin, norepinephrine, and dopamine) and their corresponding receptors (e.g., 5-HT1A, 5-HT2A, alpha-1, D2, D1). (Callaway et al. 1992; Mechan et al. 2002; Sprague et al. 2004, 2005; Blessing et al. 2006; Risbrough et al. 2006; Rusyniak et al. 2007, 2008a; Vanattou-Saifoudine et al. 2010a,b). There has also been data implicating Beta-3 receptors and skeletal muscle uncoupling protein-3 in MDMA-mediated hyperthermia in both rodents and humans (Mills et al. 2003; Sprague et al. 2005; Hysek et al. 2013b). Despite the work done to date, the brain regions regulating the responses to MDMA are still largely unknown. Potential sites include brain regions involved in thermoregulation. Two candidates are the dorsomedial hypothalamus (DMH) and the medullary raphe pallidus (mRPa). The mRPa is the site of premotor neurons regulating sympathetic outflow to iBAT and cutaneous vessels. It has been implicated in vasoconstriction and iBAT thermogenesis from cold exposure, infection, and stress (DiMicco et al. 2006; Rathner et al. 2008; Nakamura 2011). The DMH sends projections to the mRPa and has likewise been reported to be involved with control of iBAT thermogenesis and the regulation of cutaneous vascular beds (Zaretskaia et al. 2002; DiMicco and Zaretsky 2007; Yoshida et al. 2009). We have shown that the DMH is associated with the development of hyperthermia and hyperactivity from MDMA at room temperature (Rusyniak et al. 2008b).

It is not known whether the DMH and the mRPa are involved in mediating iBAT and cutaneous vascular responses to MDMA at warm ambient temperatures. This study sought to determine the role of the DMH and the mRPa in mediating iBAT, cutaneous vascular and locomotor responses to MDMA in conscious rats at an elevated ambient temperature.

Materials and Methods

Chemicals

The NIH generously provided MDMA (± 3,4-methylenedioxymethamphetamine HCl), which was dissolved in saline for all experiments. All injection volumes were 1 mL kg^{-1} body weight. Muscimol (Sigma-Aldrich, St. Louis, MO) was dissolved in artificial cerebrospinal fluid (aCSF) and stored at −20°C until the time of the experiment.

Animals

The care and use of rats were in accordance with protocols approved by the Indiana University Animal Care and Use Committee and in accordance with ARRIVE guidelines. Experiments were carried out under the supervision of veterinarians. We used single-housed male Sprague-Dawley rats (weight 300 ± 20 g; Harlan, Indianapolis, IN) that were maintained in a 12 h light/dark cycle and fed ad libitum. A total of 110 of rats were used. We conducted the experiments on fully conscious rats in isolated quiet rooms between 10:00 AM and 4:00 PM using Raturn® cages (BASi, West Lafayette, IN); by rotating in the opposite direction the rat moves, the cages allowed for the measurement of tail blood flow without the need for an electrical swivel. We kept the rats in the Raturn® for a minimum of 12 h before experiments, including an overnight stay during their active cycle to acclimate them.

Surgical preparation

All animals underwent two consecutive surgeries. We anesthetized animals with 1.5–2% isoflurane in oxygen, adjusting the concentration of isoflurane as needed and monitored heart rate and oxygen saturation during surgery using a Pulse Oximeter monitor (model LS1P-10R;

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Nonin, Plymouth, MN). In the first surgery, we placed either bilateral guide cannulas targeting the DMH, or a single guide cannula targeting the midline mRPa. After surgery, the animals recovered for 7 days. In the second surgery, we implanted either a telemetric probe measuring temperature of the iBAT and core body temperature (TT-F40; Data Sciences International, St. Paul, MN), or we placed an ultrasound Doppler probe measuring tail arterial blood flow and a telemetric probe measuring core body temperature (T-F40; Data Sciences International). During the second surgery we also placed jugular venous catheters, to allow for systemic drug administration.

Guide cannulas

We anesthetized animals and placed them in a stereotaxic apparatus with the incisor bar set at 3.3 mm below the interaural line. The skin overlying the dorsal surface of the skull was pretreated with lidocaine/epinephrine mixture, cut, and retracted followed by removal of soft tissue to expose the surface of the skull. We treated the skull with a 30% hydrogen peroxide solution using cotton-tipped applicators. This stopped bleeding, aided in sterility, and enhanced the visibility of structures used as stereotaxic landmarks. Central nervous system (CNS) targets had the following coordinates according to a Rat Brain Atlas (Paxinos and Watson 1998):

- DMH – using bregma as a reference point: AP (anterior posterior) −3.1 mm; LR (left right) ±2.0 mm; HD (height depth) −8.2 mm. We used a 10-degree angle from the sagittal plane to avoid central sinus and to allow bilateral placement of two cannulas.
- mRPa – using the interaural point as a reference point: AP −2.8 mm, LR ±0 mm, HD −1.1 mm.

Using a rotary tool (MiniMite Cordless 4.8V; Dremel, Racine, WI) equipped with a surgical carbide burr (DHP557; Miltex, Plainsboro, NJ), we made a small hole in the skull. We inserted the cannulas (26 gauge; Plastics One, Roanoke, VA) through the holes, and positioned them to target appropriate CNS sites. We placed two jeweler's screws (size 80) into the skull to facilitate attachment of the cement cap. Once inserted, we secured the cannulae using Vetbond glue (3M, St. Paul, MN) and cranioplastic cement. We inserted dummy-wire cannulas into the guides and returned the rats to their home cages for recovery.

Intravenous (i.v.) catheter implantation

A week later, we implanted jugular vein catheters to allow for the systemic administration of drugs. Rats were placed in a sterile surgery field in a dorsal recumbent position exposing the neck and shoulder area. A small longitudinal incision was made rostral to the clavicle and the jugular vein was dissected and ligated. We inserted a catheter, constructed of 3.5 cm Silastic tubing (508-007; Dow Corning, Midland, MI) connected to 10 cm of Tygon tubing (Small Parts Inc., Miami, FL,) with 1 cm of PE-50 tubing (Plastics One). The catheter was filled with saline and inserted, Silastic end first, ~3 cm into the vein. We then routed the catheter subcutaneously, exteriorizing it at the dorsal neck area. We flushed the catheter, capped it with a metal plug, and secured it to the skin with sutures. To prevent the catheter from being chewed, we looped it either through a rodent saddle (INFU-SDL1; Kent Scientific Corporation, Torrington, CT), which also held the externalized connection for tail flow probe, or to the rat collar, which connects the rat to the Raturn (BASi).

Temperature telemetric probes

After inserting jugular catheters, we placed animals in the ventral recumbent position to expose their back. In order to implant the dual thermistor telemetry transmitters (TT F40) to measure iBAT temperature we made two longitudinal skin incisions: a medial incision in the interscapular area for iBAT measurement; and another slightly aside of midline several centimeters posterior to the first to house the body of the transmitter and to tunnel the peritoneal temperature probe. We placed the tip of the iBAT thermistor under the left lobe of the iBAT through a small incision made through the connective tissue, and secured the catheter in place with a purse-string suture. We tunneled the peritoneal thermistor subcutaneously to a small cutaneous incision on the side of the abdominal cavity. The tip was placed into the abdominal cavity through the incision, and fixed to the muscle wall with purse-string suture. We sutured the skin incisions closed and applied a topical antibiotic ointment. Animals were returned to their cage and allowed to recover at least 1 week.

In the experiments measuring Doppler flow, we measured core temperature with an implanted telemetry thermistor (T-F40). After inserting jugular catheters, we placed animals in a dorsal recumbent position with abdominal skin shaved along midline. We made a 2 cm-long longitudinal medial skin incision, followed by a longitudinal incision of the muscular wall along the white line. We inserted the body of the transmitter into the abdominal cavity and sutured the muscle closed followed by the skin. Animals were returned to their cage for at least 1 week before experimentation.

Doppler cuffs

To measure tail artery blood flow we implanted a Doppler ultrasonic flow probe (Iowa Doppler Products, Iowa City,

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IA) around the proximal portion (~3 cm from the base) using slightly modified methods we have previously reported (Rusyniak et al. 2008a). Briefly, we placed anesthetized animals on their back in a sterile surgery field with the base of the tail area exposed. We made a cutaneous longitudinal incision ~1–2 cm caudally to the anus. After exposing the tail artery and placing the cuff around it, we tunneled the wires through tendons above the bone perpendicular to the artery. We stabilized the artery cuff using sutures and Vetbond glue around the polyvinyl chloride cover of the wire. We closed the connective tissues above the cuff with sutures and routed the wires subcutaneously to the base of the neck were they were externalized. The skin was closed with sutures. To prevent the wires from being chewed or pulled, we looped them through a rodent saddle. We then soldered the ends of the wires to a small three-pronged adapter (SM3H, SMH; Powell Electronics, Inc., Swedesboro, NJ) that connects to a Doppler flowmeter (Iowa Doppler). We capped the adapter and lightly fixed it to the saddle between experiments. We calculated blood flow from the flowmeter output voltage using a formula provided by the manufacturer.

Testing functionality of sensors

Before conducting iBAT experiments, we established that the iBAT probe and tail blood flow sensors were working properly. After rats were acclimated overnight at room temperature, over 40–60 min, we increased the ambient temperature to 32°C. We confirmed that animals at this temperature developed a sustained tail blood flow increase (at least 20 cm sec^{-1} maximal amplitude). Afterward, we lowered the ambient temperature to 10°C. Previously, we defined a correctly placed iBAT sensor if cooling increased the iBAT temperature at least 0.5°C (Rusyniak et al. 2008a). Furthermore, we confirmed that the tail blood flow probe was considered properly functioning if flow decreased to <5 cm sec^{-1} at 10°C.

Experimental protocol

Using a custom built environmental chamber we conducted experiments at an ambient temperature of 32°C as this temperature was easy to reach and maintain, caused a sustained vasodilation at baseline (making it easier to detect drug-induced vasoconstriction), is in the range of temperatures which others have reported hyperthermia with MDMA in rats (Gordon et al. 1991), and is similar to ambient temperatures at outdoor events in the United States (Cavaliere 2013), which were linked to deaths from MDMA use.

In an initial pilot study, we noticed that after receiving 7.5 mg/kg (i.v.) of MDMA, several animals died shortly after their body temperature approached or exceeded 42°C. To prevent animal suffering, we chose 41°C as a surrogate marker of mortality. This allowed us to stop the experiment and quickly remove the animal from the temperature chamber and cool them. No animals died during the experiment using this protocol.

To investigate whether the DMH and or the mRPa participate in MDMA-mediated increases in iBAT thermogenesis and cutaneous vasoconstriction, we conducted the following experiment. After rats were acclimating in the Raturn® overnight we increased the ambient temperature from 24 ± 0.5°C to 32 ± 0.5°C. Once ambient temperatures stabilized, we connected microinjectors (33 gauge; Plastics One) to a 10 μl Hamilton syringe with Teflon tubing (ID 0.12 mm; OD 0.65 mm; BASi), and placed microinjectors in the guide cannulas. The syringes were mounted on an infusion pump (Model 200, KD Scientific, Holliston, MA) capable of delivering, over 30 sec, a 100 nL solution of either vehicle (aCSF) or muscimol (80pmol). We left animals undisturbed for at least 1 h to reduce changes in temperature and tail blood flow related to the stress of placing the injectors (Zaretsky et al., 2011). After this, we microinjected animals with either muscimol or vehicle followed 5 min later by an i.v. injection of either MDMA (7.5 mg kg^{-1}) or saline. We chose the dose of 7.5 mg $kg^{-1}$of MDMA as it causes temperature responses reported in moderate to severe cases of human intoxication, and results in plasma concentrations similar to those seen in humans taking recreational doses (Green et al. 2009). We chose the i. v. route as it avoids the stress seen with intraperitoneal and subcutaneous injections. This was important since microinjections of muscimol in the mRPa and DMH, compared to CSF, decrease stress responses (DiMicco et al. 2006). Therefore, i.v. injections allowed us to better evaluate the role of the mRPa in DMH in responses to MDMA independent of the stress of the injections. In all experiments, physiological parameters were recorded every minute until either a core temperature reached 41°C, or for a total of 60 min after last injection. We chose this time since the duration of muscimol's inhibitory action, for the dose and volume in this study, is ~60 min (Zaretsky et al. 2003; Rusyniak et al. 2007). A representative example of single experiment can be seen in Figure 1.

Verification of injection sites

At the conclusion of each experiment, we injected rats with pentobarbital (100 mg kg^{-1} i.v.) and then transcardially perfused them with 60 ml of cold saline (4°C) followed by 60 ml of 4% paraformaldehyde. We excised the brain and post fixed it in a 4% solution of paraformaldehyde overnight at 4°C. We then placed brains in a

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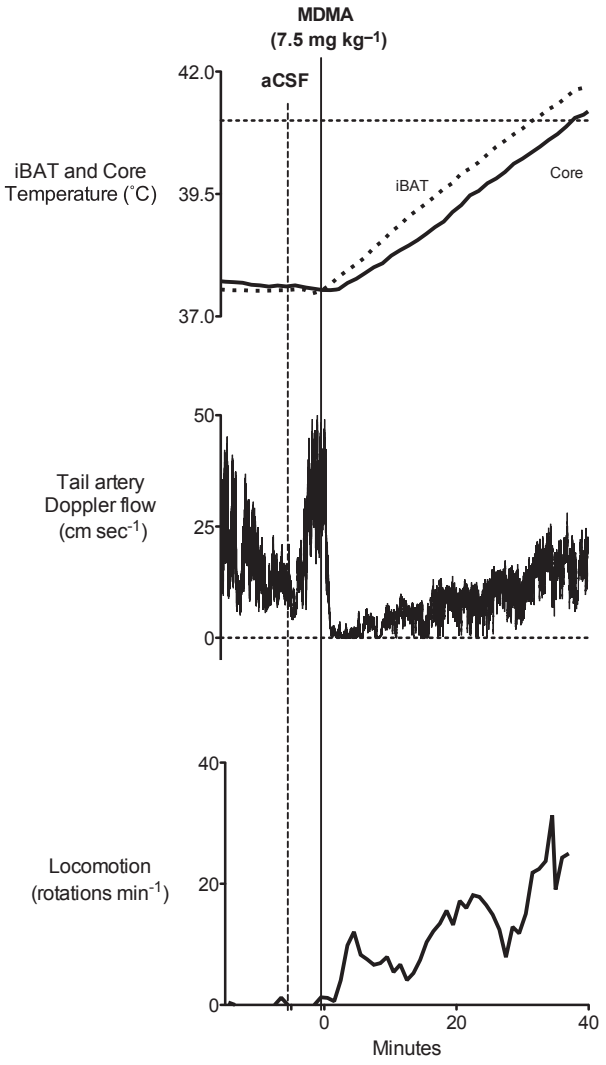

Figure 1. The following data represent the 1-min means of iBAT, Core temperature, and locomotion (obtained from one rat) and the tail blood flow (100 Hz), from a separate rat. To make the interpretation of the data more meaningful we chose two animals with nearly identical core temperature responses. aCSF was microinjected into the mRPa at $T = -5$ min and 7.5 mg kg^{-1} MDMA (7.5 mg kg^{-1}) was injected at $t = 0$ min. Ambient temp = 32°C.

solution of 23% sucrose in PBS at 4°C until sectioning. We cut coronal brain sections (40 μm) using a cryostat (Leica Microsystems, Buffalo Grove, IL). An observer blinded to group allocation determined the injection sites by visualizing the injection track under light microscopy in Neutral Red stained tissue sections.

Data analysis and statistical procedures

We conducted statistics and created graphs using Prism (Graphpad Software, San Diego, CA) software. We calculated survival using Kaplan–Meier curves plotting the percent survival (animals with core temps <41°C) over time. We compared groups using a log-rank (Mantel–Cox) test. We compiled absolute core temperature values from both iBAT and tail experiments and plotted the 5 min means. Since animals were removed from the study at different time points (depending on when and whether they reached a core temperature of 41°C), we only plotted the first 27.5 min of the experiment (at this time no animal had been removed from the study). We compared groups by conducting an analysis of variance (ANOVA) analysis, with Tukey's post hoc analysis, on temperatures at 27.5 min. We obtained locomotion data by converting the voltage output of the Raturn® to cage rotations per min – the Raturn® rotates at a speed of 31.5 rotations per minute. As with core temperature, we compiled absolute locomotion values from both the iBAT and tail experiments plotting the first 27.5 min and analyzing them at 27.5 min. Since baseline tail blood flow is more variable between animals (likely secondary to slight differences in placement and artery size), we plotted and compared the percent change from baseline. As with temperature, we plotted the first 27.5 min and analyzed the data at 27.5 min. To assess iBAT thermogenesis, we plotted the difference between iBAT and core temperatures, and analyzed these values in a manner similar to temperature.

Results

Location of injection sites

We confirmed the microinjection sites by identifying the injection needle track under light microscopy. We estimated the approximate centre of the injection site and plotted it on schematic coronal sections in which the DMH and mRPa were demarcated using a drawing adapted from the atlas of Paxinos and Watson (1998). The total area encompassing all the injections in the region of the DMH and mRPa is shown in gray (Fig. 2A and C). Injections into the DMH and the mRPa were considered successful only if the centre of injection site (or injection sites in the case of DMH) was within gray rectangle. Seven animals (3 DMH, 4 mRPa) had injection sites outside of these areas and were excluded from analysis.

Effects of inhibiting the DMH or the mRPa on survival rates in rats treated with MDMA in a warm environment

Inhibition of the DMH significantly decreased the number of animals reaching a body temperature of 41°C, and

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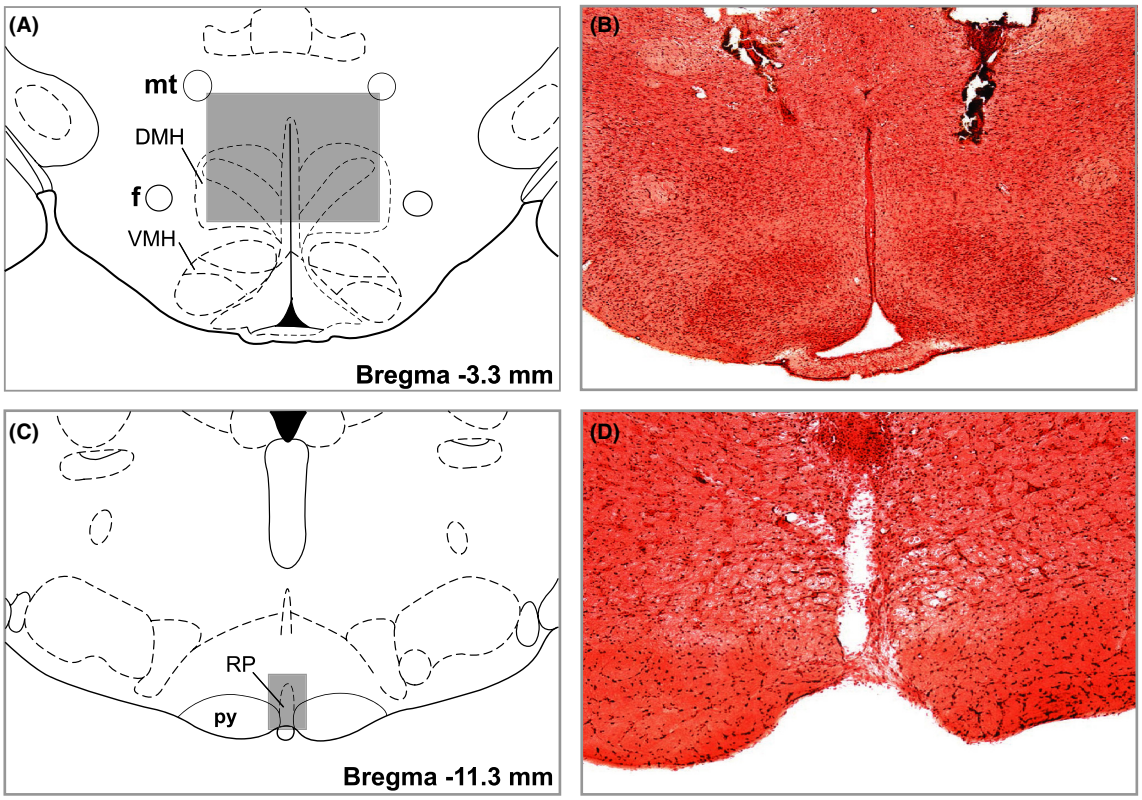

Figure 2. (A and C) show coronal brain sections, adapted from the Paxinos and Watson (1998) atlas, with the area in which all the DMH (A) and all the mRPa (C) microinjections occurred depicted by the gray rectangles. (B and D) show a representative injection site of a DMH (B) and mRPa (D) in individual rats.

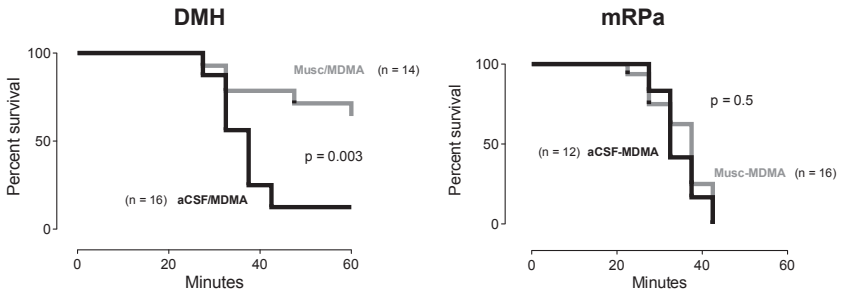

Figure 3. The figures represent Kaplan–Meyer survival curves for animals microinjected into the region of the DMH or mRPa with either aCSF or muscimol (80 pmol, 100 nL) followed by an i.v injection of MDMA (7.5 mg kg^{-1}) at an ambient temp of 32°C. The *x*-axis represents time of survival after injection of MDMA and the *y*-axis the percent of animals surviving. A core temperature of 41°C was used as a surrogate marker of mortality and animals were removed from the study when they reached this temperature. Values in parentheses are the number of animals in each group. Differences were determined using a log-rank (Mantel-Cox) test.

by extension, increased survival. In contrast, inhibition of the mRPa had no effect on animal "survival". As Figure 3 demonstrates the majority of animals microinjected with aCSF, regardless of whether injected in the DMH or mRPa, rapidly reached core temperatures of 41°C after MDMA: All of the 12 animals microinjected with aCSF in the mRPa and 14 of 16 animals (87.5%) microinjected with aCSF in the DMH achieved temperatures of 41°C before the end of a 60 min observation period. In contrast, only 5 of 14 (36%) of animals in the DMH Musc-MDMA group developed temperatures of 41°C before the end of the 60-min study period; All (16 of 16) of the

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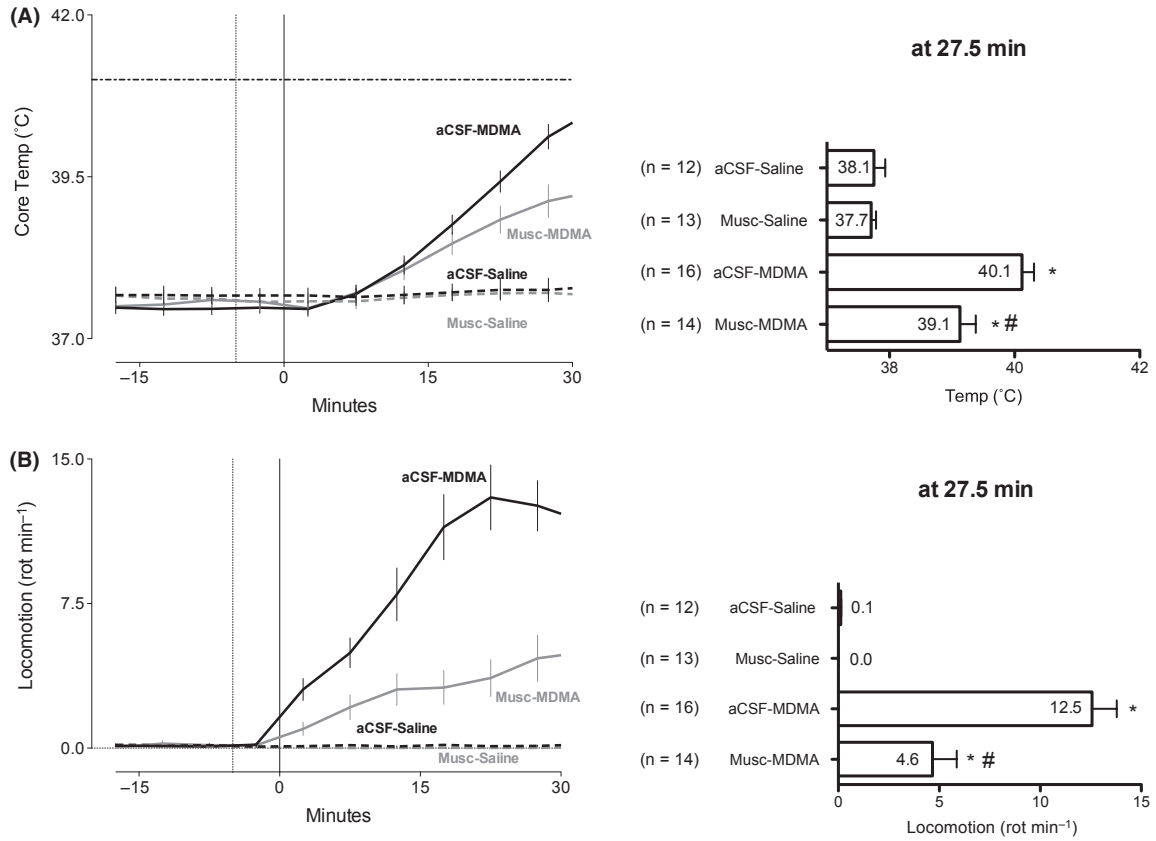

Figure 4. (A and B) show the temperature and locomotion responses, respectively, after an i.v. injection of MDMA (7.5 mg kg^{-1}) or saline in rats previously microinjected into the region of the DMH with either aCSF or muscimol (80 pmol, 100 nL). The line graphs on the left represent the 5-min means for each group over time and the bar graphs on the right show the mean for the temperature and locomotion at 27.5 min. Error bars represent SEMs. The number of animals in each group is shown in parentheses. The dashed vertical line denotes the time of microinjection and the solid vertical line denotes the time of i.v. injection. *Significant difference ($P < 0.05$) from its corresponding control and "#" significant ($P < 0.05$) difference between groups receiving MDMA. Differences were determined by ANOVA with a Tukey's post hoc test.

mRPa Musc-MDMA group developed temperatures of 41°C before the end of the observation period.

Effects of inhibiting the DMH or the mRPa on temperature and locomotor responses mediated by MDMA in a warm environment

Independent of whether we had microinjected aCSF or muscimol (80 pmol), and whether it was injected into the DMH or mRPa, MDMA caused a significant increase in core body temperature and locomotion at 30 min over controls. Compared to aCSF, however, muscimol injected into the DMH significantly reduced the increases in both temperature (Fig. 4A) and locomotion (Fig. 4B). Microinjecting muscimol into the mRPa had no effect on the development of hyperthermia or hyperactivity produced by MDMA (Fig. 5A and B). Microinjection of muscimol or saline into either the DMH or the mRPa, had no effect on core temperature or locomotion after the administration of saline. Although we have previously shown that inhibiting the mRPa (Zaretsky et al. 2003; Rusyniak et al. 2008b) and DMH decreases body temperature in control animals, these studies were conducted at room temperature. As we conducted this study at or above the thermoneutral zone for Sprague Dawley rats, we would not expect a decrease in core temperature (Gordon 1987).

Effects of inhibiting the DMH or the mRPa on iBAT temperature and tail blood flow responses mediated by MDMA in a warm environment

As previously reported, the systemic injection of MDMA increases iBAT thermogenesis while simultaneously decreasing heat dissipation through vasoconstriction (Blessing et al. 2006). We found that inhibiting either the

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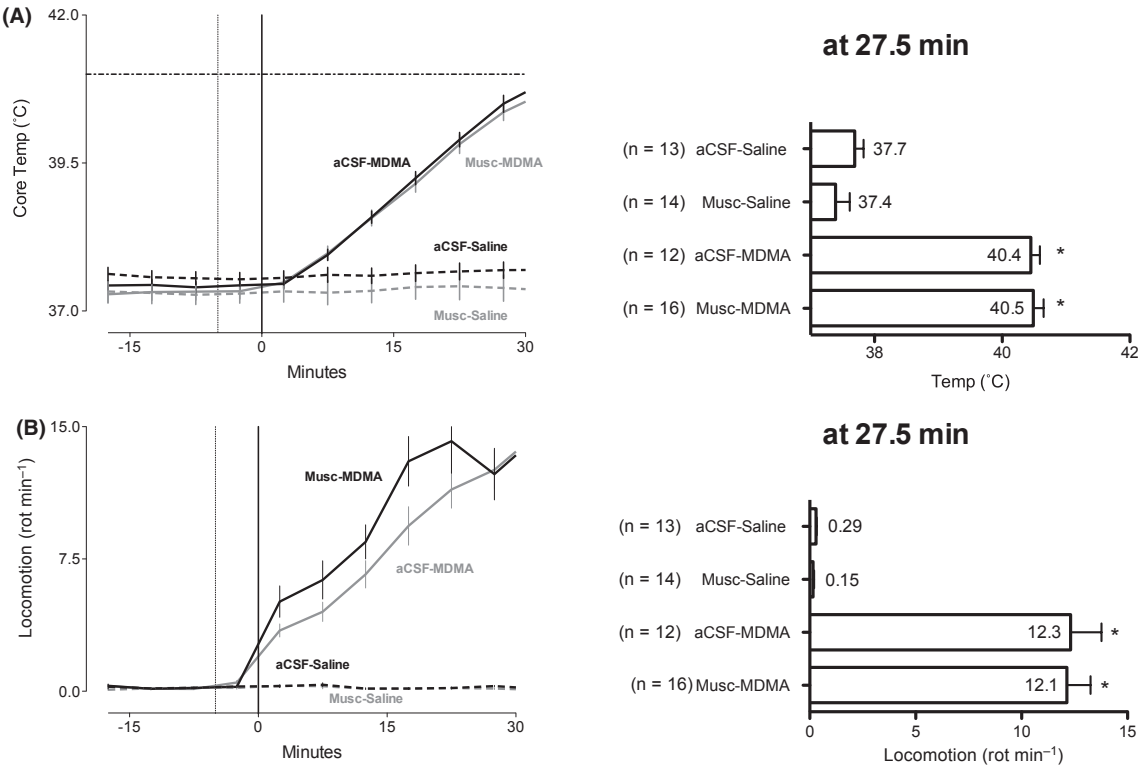

Figure 5. (A and B) show the temperature and locomotion responses, respectively, after an i.v. injection of MDMA (7.5 mg kg^{-1}) or saline in rats previously microinjected into the region of the mRPa with either aCSF or muscimol (80 pmol, 100 nL). The line graphs on the left represent the 5-min means for each group over time and the bar graphs on the right show the mean for the temperature and locomotion at 27.5 min. Error bars represent SEMs. The number of animals in each group is shown in parentheses. The dashed vertical line denotes the time of microinjection and the solid vertical line denotes the time of i.v. injection. *Significant difference ($P < 0.05$) from its corresponding control. Differences were determined by ANOVA with a Tukey's post hoc test.

DMH or the mRPa had no effect on these responses (Figs. 6 and 7). Microinjection of muscimol or saline into either the DMH or the mRPa, had no effect on iBAT temperature or tail blood flow after the administration of saline.

Discussion and Conclusions

Our findings demonstrate that neurons in the DMH, not the mRPa, mediate hyperthermia from MDMA. Furthermore, our data suggest that locomotion may be a key central mechanism mediating hyperthermia. In our previous study we showed that inhibiting the DMH attenuated both temperature and locomotor responses to MDMA (Rusyniak et al. 2008b). These experiments, however, were conducted at room temperature and resulted in peak temperature responses well below those associated with adverse outcomes in humans (Gowing et al. 2002). Furthermore, our prior work did not determine if along with decreasing locomotion, the DMH affected-iBAT and cutaneous vascular responses. Our present finding, that inhibiting the DMH decreased locomotion but had no effect on tail blood flow or iBAT thermogenesis, suggests that inhibition of locomotion alone might be sufficient to prevent hyperthermia from MDMA. This is important when viewed in the context that MDMA is commonly used at dance parties and concerts where the combination of a warm environment and exercise may be particularly dangerous. This was made even more evident by a string of deaths this summer at dance parties in the United States associated with MDMA (Cavaliere 2013). While it is not difficult to understand how skeletal muscle contractions contribute to heat generation, it remains unclear why in rats, and presumably in humans, MDMA motivates animals to continue to exert themselves even when core body temperatures are critically elevated. Sports medicine literature has shown that in both humans and rodents as core temperatures approach 40°C study subjects become exhausted and stop exercising (Fuller et al. 1998; González-Alonso et al. 1999). As evident in Figure 1, rats

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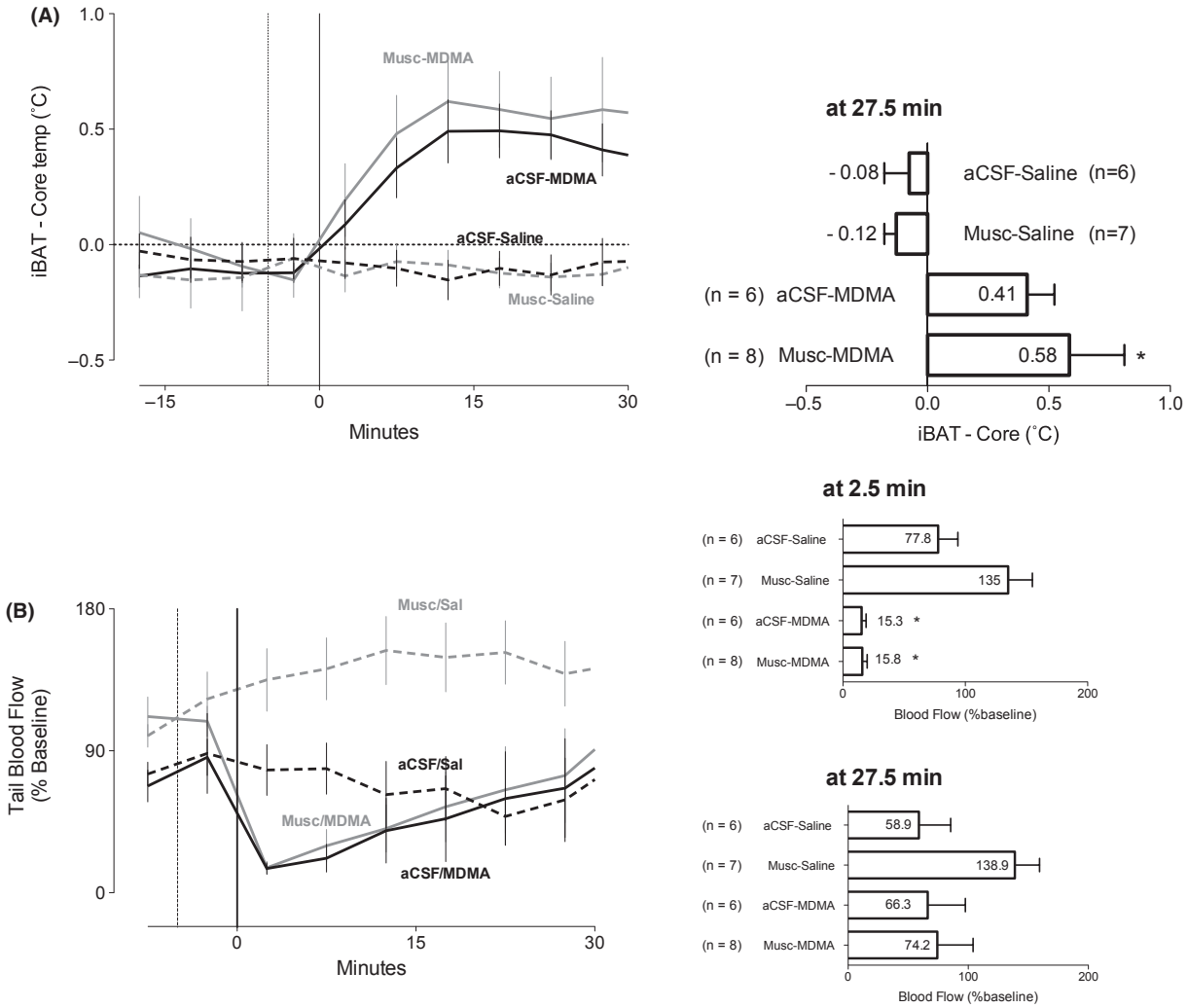

Figure 6. (A and B) show the difference between iBAT and core temperature and the change from baseline in tail Doppler blood flow, respectively, after an i.v. injection of MDMA (7.5 mg kg^{-1}) or saline in rats previously microinjected into the region of the DMH with either aCSF or muscimol (80 pmol, 100 nL). The line graphs on the left represent the 5-min means for each group over time and the bar graphs on the right show the mean for the iBAT – Core at 27.5 min and tail blood flow at 2.5 and 27.5 min. Error bars represent SEMs. The number of animals in each group is represented in parentheses. The dashed vertical line denotes the time of microinjection and the solid vertical line denotes the time of i.v. injection. *Significant difference ($P < 0.05$) from its corresponding control. Differences were determined by ANOVA with a Tukey's post hoc test.

continue running at a high rate even when core temperatures are above 40°C. We chose 41°C as the temperature to remove animals from the experiment, as some animals in our pilot study appeared to literally run to death. This suggests that key contributors to hyperthermia from MDMA are spontaneous locomotion and the failure to stop activity despite critical elevations in temperature. While we showed that inhibiting the DMH decreases locomotion, it remains unclear if the DMH is also involved in centrally mediated exhaustion. It is important to note that the DMH is a complex region of the brain involved in a variety of physiologic functions. So while our data show a strong association between locomotion mediated through the DMH and MDMA-evoked hyperthermia, further work is needed to confirm this link.

If locomotion is a significant contributor to MDMA hyperthermia, it is contrary to what others, including ourselves, have previously published (Dafters 1995; Green et al. 2004; O'Shea et al. 2005; Rusyniak et al. 2007; Docherty and Green 2010). These prior assertions were largely based on the fact that MDMA caused similar amounts of locomotion in a cold and warm environment but opposite temperature responses (Dafters 1994, 1995). The lack of correlation between locomotor activity and

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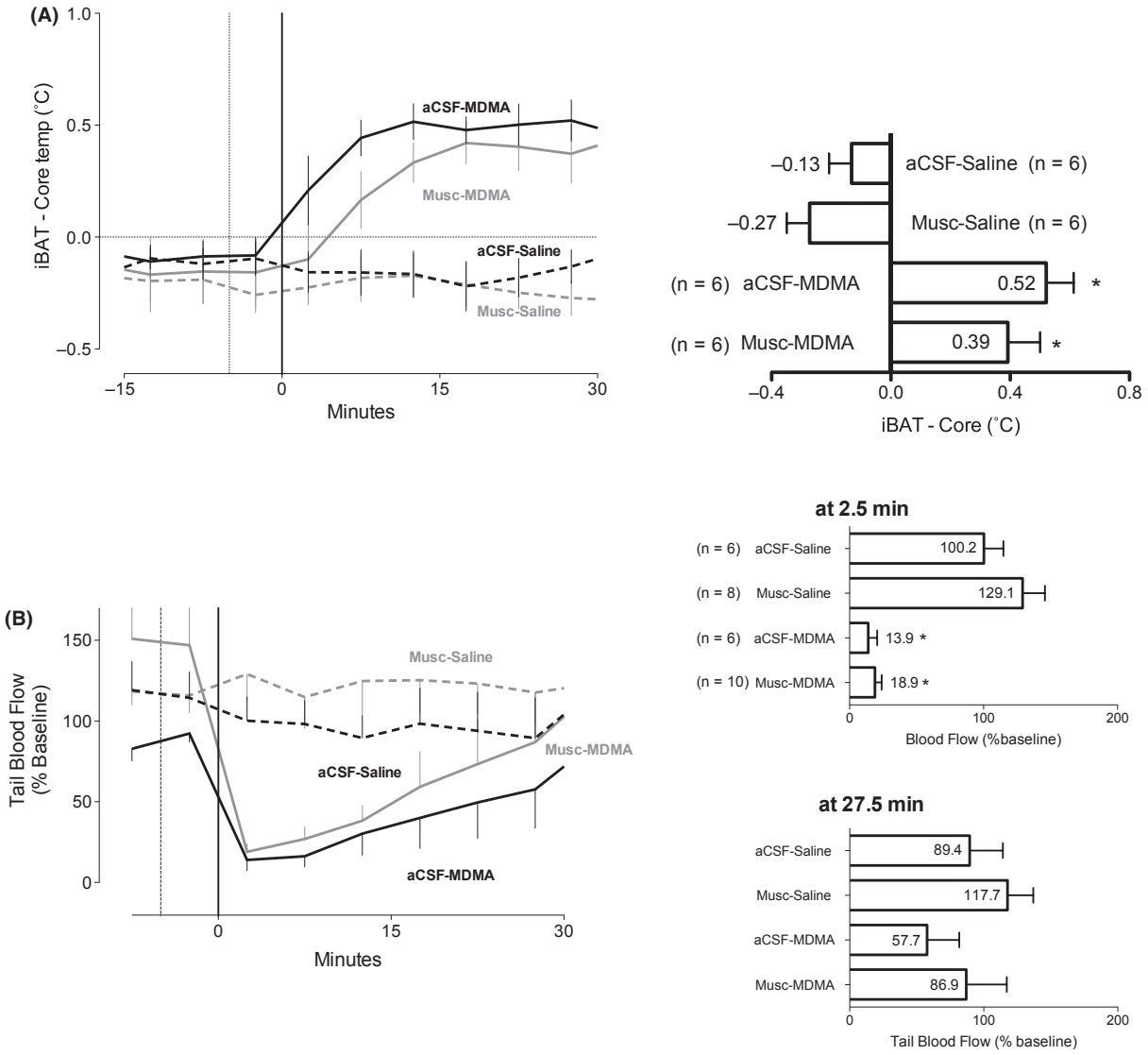

Figure 7. (A and B) show the difference between iBAT and core temperature and the change from baseline in tail Doppler blood flow, respectively, after an i.v. injection of MDMA (7.5 mg kg^{-1}) or saline in rats previously microinjected into the region of the mRPa with either aCSF or muscimol (80 pmol, 100 nL). The line graphs on the left represent the 5-min means for each group over time and the bar graphs on the right show the mean for the iBAT – Core at 27.5 min and tail blood flow at 2.5 and 27.5 min. Error bars represent SEMs. The number of animals in each group is shown in parentheses. The dashed vertical line denotes the time of microinjection and the solid vertical line denotes the time of i.v. injection. *Significant difference ($P < 0.05$) from its corresponding control. Differences were determined by ANOVA with a Tukey's post hoc test.

core temperature does not, however, mean that locomotion is not a significant contributor to hyperthermia. A corollary to this is a marathon. While the distance run is the same whether the event is held on a cold or warm day, the risk for exertional heat stroke is greater in warm. This does not mean, however, that the heat generated by running does not contribute to heat stroke. What previous rat studies with MDMA, which suggest locomotion does not contribute to hyperthermia, may not account for is that heat dissipation will be more efficient in a cold or cool environment than a warm environment. This would be expected to affect not only radiant heat loss through skin but also evaporative heat loss by saliva spreading (Hainsworth 1967), as well as loss of heat through respiration (both heat transfer and evaporation). Therefore, heat stroke from MDMA occurs when heat generation is greater than heat dissipation.

The assumption that locomotion does not contribute to hyperthermia from MDMA has led to the belief that thermogenesis from MDMA was mediated primarily through nonshivering thermogenesis (via iBAT and possibly skeletal muscle). This idea is supported by data

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showing that drugs that decrease hyperthermia from MDMA also decrease nonshivering thermogenesis. Clozapine, for instance, decreases hyperthermia from MDMA and has also been shown to decrease iBAT thermogenesis (Blessing et al. 2006). Clozapine, however, also decreases locomotion produced by MDMA (Kehne et al. 1996). In fact, the majority of compounds that have been shown to decrease or prevent hyperthermia induced by MDMA also decrease locomotion – e.g., the 5-HT-2A antagonists M 100907; the D2 antagonist haloperidol; the D1 antagonist SCH 23390; and the alpha-1 antagonist prazosin (Kehne et al. 1996; Fantegrossi et al. 2004). While nonshivering thermogenesis likely contributes to hyperthermia produced by MDMA, it may not be the primary means by which heat is generated.

MDMA impairs heat dissipation. Our data, similar to what others have reported, show that MDMA causes vasoconstriction. In this study, vasoconstriction was maximum immediately after the MDMA was administered and returned to baseline by 30 min. This is different from what others have reported with MDMA. In their article, Blessing et al. 2003 showed sustained reductions in tail blood flow for as long as 90 min after the injection. There are several factors that might account for these differences. They conducted their experiments between 26°C and 28°C, while we conducted our studies at 32°C. In their graphs, core temperature rises slowly and does not exceed 40°C. Since our experiments were conducted at a higher temperature, our rats had higher core and skin temperatures. This may have increased the peripheral and central afferent signals activating warm-sensitive neurons causing vessels to vasodilate sooner. Another difference with this study is the route of administration. We administered MDMA i.v., which would create higher blood concentrations that more rapidly distribute out of the vascular system. This could explain our rapid responses and recovery. In addition, i.v. administration would eliminate drug first-pass effect; as MDMA has numerous active metabolites this could also account for differences seen in this study (Baumann et al. 2009). Independent of this, our data clearly show that even though vasoconstriction returned to baseline levels by 30 min, core temperatures in these animals continued to rise. This suggests that in our experiments heat generation contributes more to a core temperature change than impaired heat dissipation.

Another surprising finding in our data is that MDMA-mediated increases in iBAT thermogenesis, and decreases in tail blood flow, were not mediated by neurons in either the DMH or mRPa. These data clearly demonstrate that hyperthermia mediated by MDMA is not the result of altering thermoregulatory circuits in the brain as has been previously suggested (Hargreaves et al. 2007; Benamar et al. 2008). Rather, our data suggest that monoamines released by MDMA act either directly at the level of the spinal cord or peripherally on blood vessels and iBAT. Ootsuka et al. (2004) showed that inhibiting neurons in the region of the mRPa, in anesthetized rabbits, did not prevent increases in sympathetic outflow to the ear caused by MDMA. Increases in sympathetic nerve activity were, however, reversed by the systemic injection of SR46349B, a 5-HT2A antagonist. They suggested that MDMA mediates its cutaneous effects through serotonin receptors in the spinal cord. These experiments, however, were conducted in anesthetized animals and the effect on blood flow was not measured. Our data are the first to measure the effect of inhibiting brain regions on tail blood flow in a conscious freely moving animal. Whether or not MDMA is acting at the level of the spinal cord requires further research. It is also possible that MDMA could have direct effects on tail vasoconstriction. In the rat MDMA has weak agonist properties on numerous adrenergic receptors in rats, including alpha-1, alpha-2, trace amine, and 5-HT2A (Nash et al. 1994; Bexis and Docherty 2006) (Broadley et al. 2013). Although these effects are relatively weak, at high doses, similar to those in our studies, it is possible that MDMA may directly constrict cutaneous vessels through these receptors, which are also located on vascular smooth muscle and cause vasoconstriction (Villalön and Centuriön 2007). If MDMA causes direct vasoconstriction in humans, it is most likely through 5-HT2A receptors (Liechti et al. 2000). In both humans and rodents, MDMA has also been shown to cause large increases in circulating levels of norepinephrine and epinephrine, which can cause vasoconstriction through alpha-1 receptors (Sprague et al. 2005; Hysek et al. 2011, 2013a).

In conclusion, this study suggests that locomotion mediated by MDMA, through neurons located in the DMH, may contribute to fatal hyperthermia in warm environment. If true, drugs or strategies that decrease locomotion may prevent mortality. Confirming the role of locomotion, and the central mechanisms through which it is controlled, is an important next step in understanding how MDMA causes temperature-related deaths.

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Disclosure

None declared.

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