Chronic Stress Enhances the Corticosterone Response and Neurotoxicity to +3,4-Methylenedioxymethamphetamine (MDMA): The Role of Ambient Temperature

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ABSTRACT

Stress facilitates drug abuse by humans. In rodents, stress enhances the neurochemical, neuroendocrine, and behavioral responses to psychostimulants. Although chronic unpredictable stress (CUS) enhances the acute hyperthermic and long-term monoamine-depleting effects of the psychostimulant +3,4-methylenedioxymethamphetamine (MDMA), the roles of hyperthermia and corticosterone (CORT) in mediating the stress-induced enhancement of MDMA-induced serotonin (5-HT) and dopamine (DA) depletions are unknown. Rats were exposed to 10 days of CUS and then challenged with MDMA (5 mg/kg i.p. once every 2 h for a total of four injections). Prior exposure to CUS augmented MDMA-induced hyperthermia and plasma CORT secretion and the long-term depletions in 5-HT content in striatum, hippocampus, and frontal cortex and DA content in striatum. A reduced ambient temperature of 21°C

attenuated the hyperthermia, CORT secretion, and 5-HT decreases after MDMA in nonstressed rats. The lower ambient temperature also prevented the augmented hyperthermia, CORT secretion, and enhanced 5-HT and DA depletions after MDMA in chronically stressed rats to levels exhibited by nonstressed, MDMA-treated rats. To investigate the role of CORT on monoamine depletions in response to MDMA, stressed and nonstressed rats were treated with the CORT synthesis inhibitor metyrapone during exposure to MDMA. Metyrapone prevented CORT secretion in both stressed and nonstressed rats but did not modify 5-HT or DA depletions in any brain region examined. This study suggests that enhanced CORT is a consequence of enhanced hyperthermia and the CUS-induced enhancements of MDMA-induced monoamine depletions may be mediated by hyperthermia but not CORT.

Introduction

Drug abuse by humans is often precipitated by stressful life events (Sinha, 2008). Repeated exposure to stressors also alters the neurobehavioral responses to drugs of abuse in rodent models such that prior exposure to stress facilitates psychostimulant self-administration and reinstates drug-seeking behavior in extinguished animals (Piazza and LeMoal, 1998). The facilitation and reinstatement of drug use can be prevented by adrenalectomy or corticosterone (CORT) synthesis inhibition (Marinelli and Piazza, 2002), suggesting a role for CORT in addiction and relapse. Recently, we demonstrated that prior exposure to chronic unpredictable stress (CUS) enhances +3,4-methylenedioxymethamphetamine (MDMA; Ecstasy)-

induced hyperthermia and serotonin (5-HT) depletions and causes a dopamine (DA) depletion in the rat brain, all of which depended on chronic stress-induced elevations in CORT (Johnson and Yamamoto, 2009). These results show that the acute and long-term consequences of MDMA are enhanced by stress in a CORT-dependent manner.

Persistent elevations in CORT result in increased susceptibility not only to substance abuse but also to depression, disease, and cognitive deficits in humans (McEwen 2000; de Kloet et al., 2005; Sinha, 2008). Likewise, chronic stress produces deficits in hippocampal-dependent cognition in rodents (McEwen, 2000) which may be caused by CORT-dependent dendritic atrophy in this region (Magariños and McEwen, 1995). Moreover, chronic stress or exogenous administration of glucocorticoids increases vulnerability to and potentiates hippocampal neuronal damage produced by neurotoxins (Sapolsky, 1985; Stein-Behrens et al., 1994) and enhances monoamine depletions induced by MDMA (Johnson and Yamamoto, 2009). The mechanisms of how prolonged stress and neurotoxins in-

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ABBREVIATIONS: CUS, chronic unpredictable stress; ANOVA, analysis of variance; AUC, area under the curve; CORT, corticosterone; DA, dopamine; MDMA, 3,4-methylenedioxymethamphetamine; NO, nitric oxide; PO, preoptic area of the hypothalamus; 5-HT, serotonin; q2h×4, once every 2 h for a total of four injections.

teract to enhance neuronal damage have not been fully elucidated but may involve CORT.

MDMA is a widely abused "club drug" and member of the amphetamine family of compounds. High-dose MDMA administration to rodents and nonhuman primates results in long-term, selective decreases in 5-HT nerve terminal markers, such as 5-HT tissue content, activity of tryptophan hydroxylase, and the density of 5-HT reuptake sites in several brain regions including the hippocampus, striatum, and cortex (for review see Baumann et al., 2007). Depletions of 5-HT have been reported to endure for as long as 52 weeks after exposure to MDMA, with the most pronounced and long-lasting decreases occurring in the hippocampus and frontal cortex (Sabol et al., 1996). High-dose MDMA produces hippocampal-dependent cognitive deficits in rodents (Baumann et al., 2007), and cognitive deficits are present in abstinent human MDMA abusers along with decreased 5-HT transporter binding in several regions including the hippocampus (McCann et al., 2008).

Hyperthermia is a major contributor to MDMA-induced 5-HT depletions. The prevention of hyperthermia during MDMA administration attenuates 5-HT depletions, whereas increasing ambient temperature during MDMA administration enhances depletions of 5-HT (Malberg and Seiden, 1998). In addition to hyperthermia, CORT secretion is increased by MDMA (Nash et al., 1988), but its role in 5-HT depletions has been controversial. Johnson et al. (1989) demonstrated that adrenalectomized rats supplemented with high CORT and treated with MDMA had enhanced depletions of 5-HT in the hippocampus. Fernandez et al. (2002) found that adrenalectomy attenuated both MDMA-induced hyperthermia and depletions of 5-HT in striatum. In contrast, Aguirre et al. (1997) found that adrenalectomy did not significantly alter MDMA-induced 5-HT depletions. We have shown that prior exposure to chronic stress potentiates MDMA-induced hyperthermia (Johnson and Yamamoto, 2009), and others have found increases in temperature and CORT in response to a novel stressor after chronic stress exposure (Bhatnagar et al., 2006). However, the effects of CUS on CORT responses to repeated MDMA administration and the roles of hyperthermia and CORT in the enhanced 5-HT and DA depletions produced by CUS exposure before MDMA are unknown.

The current studies investigated the effects of prior exposure to CUS on MDMA-induced hyperthermia, CORT secretion, and long-term changes in 5-HT and DA tissue content. The roles of hyperthermia and CORT were investigated in both nonstressed and chronically stressed rats by determining the effect of treatment with MDMA at a reduced ambient temperature of 21°C compared with a typical ambient temperature of 24°C and pharmacologically inhibiting the synthesis of CORT during MDMA administration.

Materials and Methods

Animals and Stress Exposure. Male Sprague-Dawley rats (175–250 g) were purchased from Harlan (Indianapolis, IN). Rats were group housed in clear plastic containers (45 × 24 × 20 cm) with food and water available ad libitum, in a temperature (23 ± 1°C)-controlled environment on a 12-h light/dark cycle (lights on 7:00 AM and off 7:00 PM). All procedures were carried out in accordance with the Boston University and the University of Toledo Institutional Animal Care and Use Committees and the National Institutes of

Health Guide for the Care and Use of Laboratory Animals to ensure that animal numbers and suffering were kept to a minimum.

Stressed rats were exposed to stressors that varied by day and time, two per day, for 10 days. The chronic unpredictable stress model varies the type, time, and exposure length of each stressor to avoid adaptation to the stressors (Herman et al., 1995). This model better mimics unexpected stressful life events encountered by humans. The following schedule was used: day 1, 10:00 AM 50-min cold room (4°C) and 1:00 PM 60-min restraint; day 2, 11:00 AM 60-min cage agitation and 6:00 PM lights on overnight; day 3, 10:00 AM 3-h lights off and 3:00 PM 3-min swim stress (23°C); day 4, 11:00 AM 50-min restraint and 5:00 PM food and water deprivation overnight; day 5, 12:00 PM 15-min cold room isolation (4°C) and 12:30 PM isolation housing overnight; day 6, 10:00 AM 4-min swim stress (23°C) and 6:00 PM lights on overnight; day 7, 9:00 AM 2-h lights off and 6:00 PM food and water deprivation overnight; day 8, 10:00 AM 30-min restraint and 3:00 PM 40-min cage agitation; day 9, 11:00 AM 3-min swim stress (23°C) and 6:00 PM lights on overnight; day 10, 10:00 AM 3-h lights off and 1:00 PM 20-min cage agitation. Non-stressed rats were transported daily to the area where stressors were administered but they were not exposed to the stressors. After the completion of each stressor, rats were returned to the housing room.

Drugs and Drug Administration. +3,4-Methylenedioxymethamphetamine (MDMA) hydrochloride was provided by the National Institute on Drug Abuse (Rockville, MD). The day after the last stressor (day 11), 5 mg/kg MDMA, dissolved in 0.9% NaCl (saline), or saline was injected intraperitoneally once every 2 h for a total of four injections (q2h×4) (5 mg/kg i.p.) at a volume of 1 ml/kg. MDMA or saline injections were administered in a room with an ambient temperature of 24°C unless otherwise specified. This lower repeated dosing regimen of MDMA was selected for its lesser impact on 5-HT tissue content depletions as determined in preliminary experiments.

2-Methyl-1,2-di-3-pyridyl-1-propanone (metyrapone) was purchased from Sigma-Aldrich (St. Louis, MO). Metyrapone inhibits corticosteroid biosynthesis by binding to 11 β -hydroxylase, the enzyme that converts 11-deoxycorticosterone to CORT in the adrenal glands (Sonino, 1982). Metyrapone was dissolved in 10% ethanol and administered intraperitoneally in a volume of 1 ml/kg. Metyrapone (50 mg/kg) or its vehicle, 10% ethanol (1 ml/kg), was administered 2 and 1 h before the first injection of MDMA and then 1 h before the second, third, and fourth injections of MDMA. This treatment regimen was effective at preventing MDMA-induced CORT secretion in preliminary experiments.

Temperature Measurements and Manipulation. On day 10 after the last stressor (5:00 PM), rats were moved to treatment rooms maintained at either 21 or 24°C where they were housed overnight. For the metyrapone experiment, all rats were treated in a room maintained at 24°C. The day after the last stressor (day 11), rats received repeated MDMA (5 mg/kg i.p. q2h×4) or saline injections beginning at 9:00 AM. Body temperature was measured with a rectal probe digital thermometer (Thermalert TH-8 monitor; Physitemp Instruments, Inc., Clinton, NJ). Temperatures were recorded before the first injection of MDMA or saline and 1 h after each injection by holding each rat at the base of the tail and inserting a probe (RET-2) 4.6 cm past the rectum into the colon for 6 to 8 s until rectal temperature was maintained for 3 s. The enhanced MDMA-induced hyperthermia in rats pre-exposed to CUS was effectively reduced to temperatures similar to those of nonstressed, MDMA-treated rats by treatment in a room maintained at 21°C compared with 24°C as determined in preliminary experiments. Rats were either killed 1 h after the fourth MDMA or saline injection for CORT measurement or returned to the housing colony the morning after MDMA or saline injections.

In a preliminary experiment to determine the dosing of metyrapone during MDMA treatment that would effectively prevent increases in CORT secretion, it was determined that metyrapone enhanced MDMA-induced body temperature. To eliminate body temperature effects on 5-HT depletions, both nonstressed and stressed rats administered metyrapone during MDMA treatment

were maintained at body temperatures typical of nonstressed and stressed MDMA-treated rats, respectively, by placing cages on ice water for brief periods of time. This procedure effectively maintained rectal temperatures similar to those of either nonstressed or stressed rats treated with MDMA.

Measurement of Plasma Corticosterone. Half of the rats from each treatment group were killed by rapid decapitation 1 h after the fourth injection of MDMA or saline. Trunk blood was collected into microcentrifuge tubes on ice and centrifuged at 800g for 14 min at 4°C, and the plasma was collected and centrifuged further at 800g for 7 min at 4°C. Plasma was stored at -80°C until analysis. Plasma CORT was analyzed with a commercially available EIA (Diagnostic Systems Laboratories, Webster, TX). Data are presented as ng/ml.

High-Performance Liquid Chromatography for Tissue 5-HT and DA Content. Rats from the other half of each treatment group were killed by rapid decapitation 5 days after MDMA or saline treatment. The brains were quickly removed, and whole striata, hippocampi, and frontal cortices were dissected and frozen on dry ice. The tissue was stored at -80°C for later analysis of 5-HT and DA tissue content. Tissues were sonicated in cold 0.25 M perchloric acid and centrifuged at 14,000g for 20 min at 4°C. The supernatant was analyzed with high-performance liquid chromatography with electrochemical detection. Separation of biogenic amines from their metabolites was achieved with a 3- μ m particle-size reverse-phase C-18 column (100 $\times$ 2.0 mm; Phenomenex, Torrance, CA) and a mobile phase consisting of 32 mM citric acid, 54.3 mM sodium acetate, 0.074 mM EDTA, 0.22 mM octyl sodium sulfate, and 3% methanol (pH 3.1). Compounds were detected with an LC-4B amperometric detector (BAS Bioanalytical Systems, West Lafayette, IN) with a 6-mm glassy carbon working electrode maintained at a potential of +0.6 V relative to an Ag/AgCl reference electrode. Data were collected by using EZChrom Elite software (Agilent Technologies, Santa Clara, CA). The pellet was dissolved in 1 N NaOH, and protein content was determined by using a Bradford assay (Bio-Rad Laboratories, Hercules, CA). Data are presented as pg/ μ g protein.

Statistical Analyses. Rectal temperatures were analyzed by using a three-way ANOVA with repeated measures with treatment as the between-subjects factor and time as the repeated-measures factor. Three-way ANOVAs were used for the analysis of area under the curve (AUC) for rectal temperatures, CORT, and 5-HT and DA tissue content. ANOVAs were followed by Tukey's post hoc comparisons. Statistical significance was set at $p < 0.05$ for all tests. Figure legends contain the sample sizes.

Results

Impact of CUS on MDMA-Induced Hyperthermia at Ambient Temperatures of 21 and 24°C

Figure 1A illustrates body temperatures in response to MDMA (5 mg/kg i.p. q2h $\times$ 4) or saline administered at ambient temperatures of 21 or 24°C to stressed and nonstressed rats. A three-way repeated measures ANOVA showed the main effects of treatment ($F_{7,409} = 169.76$, $p < 0.001$) and time ($F_{4,409} = 74.39$, $p < 0.001$) and a treatment $\times$ time interaction ($F_{28,409} = 16.99$, $p < 0.001$). Figure 1B illustrates body temperatures as an AUC. An AUC was generated for each rat by using a trapezoidal AUC analysis. A three-way ANOVA revealed the main effects of stress ($F_{1,80} = 58.026$, $p < 0.001$), MDMA ($F_{1,80} = 494.5$, $p < 0.001$), and ambient temperature ($F_{1,80} = 128.2$, $p < 0.001$) and significant stress $\times$ MDMA ($F_{1,80} = 56.163$, $p < 0.001$), MDMA $\times$ ambient temperature ($F_{1,80} = 28.786$, $p < 0.001$), and stress $\times$ MDMA $\times$ ambient temperature ($F_{1,80} = 5.371$, $p < 0.05$) interactions. A two-way ANOVA within the MDMA-treated groups showed a stress $\times$ ambient temperature interaction ($F_{1,42} = 4.442$, $p < 0.05$). The post hoc analysis

Fig. 1. Effect of MDMA (5 mg/kg i.p. q2h $\times$ 4) or saline administration at ambient temperatures of 21 or 24°C on body temperature of stressed and nonstressed rats. A, body temperatures (°C) were measured before and 1 h after each MDMA or saline injection. Arrows indicate each injection of MDMA or saline. Solid lines represent treatment at 24°C, and dashed lines represent treatment at 21°C. B, AUC values for all treatment groups. *, $p < 0.05$ compared with saline controls; #, $p < 0.05$ compared with No Stress/MDMA, & $p < 0.05$ for 24°C compared with 21°C. Values are expressed as means $\pm$ S.E.M. ($n = 10$ –11 per group).

revealed significant enhancements in body temperature between nonstressed and stressed rats treated with MDMA at both 21°C ($p < 0.001$) and 24°C ($p < 0.001$) and significant attenuation of increases in body temperatures between nonstressed rats treated with MDMA at 21°C compared with 24°C ($p < 0.001$) and between stressed rats treated with MDMA at 21°C compared with 24°C ($p < 0.001$).

Impact of CUS on MDMA-Induced Plasma CORT at Ambient Temperatures of 21°C and 24°C

Figure 2 illustrates plasma CORT levels measured 1 h after the last injection of MDMA (5 mg/kg i.p. q2h $\times$ 4) or saline administered at ambient temperatures of 21 or 24°C to stressed and nonstressed rats. A three-way ANOVA showed the main effects of stress ($F_{1,49} = 5.111$, $p < 0.05$), MDMA ($F_{1,49} = 94.941$, $p < 0.001$), ambient temperature ($F_{1,49} = 17.666$, $p < 0.001$), significant stress $\times$ MDMA interaction ($F_{1,49} = 21.301$, $p < 0.001$), stress $\times$ ambient temperature interaction ($F_{1,49} = 9.641$, $p < 0.01$), and MDMA $\times$ ambient temperature interaction ($F_{1,49} = 23.921$, $p < 0.001$); however, the stress $\times$ MDMA $\times$ ambient temperature interaction was not significant ($F_{1,49} = 0.105$, $p > 0.05$). Post hoc comparisons revealed that MDMA had a greater effect on CORT compared

Fig. 2. Plasma CORT (ng/ml) measured 1 h after the fourth injection of MDMA (5 mg/kg i.p. q2h $\times$ 4) or saline administration at ambient temperatures of 21 or 24°C to stressed and nonstressed rats. *, $p < 0.05$ compared with saline controls; #, $p < 0.05$ compared with No Stress/MDMA; &, $p < 0.05$ for 24°C compared with 21°C. Values are expressed as means $\pm$ S.E.M. ($n = 5$ –6 per group).

with saline in both nonstressed ($p < 0.05$) and stressed ($p < 0.001$) rats. MDMA had a greater effect on CORT in stressed rats compared with nonstressed rats ($p < 0.001$). MDMA also had a greater effect on CORT among rats treated at 24°C compared with rats treated with MDMA at 21°C ($p < 0.01$). MDMA administration produced a greater effect on CORT compared with saline administration among rats treated at either 21°C ($p < 0.001$) or 24°C ($p < 0.01$). Within the saline-treated rats there was no effect of stress compared with no stress on CORT ($p > 0.05$) and no effect of ambient temperature (21°C versus 24°C) on CORT ($p > 0.05$). A two-way ANOVA within MDMA-treated rats revealed a significant stress $\times$ ambient temperature interaction ($F_{1,27} = 4.259$, $p = 0.05$). Post hoc comparisons indicated that MDMA caused a greater increase in CORT in stressed compared with nonstressed rats at both 21°C ($p < 0.001$) and 24°C ($p < 0.001$) and elevated CORT levels after MDMA treatment were reduced at 21°C compared with 24°C in both nonstressed ($p < 0.01$) and stressed ($p < 0.001$) rats.

Impact of CUS on MDMA-Induced 5-HT and DA Depletions at Ambient Temperatures of 21 and 24°C

Figure 3 illustrates 5-HT and DA tissue content in the striatum, hippocampus, and frontal cortex measured 5 days after MDMA (5 mg/kg i.p. q2h $\times$ 4) or saline administration at ambient temperatures of 21 or 24°C to stressed and nonstressed rats. High-performance liquid chromatography with electrochemical detection peaks for DA in the hippocampus were below the detection limit and therefore were not measured.

Striatum. A three-way ANOVA of 5-HT tissue content in the striatum (Fig. 3A) revealed main effects of stress ($F_{1,43} = 10.908$, $p < 0.01$), MDMA ($F_{1,43} = 107.577$, $p < 0.001$), ambient temperature ($F_{1,43} = 14.789$, $p < 0.001$), stress $\times$ MDMA interaction ($F_{1,43} = 13.784$, $p < 0.001$), and MDMA $\times$ ambient temperature interaction ($F_{1,43} = 5.87$, $p < 0.05$). There was no stress $\times$ MDMA $\times$ ambient temperature interaction ($F_{1,43} = 0.032$, $p > 0.05$). Post hoc comparisons confirm that MDMA produced a significant depletion in 5-HT content compared with saline controls ($p < 0.001$) and prior exposure to CUS enhanced this depletion ($p < 0.001$). A two-way ANOVA within MDMA-treated groups confirmed the ability of CUS to increase 5-HT depletions ($F_{1,21} = 35.495$, $p <$

0.001) and revealed a main effect of ambient temperature ($F_{1,21} = 28.339$, $p < 0.001$). Post hoc comparisons showed that treatment with MDMA at 21°C prevented the enhancements in 5-HT depletions compared with treatment at 24°C ($p < 0.001$). Analysis of DA content in the striatum (Fig. 3B) revealed the main effects of stress ($F_{1,43} = 7.735$, $p < 0.01$), MDMA ($F_{1,43} = 14.095$, $p < 0.001$), ambient temperature ($F_{1,43} = 6.095$, $p < 0.05$), stress $\times$ ambient temperature interaction ($F_{1,43} = 5.989$, $p < 0.05$), and MDMA $\times$ ambient temperature interaction ($F_{1,43} = 3.798$, $p = 0.05$), but did not show a stress $\times$ MDMA $\times$ ambient temperature interaction ($F_{1,43} = 1.492$, $p > 0.05$). Post hoc comparisons indicated that MDMA had no effect on DA content in nonstressed rats compared with saline controls ($p > 0.05$), but MDMA produced a significant depletion in DA content in stressed rats ($p < 0.001$). A two-way ANOVA within MDMA-treated groups confirmed the ability of CUS to produce a DA depletion in stressed rats compared with nonstressed rats ($F_{1,21} = 8.127$, $p < 0.05$) and indicated a main effect of ambient temperature ($F_{1,21} = 7.12$, $p < 0.05$). Post hoc comparisons revealed that treatment with MDMA at 21°C prevented enhanced DA depletions compared with treatment at 24°C ($p < 0.01$).

Hippocampus. A three-way ANOVA of 5-HT tissue content in the hippocampus (Fig. 3C) revealed the main effects of stress ($F_{1,43} = 9.565$, $p < 0.01$), MDMA ($F_{1,43} = 142.347$, $p < 0.001$), ambient temperature ($F_{1,43} = 11.155$, $p < 0.01$), stress $\times$ MDMA interaction ($F_{1,43} = 4.753$, $p < 0.05$), and MDMA $\times$ ambient temperature interaction ($F_{1,43} = 6.217$, $p < 0.05$), but did not find a stress $\times$ MDMA $\times$ ambient temperature interaction ($F_{1,43} = 0.285$, $p > 0.05$). Post hoc comparisons confirmed that MDMA produced a significant depletion in 5-HT content compared with saline controls ($p < 0.001$) and prior exposure to CUS enhanced this depletion ($p < 0.001$). A two-way ANOVA within MDMA-treated groups confirmed the ability of CUS to increase 5-HT depletions ($F_{1,21} = 11.6$, $p < 0.01$) and showed a main effect of ambient temperature ($F_{1,21} = 14.198$, $p < 0.01$). Post hoc comparisons revealed that treatment with MDMA at 21°C prevented enhancements in 5-HT depletions compared with treatment at 24°C ($p < 0.01$).

Frontal Cortex. A three-way ANOVA of 5-HT tissue content in the frontal cortex (Fig. 3D) revealed the main effects of stress ($F_{1,43} = 9.385$, $p < 0.01$), MDMA ($F_{1,43} = 212.887$, $p < 0.001$), ambient temperature ($F_{1,43} = 9.154$, $p < 0.01$), stress $\times$ MDMA interaction ($F_{1,43} = 12.262$, $p < 0.01$), and MDMA $\times$ ambient temperature interaction ($F_{1,43} = 8.626$, $p < 0.01$). There was no stress $\times$ MDMA $\times$ ambient temperature interaction ($F_{1,43} = 1.41$, $p > 0.05$). Post hoc comparisons confirmed that MDMA produced a significant depletion in 5-HT content compared with saline controls ($p < 0.001$) and prior exposure to CUS enhanced this depletion ($p < 0.001$). A two-way ANOVA within MDMA-treated groups confirmed the ability of CUS to increase 5-HT depletions ($F_{1,21} = 31.438$, $p < 0.001$) and revealed a main effect of ambient temperature ($F_{1,21} = 25.933$, $p < 0.001$). Post hoc comparisons showed that treatment with MDMA at 21°C prevented enhancements in 5-HT depletions compared with treatment at 24°C ($p < 0.001$). There were no main effects of stress ($F_{1,43} = 0.128$, $p > 0.05$), MDMA ($F_{1,43} = 0.277$, $p > 0.05$), ambient temperature ($F_{1,43} = 0.0238$, $p > 0.05$),

Fig. 3. 5-HT and DA tissue concentrations (pg/μg protein) in the striatum (A and B), hippocampus (C), and frontal cortex (D and E) measured 5 days after injections of MDMA (5 mg/kg i.p. q2h×4) or saline administered at ambient temperatures of 21 or 24°C to stressed and nonstressed rats. *, $p < 0.05$ compared with saline controls; #, $p < 0.05$ compared with No Stress/MDMA; &, $p < 0.05$ for 24°C compared with 21°C. Values are expressed as means $\pm$ S.E.M. ($n = 5-6$ per group).

or a stress $\times$ MDMA $\times$ ambient temperature interaction ($F_{1,43} = 0.0002$, $p > 0.05$) on DA content in the frontal cortex (Fig. 3E).

Effect of Metyrapone Administration during MDMA on Body Temperature

Figure 4 illustrates rectal temperatures in response to MDMA (5 mg/kg i.p. q2h×4) or saline administration of stressed and nonstressed rats also receiving vehicle (10% EtOH, 1 ml/kg i.p.) or metyrapone (50 mg/kg i.p.) 2 and 1 h before the first MDMA or saline injection and 1 h before the second, third, and fourth injections. The body temper-

atures of MDMA-treated and nonstressed and stressed rats that received metyrapone were maintained at body temperatures typical of rats in the treatment groups that received vehicle instead of metyrapone. This was necessary because of a preliminary finding that metyrapone enhanced hyperthermia in MDMA-treated rats. A three-way repeated measures ANOVA showed the main effects of treatment ($F_{7,424} = 197.15$, $p < 0.001$) and time ($F_{4,424} = 136.69$, $p < 0.001$) and a treatment $\times$ time interaction ($F_{28,424} = 25.24$, $p < 0.001$). Post hoc comparisons indicated that MDMA administration to nonstressed rats re-

Fig. 4. Effect of metyrapone (50 mg/kg i.p.) or vehicle (10% EtOH, 1 ml/kg i.p.) treatment during MDMA (5 mg/kg i.p. q2h $\times$ 4) or saline administration on body temperatures of stressed and nonstressed rats. Metyrapone was administered 2 and 1 h before the first MDMA or saline injection and 1 h before the second, third, and fourth injections. Body temperatures ($^{\circ}$ C) were measured before and 1 h after each MDMA or saline injection. *, $p < 0.05$ compared with saline controls; #, $p < 0.05$ compared with No Stress/MDMA Vehicle and No Stress/MDMA Metyrapone. Small arrows indicate each metyrapone or vehicle injection, and large arrows indicate each injection of MDMA or saline. Values are expressed as means $\pm$ S.E.M. ($n = 10$ –12 per group).

sulted in increases in body temperature compared with saline-treated rats 1 h after the second ($p < 0.001$), third ($p < 0.001$), and fourth ($p < 0.001$) injections, and administration of MDMA to stressed rats resulted in increases in body temperature compared with saline-treated rats 1 h after the first ($p < 0.05$), second ($p < 0.001$), third ($p < 0.001$), and fourth ($p < 0.001$) injections. Stressed, MDMA-treated rats exhibited significant increases in body temperature compared with nonstressed, MDMA-treated rats 1 h after the second ($p < 0.001$), third ($p < 0.001$), and fourth ($p < 0.001$) injections of MDMA. There were no temperature differences between vehicle and metyrapone treatments in the nonstressed, MDMA-treated ($p > 0.05$ for all time points) or stressed, MDMA-treated ($p > 0.05$ for all time points) groups. There were no differences between any of the saline-treated groups whether the rats were stressed or nonstressed or treated with vehicle or metyrapone during saline treatment ($p > 0.05$ for all time points).

Effect of Metyrapone Administration during MDMA on Plasma CORT

Figure 5 illustrates plasma CORT in response to MDMA (5 mg/kg i.p. q2h $\times$ 4) or saline administration of stressed and nonstressed rats also receiving vehicle (10% EtOH, 1 ml/kg i.p.) or metyrapone (50 mg/kg i.p.) 2 and 1 h before the first MDMA or saline injection and 1 h before the second, third, and fourth injections. A three-way ANOVA revealed a significant stress $\times$ MDMA $\times$ metyrapone interaction ($F_{1,41} = 7.744$, $p < 0.01$), indicating an increase in CORT after MDMA that was enhanced by prior exposure to CUS and prevented by metyrapone administration during MDMA. Metyrapone administration during MDMA to both nonstressed and stressed rats prevented increases in CORT. Analysis within MDMA-treated groups revealed a stress $\times$ metyrapone interaction ($F_{1,21} = 16.622$, $p < 0.001$), confirming the

Fig. 5. Plasma CORT (ng/ml) measured 1 h after the fourth injection of MDMA (5 mg/kg i.p. q2h $\times$ 4) or saline to stressed and nonstressed rats also receiving metyrapone or vehicle during MDMA or saline injections. *, $p < 0.05$ compared with saline controls; #, $p < 0.05$ compared with No Stress/MDMA Vehicle; &, $p < 0.05$ compared with No Stress/MDMA Metyrapone and Stress/MDMA Metyrapone. Values are expressed as means $\pm$ S.E.M. ($n = 5$ –6 per group).

stress-induced enhancement in MDMA-induced CORT and the prevention of MDMA-induced CORT with metyrapone. A post hoc comparison shows that prior exposure to CUS enhanced CORT compared with nonstressed, MDMA-treated rats ($p < 0.001$). Metyrapone administration during MDMA prevented increases in CORT in nonstressed rats ($p < 0.01$) and the enhancements in CORT in stressed rats ($p < 0.001$).

Effect of Metyrapone Administration during MDMA on 5-HT and DA Depletions

Figure 6 illustrates 5-HT and DA tissue content in the striatum, hippocampus, and frontal cortex 5 days after MDMA (5 mg/kg i.p. q2h $\times$ 4) or saline administration to stressed and nonstressed rats also receiving vehicle (10% EtOH, 1 ml/kg i.p.) or metyrapone (50 mg/kg i.p.) 2 and 1 h before the first MDMA or saline injection and 1 h before the second, third, and fourth injections.

Striatum. Analysis of 5-HT tissue content in the striatum (Fig. 6A) revealed a significant effect of MDMA on 5-HT depletion that was enhanced by CUS as noted by a main effect of MDMA ($F_{1,42} = 134.238$, $p < 0.001$) and a stress $\times$ MDMA interaction ($F_{1,42} = 16.295$, $p < 0.001$). The post hoc comparisons confirmed that MDMA produced a 5-HT depletion compared with saline controls ($p < 0.001$) and chronically stressed rats treated with MDMA had enhanced 5-HT depletions compared with nonstressed, MDMA treated rats ($p < 0.001$). The three-way ANOVA did not find a stress $\times$ MDMA $\times$ metyrapone interaction ($F_{1,42} = 0.0576$, $p = 0.812$), and the post hoc analysis did not reveal an effect of metyrapone compared with vehicle treatment during MDMA on 5-HT depletions in either nonstressed ($p > 0.05$) or stressed ($p > 0.05$) rats. Metyrapone had no effect on 5-HT concentrations in either nonstressed ($p > 0.05$) or stressed ($p > 0.05$) rats administered saline. A three-way ANOVA of DA tissue content in the striatum (Fig. 6B) did not find a stress $\times$ MDMA $\times$ metyrapone interaction ($F_{1,42} = 1.211$, $p = 0.279$); however, there was a significant stress $\times$ MDMA interaction ($F_{1,42} = 16.748$, $p < 0.001$). Post hoc comparisons revealed that MDMA had no effect on DA content in nonstressed rats compared with saline controls ($p > 0.05$) but MDMA caused a DA depletion in chronically stressed rats compared with nonstressed rats similarly treated with MDMA ($p < 0.001$).

Fig. 6. 5-HT and DA tissue concentrations (pg/μg protein) in the striatum (A and B), hippocampus (C), and frontal cortex (D and E) measured 5 days after injections of MDMA (5 mg/kg i.p. q2h×4) or saline to stressed and nonstressed rats also receiving metyrapone or vehicle during MDMA or saline injections. *, $p < 0.05$ compared with saline controls; #, $p < 0.05$ compared with No Stress/MDMA Vehicle. Values are expressed as means $\pm$ S.E.M. ($n = 5-6$ per group).

There was no effect of metyrapone compared with vehicle treatment during MDMA on DA depletions in either nonstressed ($p > 0.05$) or stressed ($p > 0.05$) rats. Metyrapone had no effect on DA concentrations in either nonstressed ($p > 0.05$) or stressed ($p > 0.05$) rats administered saline.

Hippocampus. Analysis of 5-HT tissue content in the hippocampus (Fig. 6C) revealed a significant effect of MDMA on 5-HT depletion that was enhanced by CUS as noted by a main effect of MDMA ($F_{1,42} = 274.526$, $p < 0.001$) and a stress $\times$ MDMA interaction ($F_{1,42} = 9.984$, $p < 0.01$). The post

hoc comparisons confirmed that MDMA produced a 5-HT depletion compared with saline controls ($p < 0.001$) and chronically stressed rats treated with MDMA had enhanced 5-HT depletions compared with nonstressed, MDMA treated rats ($p < 0.001$). The three-way ANOVA did not find a stress $\times$ MDMA $\times$ metyrapone interaction ($F_{1,42} = 0.0990, p = 0.755$), and the post hoc analysis did not reveal an effect of metyrapone compared with vehicle treatment during MDMA on 5-HT depletions in either nonstressed ($p > 0.05$) or stressed ($p > 0.05$) rats. Metyrapone had no effect on 5-HT concentrations in either nonstressed ($p > 0.05$) or stressed ($p > 0.05$) rats administered saline.

Frontal Cortex. Analysis of 5-HT tissue content in the frontal cortex (Fig. 6D) revealed a significant effect of MDMA on 5-HT depletion that was enhanced by CUS as noted by a main effect of MDMA ($F_{1,42} = 133.571, p < 0.001$) and a stress $\times$ MDMA interaction ($F_{1,42} = 5.202, p < 0.05$). The post hoc comparisons confirmed that MDMA produced a 5-HT depletion compared with saline controls ($p < 0.001$) and chronically stressed rats treated with MDMA had enhanced 5-HT depletions compared with nonstressed, MDMA-treated rats ($p < 0.001$). The three-way ANOVA did not find a stress $\times$ MDMA $\times$ metyrapone interaction ($F_{1,42} = 1.166, p = 0.288$), and the post hoc analysis did not reveal an effect of metyrapone compared with vehicle treatment during MDMA on 5-HT depletions in either nonstressed ($p > 0.05$) or stressed ($p > 0.05$) rats. Metyrapone had no effect on 5-HT concentrations in either nonstressed ($p > 0.05$) or stressed ($p > 0.05$) rats administered saline. There were no stress $\times$ MDMA ($F_{1,36} = 0.165, p = 0.687$) or stress $\times$ MDMA $\times$ metyrapone ($F_{1,36} = 0.159, p = 0.693$) interactions on DA tissue content in the frontal cortex (Fig. 6E).

Discussion

Prior exposure to CUS enhances acute hyperthermia and plasma CORT responses produced by repeated administration of MDMA, enhancing long-term 5-HT tissue content depletions in striatum, hippocampus, and frontal cortex and reducing DA tissue content in striatum. Reduction of ambient temperature attenuates MDMA-induced hyperthermia, CORT secretion, and 5-HT depletions in rats with and without prior CUS exposure and prevents MDMA-induced DA depletions in stressed rats. Administration of the CORT synthesis inhibitor, metyrapone, during MDMA administration to nonstressed and stressed rats prevents CORT secretion, but does not alter 5-HT or DA depletions. These findings indicate that the increase in CORT after MDMA and the potentiated CORT response to MDMA after CUS are mediated by hyperthermia. Furthermore, the augmented monoamine depletions in MDMA-treated rats with prior CUS exposure are mediated by the hyperthermic response to MDMA and are not directly related to the ability of CUS to enhance MDMA-induced CORT.

CUS enhanced the hyperthermia (Figs. 1 and 4) and long-term 5-HT depletions in striatum (Figs. 3A and 6A), hippocampus (Figs. 3C and 6C), and frontal cortex (Figs. 3D and 6D) to MDMA administered in an ambient temperature of 24°C. MDMA caused a DA depletion in striatum (Figs. 3B and 6B) but not in cortex (Figs. 3E and 6E) of stressed rats compared with nonstressed rats treated at 24°C. These findings replicate previous results (Johnson and Yamamoto,

2009). Although a three-way interaction was not statistically significant between MDMA treatment, CUS history, and ambient temperature, when analyzed with a two-way ANOVA within MDMA-treated groups, treatment with MDMA at a reduced ambient temperature of 21°C attenuated the acute hyperthermia and long-term 5-HT depletions in nonstressed rats and prevented stress-induced enhancements in hyperthermia and depletions of 5-HT and DA compared with rats treated at an ambient temperature of 24°C (Figs. 1 and 3). Stressed and nonstressed rats treated with saline at 21°C exhibited a decline in body temperatures (Fig. 1); however, stress or the ambient temperature condition did not change 5-HT or DA content in saline-treated rats (Fig. 3). These findings agree with studies showing an association between MDMA-induced 5-HT depletions and the degree of hyperthermia (Malberg and Seiden, 1998) and support a role for hyperthermia in monoamine depletions produced by MDMA in CUS-exposed rats. These data also indicate that DA terminals in striatum are sensitive to the enhanced hyperthermic response to MDMA after CUS. The lack of effect on DA terminals in cortex (Fig. 3E) may be explained by findings that these terminals are also relatively insensitive to the known striatal DA neurotoxin, methamphetamine (Gehrke et al., 2003).

An interaction between stress and 5-HT_{2A} receptors within the preoptic area of the hypothalamus (PO) may mediate the ability of CUS to enhance MDMA-induced hyperthermia. Microinjection of the 5-HT_{2A/C} agonist, ± 1 -(2,5-dimethoxy-4-iodophenyl)-2-amino-propane, into the rat PO produces hyperthermia (Lin et al., 1998). Exposure to 10 days of CUS increases 5-HT_{2A} mRNA in the PO (Raudensky and Yamamoto, 2008), and ± 1 -(2,5-dimethoxy-4-iodophenyl)-2-amino-propane-induced hyperthermia is enhanced in rats pre-exposed to 10 days of CUS (Matuszewich and Yamamoto, 2003). The CUS-induced enhancement in methamphetamine-induced hyperthermia is prevented by 5-HT_{2A/C} antagonism with ketanserin during methamphetamine exposure (Doyle and Yamamoto, 2010). 5-HT_{2A/C} receptor activation also mediates MDMA-induced hyperthermia (Schmidt et al., 1990). Therefore, increased 5-HT_{2A} receptors in the PO may also produce enhanced hyperthermia to MDMA after CUS.

The mechanisms by which MDMA and hyperthermia enhance 5-HT depletions after CUS are unknown. One possibility is the role of CORT. MDMA increases CORT secretion in rodents (Nash et al., 1988) and humans (Mas et al., 1999), indicating MDMA itself is a stressor. Furthermore, both body temperature and CORT increase in response to a novel stressor after chronic stress (Bhatnagar et al., 2006). Based on analysis within the MDMA-treated groups, the current findings are the first to demonstrate that not only is MDMA-induced CORT secondary to the hyperthermic response, but prior exposure to CUS enhances MDMA-induced CORT secretion in a hyperthermia-dependent manner (Fig. 2). Whereas CORT in stressed rats is elevated the morning after the last stressor (Johnson and Yamamoto, 2009), CORT levels of stressed rats after injections of saline were similar to those of nonstressed, saline-treated rats at 24°C (Figs. 2 and 5). This can be explained by the finding that whereas chronic stress elevates basal CORT the morning after the last stressor, CORT levels of stressed rats are equivalent to nonstressed rats by evening (Ottenweller et al., 1994). Injections of saline at 21°C seem to increase CORT compared with the same treatment at 24°C (Fig. 2). These data suggest that

reduced ambient temperature is stressful to naive rats, but stressed rats that have been exposed to cold during CUS are tolerant to decreases in ambient temperature. Whereas CORT secretion and monoamine depletions were attenuated by the reduction in ambient temperature, a specific role for CORT in MDMA-induced monoamine depletions that is independent of hyperthermia could not be established.

The role of CORT in MDMA-induced 5-HT depletions has been investigated by using adrenalectomized rats, but the effects were variable. Johnson et al. (1989) found that adrenalectomy attenuated MDMA-induced decreases in tryptophan hydroxylase activity and adrenalectomy combined with CORT supplementation enhanced MDMA-induced 5-HT depletions. In contrast, Aguirre et al. (1997) found that adrenalectomy did not alter MDMA-induced 5-HT depletions. However, body temperatures were not recorded in either study; therefore the impact of adrenalectomy on MDMA-induced hyperthermia could not be determined.

Fernandez et al. (2002) found that MDMA elicited a lesser hyperthermic response in adrenalectomized rats compared with sham-operated rats and attenuated 5-HT depletions. However, adrenalectomy removes both the adrenal medulla and adrenal cortex, resulting in decreased sympathetic responses to MDMA and, consequently, hyperthermia. The hyperthermic response to MDMA involves activation of the sympathetic nervous system and the release of norepinephrine, causing cutaneous vasoconstriction and increased body temperature (Sprague et al., 2003). Sympathectomy, ganglionic blockade, or norepinephrine α 1- and β 3-receptor antagonism attenuate MDMA-induced hyperthermia and 5-HT depletions (Pedersen and Blessing, 2001; Fernandez et al., 2002; Sprague et al., 2003), suggesting that sympathetic blockade rather than CORT reduction after adrenalectomy participates in MDMA-induced hyperthermia and neurotoxicity. To address this possible confound, a pharmacological approach to prevent CORT secretion during MDMA administration was used. Administration of the 11β -hydroxylase inhibitor metyrapone diminishes stress-induced elevations in plasma CORT without affecting basal levels (Piazza et al., 1994). In the current study, metyrapone administration during MDMA prevented increases in CORT measured 1 h after the final MDMA injection in both nonstressed and stressed rats without affecting basal concentrations (Fig. 5). Although metyrapone blocked MDMA-induced CORT, it did not prevent MDMA-induced 5-HT depletions in either nonstressed or stressed rats in any brain region examined (Fig. 6). The MDMA-induced DA depletion in the striatum of stressed rats was also unaffected by metyrapone (Fig. 6B). Therefore, CORT secreted during MDMA or the enhancement of CORT by prior exposure to CUS did not produce or enhance monoamine depletions. In contrast, CORT may enhance the stimulant and rewarding properties of MDMA similar to amphetamine and cocaine (Marinelli and Piazza, 2002).

The enhancement of MDMA-induced hyperthermia after CUS may produce monoamine depletions via metabolic and oxidative stress. Environmental hyperthermia decreases endogenous antioxidants and increases nitric oxide (NO), superoxide production, and consequent lipid peroxidation (Sharma et al., 2000; Chang et al., 2007). Stress causes hyperthermia in both humans (Marazziti et al., 1992) and rodents (Olivier et al., 2003), and chronic stress decreases endogenous antioxidants and mitochondrial function, increases NO production, and pro-

duces lipid peroxidation in rodents (Madrigal et al., 2006). MDMA decreases endogenous antioxidants and mitochondrial function, increases the production of free radicals and NO, depletes ATP, and causes lipid peroxidation (Quinton and Yamamoto, 2006). The prevention of MDMA-induced oxidative stress with antioxidants or through NO synthase inhibition blocks 5-HT depletions without affecting hyperthermia (Quinton and Yamamoto, 2006), suggesting oxidative stress is a consequence of hyperthermia. Coadministration of MDMA and the mitochondrial complex II inhibitor malonate produces a DA depletion in striatum (Nixdorf et al., 2001), implicating enhanced oxidative stress as a cause of DA depletions in stressed rats. Thus, the augmented hyperthermic response to MDMA in stressed rats may enhance 5-HT depletions and produce a DA depletion via enhanced production of metabolic and oxidative stress. The ability of CUS exposure to enhance metabolic and oxidative stress in response to MDMA warrants further investigation.

The present study demonstrates that the CUS-induced enhancement in the long-term neurotoxic effects of MDMA is influenced by hyperthermia. This study is the first demonstration that the CORT response to repeated injections of MDMA is enhanced by previous CUS exposure and mediated by hyperthermia. Although the precise mechanisms by which MDMA-induced monoamine depletions are enhanced by CUS remain unknown, our studies demonstrate that although hyperthermia may play some role, acute increases in CORT do not directly affect monoamine depletions. Given the coincidence of stress and drug abuse (Sinha, 2008) and findings that stress enhances the long-term neurotoxic effects of psychostimulants (Matuszewich and Yamamoto, 2004; Johnson and Yamamoto, 2009; Doyle and Yamamoto, 2010), the current study emphasizes the importance of understanding how stress and psychostimulants interact to contribute to enhanced drug use and associated long-term deleterious consequences.

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