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ORIGINAL INVESTIGATION

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Brain concentrations of d-MDMA are increased after stress

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Abstract *Rationale:* In the mouse but not the rat, d-3,4-methylenedioxymethamphetamine (d-MDMA) is a dopaminergic neurotoxicant. Various stressors and hypothermia protect against d-MDMA-induced neurotoxicity through unknown mechanisms, one of which could be a reduction in the distribution of d-MDMA to the brain. *Objectives:* We determined striatal levels of d-MDMA in relation to body temperature in mice exposed to a neurotoxic regimen of d-MDMA in the presence or absence of various stressors. *Methods:* Female C57BL6/J mice received a neurotoxic regimen of d-MDMA (15.0 mg/kg s.c. as the base every 2 h×4) alone or in combination with manipulations with a known neuroprotective status. d-MDMA levels were determined by HPLC with fluorometric detection while rectal temperature provided core temperature status. Levels of dopamine, tyrosine hydroxylase and GFAP were used to assess neurotoxicity. *Results:* Restraint, ethanol co-treatment and cold stress were neuroprotective, caused hypothermia and increased striatal d-MDMA levels by 4- to 7-fold. Corticosterone treatment, as a stress mimic, did not alter striatal d-MDMA or temperature and was not protective. The protective glutamate receptor antagonist, MK-801, doubled striatal d-MDMA levels and caused hypothermia. *Conclusions:* Although stress and other protective manipulations can alter the striatal concentration of d-MDMA their hypothermia-inducing properties appear a more likely determinant of their neuroprotection against the striatal dopaminergic neurotoxicity of d-MDMA.

Keywords Mouse · MDMA · Striatum · Dopaminergic neurotoxicity · Stress · GFAP

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Introduction

Stress can alter toxicity and several stressors, including restraint, housing at 15°C, or treatment with agents known to release glucocorticoids (e.g. ethanol; Carson and Pruett 1996; Pruett et al. 1998), can protect the mouse brain from d-MDMA induced dopaminergic neurotoxicity (Miller and O'Callaghan 1994; Johnson and Miller 2001). While other protective manipulations against d-MDMA induced neurotoxicity have been reported for which the mechanisms are known [e.g. protection afforded by NOS inhibitors (Camarero et al. 2002) or protection by dopamine reuptake inhibitors (O'Shea et al. 2001)], the mechanism of stress-induced protection remains unclear. In this regard, it is known that protective stress manipulations induce a hypothermic response (Miller and O'Callaghan 1994; Johnson et al. 2000; Johnson and Miller 2001; Colado et al. 2001).

The simplest mechanism of stress-induced protection against d-MDMA-induced neurotoxicity might be to reduce delivery of d-MDMA to the target site, as has been demonstrated for other dopaminergic neurotoxins in the mouse (Chiba et al. 1984; Markey et al. 1984). However, no studies have been conducted to examine the effect of stress-lowered body temperature on d-MDMA levels in mouse brain. Therefore, we determined the concentration of d-MDMA in mouse striatum after several stressful neuroprotective manipulations while determining in the same mice the effects of each manipulation on rectal temperature. Stressful manipulations included restraint stress, low ambient temperature

stress and ethanol co-administration. As a stress mimic, we also examined the effects of administration of the stress hormone corticosterone. The glutamate receptor antagonist MK-801, previously reported to protect mice against the dopaminergic neurotoxicity of d-MDMA and methamphetamine (Miller and O'Callaghan 1994), is known to induce a hypothermic response when co-administered with d-MDMA (Miller and O'Callaghan 1994). Rather than its glutamate antagonist properties, the body temperature lowering effect of MK-801 is more likely responsible for its neuroprotective effect, since elevation of ambient temperature markedly attenuated the neuroprotective effect of MK-801 (Miller and O'Callaghan 1994). Therefore, the effect of this treatment on brain d-MDMA concentrations was also examined. To assess the relative contributions of body temperature reduction and altered drug distribution to neuroprotection, we determined correlations between the neuroprotective effect of each manipulation and its ability to alter mouse rectal temperature and striatal d-MDMA concentration.

Materials and methods

Materials

The following drugs and chemicals were obtained from the sources indicated: HPLC standards, corticosterone (Sigma Chemical, St Louis, Mo., USA); HPLC reagents (ESA, Chelmsford, Mass., USA); MK-801, 2-hydroxypropyl-beta-cyclodextrin (Research Biochemicals, Natick, Mass., USA); d-MDMA (Research Technology Branch, National Institute on Drug Abuse, Rockville, MD, USA); piperonyl amine was purchased from Aldrich Chemical Company(Milwaukee, Wisc., USA) and converted to its HCl salt.

Animals

All procedures were carried out according to protocols approved by the Institutional Animal Care and Use Committee and in accordance with the NRC Guide for the Care and Use of Laboratory Animals (1996). In accord with our previous neurotoxicity studies, female C57Bl/6J mice (8–10 weeks) were purchased from Jackson Laboratories (Bar Harbor, Maine, USA) and maintained in a colony certified by the American Association for Accreditation of Laboratory Animal Care (Miller and O'Callaghan 1994; O'Callaghan and Miller 1994). The mice were housed (6–8 per group) in plastic tub cages (30.5×30.5×15 cm) with heat treated pine shaving bedding (4 cm depth). The colony room was maintained under positive pressure ventilation at 21±1°C temperature and 50±10% humidity on a 12-h light/12-h dark cycle beginning at 0600 hours. Food containing 6% fat (ProLab ISOPRO RMH 3000) and water were available ad libitum.

Drug or vehicle administration

Throughout this series of experiments, animals weighed 20 g or more, and group sizes were a minimum of 10 animals per group (see specific *n* for each group in figure legends). d-MDMA 15 mg/kg (calculated as free base) or saline 200 µl were administered, by s.c. injection every 2 h for a total of four doses. This regimen causes reproducible alterations in markers of striatal dopaminergic neurotoxicity (Johnson and Miller 2001). The first injection was always given between 0900 and 1000 hours to minimize circadian influences on toxicity. Pre-treatments and co-treatments were timed according to the first d-MDMA injection as described below. When animals were pretreated with ethanol (3 g/kg), or MK-801 (1 mg/kg), these were administered by s.c. injection 30 min before the first and third injections of d-MDMA. Ethanol (USP grade, 200 proof) was diluted (2.5-fold) in saline to supply the dose of 3 g ethanol/kg body weight. The diluted ethanol was injected in a volume of 10 ml/kg body weight. MK-801 maleate salt was dissolved in sterile saline and the dose (1 mg/kg as the free base) was delivered in a volume of 10 ml/kg body weight. The doses of ethanol and MK-801 were chosen because they had been previously shown to protect mice from d-MDMA-induced neurotoxicity (Miller and O'Callaghan 1994). Corticosterone was delivered at a dosage of 20 mg/kg, s.c., given 30 min before the first, second and third saline or d-MDMA injection for a total of 3 injections in a volume of 10 ml/kg body weight. This regimen was chosen based on preliminary experiments indicating that it resulted in plasma levels similar to those caused by restraint. For example, corticosterone administration resulted in sustained plasma levels between 2000 and 5000 ng/ml over a 6-h period, while restraint caused sustained plasma levels of 400–900 ng/ml over an 8-h period (Johnson and Miller, unpublished data). Though corticosterone injection produced higher overall levels, our primary goal of achieving sustained elevated levels was achieved by the injections. Corticosterone (2 mg/ml) was suspended in a vehicle consisting of 2% 2-hydroxypropyl-beta-cyclodextrin (Research Biochemicals Inc., Natick, Mass., USA) in normal saline. Control animals received saline or corresponding vehicle at all corresponding injection times.

Restraint

Thirty minutes before the first dose of saline or d-MDMA, mice (20 g or greater) were secured into a 35-ml centrifuge tube (Nalgene Ultraplus thin wall 25 mm-inner diameter×89 mm length; Nalge Nunc International, Rochester N.Y., USA) which had been perforated in the round end to provide air flow from the front to the back of the tube. A small binder clip (0.75 in wide) held in place by a 20 g 1-in needle penetrating through the tube walls and the center of the clip secured the animal in the tube. This device allowed them to rotate from supine to prone position but did not allow them to rotate in the rostral/caudal axis. These devices allowed free mobility of the animal's tail, an important thermoregulatory structure in rodents (Gordon 1993). The restraint lasted 8.5 h and the animals were removed from the restrainer for each injection and temperature measurement.

15°C ambient temperature

Animals were singly housed in cages (23 cm length×15 cm width×16 cm height) with food and water but no bedding. Thirty minutes before the first injection of saline or d-MDMA, cages were placed into an environmental control chamber (Model #6030; Caron Products and Services, Marietta, Ohio, 15°C, 50% relative humidity) for 8.5 h. Room temperature control animals were singly housed as above but at 22°C and injected with saline or d-MDMA 15 mg/kg every 2 h for a total of four doses.

Rectal temperature measurements

Rectal temperature was measured using a rectal probe (RET-3) coupled to a thermometer (Bat-10; Physitemp, Inc. Clifton, N.J., USA) lubricated with mineral oil. To facilitate temperature measurements, mice were placed under a "Quonset" hut-shaped piece of foam that was approximately the length of the mouse and that was blocked at the front end. Each mouse was held by the base of the tail while the temperature probe was inserted to a pre-marked depth of 2.0 cm. This method minimized handling, and in conjunction with the fast-rise time of the rectal probe made it possible to obtain reliable measurements of rectal temperature in less than 30 s per mouse. Temperature was measured immediately

before the first injection of pretreatment or initiation of restraint or cold (baseline, i.e. 30 min before the first d-MDMA), again immediately before the first d-MDMA injection (time 0), at 2-h intervals through the treatment period before each subsequent d-MDMA injection (times 2, 4, 6, and 8 h) and 24 h after the first injection. Data are presented as temp-time course curves and as TAUCs to facilitate comparisons between the groups. The TAUC is an area under the curve measure calculated by Simpson's Rule, which integrates the temperature versus time curve (see Johnson et al. 2000 for a complete discussion concerning use of the TAUC). The TAUC for each mouse was calculated by the application of Simpson's Rule (Tallarida and Murray 1991) to temperatures measured at times 0, 2, 4, 6, and 8 h. This measure is expressed in units of °C-h and presented as a numerical value that facilitates comparison among the various treatment groups.

Brain dissection and tissue preparation

Brain tissue was collected at 2 h after the last injection to determine brain levels of d-MDMA, or 72 h after the first injection to determine neurotoxicity (DA, DOPAC, HVA, TH or GFAP levels). Striatal dopaminergic neurotoxicity is evident at 72 h, as indicated by a decrease in DA and TH content accompanied by an increase in GFAP (see O'Callaghan and Miller 1994 for a complete time course). Immediately after cervical dislocation and decapitation, whole brains were removed from the skull with the aid of blunt curved forceps. Striatum and hippocampus were dissected free hand on a thermoelectric cold plate (Model TCP-2; Aldrich Chemical, Milwaukee, Wisc., USA), frozen on dry ice and kept at −80°C until assay.

Determinations of striatal d-MDMA concentrations

The concentration of d-MDMA in striatum was determined from homogenized tissue using HPLC with fluorescence detection (excitation wavelength 285 nm, emission wavelength 320 nm). Preliminary experiments had indicated that d-MDMA concentration was stable in the striatum if the tissue was frozen immediately after sacrifice and dissection if maintained at −80°C. Therefore, samples were prepared by sonication of the previously frozen tissue in 200 µl 0.1 M HCl containing piperonyl amine (25 ng) as an internal standard. After centrifugation, samples were analyzed on a reversed phase HPLC column (Waters Symmetry, C_{18}, 250 mm×4.6 mm) using a mobile phase of 20 mM Na_2HPO_4, pH 3.8 with 0.5 mM octane sulfonic acid, 15% acetonitrile, and a flow rate of 0.8 ml per min. The elution time for d-MDMA and internal standard are 22.8 and 8.86 min, respectively. The limit of detection for d-MDMA is 1.0 ng per 100 µl injection (~2.0 ng in whole striatum) with an intra-assay variability of 3%.

Neurochemical assays

DA, DOPAC, HVA, 5-HT and 5-HIAA concentrations were determined in the left striatum or left hippocampus by HPLC with electrochemical detection as previously described (Johnson et al. 2000)

GFAP assay

Frozen (−80°C) right striatum or hippocampus was homogenized by sonification in 10 vol hot (90–95°C) 1% SDS. GFAP then was assayed by sandwich ELISA (O'Callaghan 2002). Total protein concentration of the SDS homogenate was determined by the method of Smith et al. (1985).

TH immunoassay

TH was assayed from the same SDS-homogenates using a fluorescence-based ELISA adapted from the GFAP assay (O'Callaghan 2002). Briefly, a mouse anti-TH monoclonal antibody (Calbiochem-EMD Biosciences, Darmstadt, Germany) was used to capture TH from dilutions of the SDS-homogenates. The detection antibody was a rabbit anti-TH polyclonal antibody (Calbiochem-EMD Biosciences) and quantification was achieved using anti-rabbit antibody conjugated with horseradish peroxidase (Amersham Biosciences, Uppsala, Sweden) using Quantablu Substrate (Pierce-Endogen, Rockford, Ill., USA) as the peroxidase substrate. Values were generated with an Fmax Plate Reader (Molecular Devices Corp., Sunnyvale, Calif., USA) set at 305/405 nm.

Statistical analysis

The JMP (version 4.04) statistical analysis software package (SAS Institute Inc., Cary, N.C., USA) was used for all data analysis. Individual variables (DA, GFAP, etc.) were evaluated by analysis of variance (ANOVA) followed by the Tukey-Kramer honestly significant difference test (alpha level set at 0.05).

Results

Selective dopaminergic neurotoxicity caused by d-MDMA in female C57Bl/6J mice

Mice treated with d-MDMA exhibited a robust and reliable dopaminergic neurotoxicity as evidenced by a depletion of DA (87%) and its metabolites (DOPAC 83% and HVA 54%), a loss of TH protein (86%) and a robust elevation in GFAP (~400%) (Fig. 1A). These data are commensurate with our previous work (Miller and O'Callaghan 1994; Johnson et al. 2000, 2002) although a lower dosage, (15 mg/kg s.c. every 2 h×4) was utilized.

The dopaminergic neurotoxicity of d-MDMA was not accompanied by decreased 5-HT levels in mouse striatum or hippocampus nor was hippocampal GFAP elevated by treatment with d-MDMA (Fig. 1B). These data confirm previous reports of the lack of d-MDMA-induced serotonergic changes in the mouse (O'Callaghan and Miller 1994; O'Shea et al. 2001; Johnson et al. 2002).

Effect of stress manipulations as modulators of d-MDMA-induced dopaminergic neurotoxicity

Restraining or singly housing mice at 15°C during the time of d-MDMA administration, or administering ethanol (3 g/kg, s.c.) 30 min before the first and third injections of d-MDMA, provided significant protection against d-MDMA-induced dopaminergic neurotoxicity. Each manipulation blocked d-MDMA-induced depletions of striatal DA, DA metabolites, and TH and also blocked the elevation of striatal GFAP (Fig. 2A–E). By contrast, corticosterone at levels similar to those caused by restraint, did not block d-MDMA induced neurotoxicity (Fig. 2A–E). Furthermore, none of the stress manipulations, including corticosterone administration, altered striatal DA, TH, DOPAC, HVA or GFAP in the absence

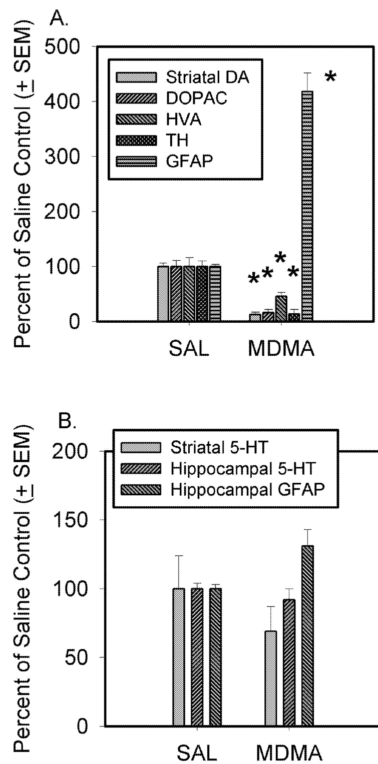

Fig. 1 Effect of d-MDMA treatment on striatal and hippocampal neurotransmitters, TH and GFAP. Mice received saline (200 µl every 2 h×4) or d-MDMA (15 mg/kg every 2 h×4) s.c. Tissue was collected 72 h after the first injection. The mean (±SEM) striatal DA, DOPAC, HVA, TH and GFAP values for grouped saline injected controls are as follows: DA 15.45 µg/g tissue (±0.73), DOPAC 2.71 µg/g tissue (±0.90), HVA 1.62 µg/g tissue (±0.26), TH 0.56 µg/mg total protein (±0.13), GFAP 0.140 µg/mg total protein (±0.040). Striatal 5-HT 0.435 µg/g tissue (±0.106), hippocampal 5-HT 1.34 µg/g tissue (±0.06), hippocampal GFAP 1.09 µg/mg total protein (±0.032). *Bars* indicate the mean±SEM. Saline treated, *n*=39, d-MDMA treated, *n*=69. *Asterisks* indicate a significant difference from saline injected control (ANOVA followed by Tukey's HSD, *P*<0.05)

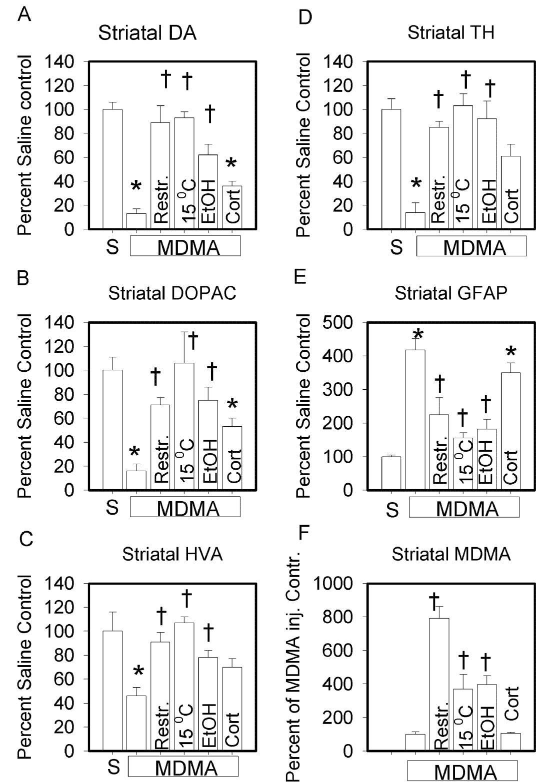

Fig. 2 Effect of stress manipulations on d-MDMA-induced dopaminergic neurotoxicity and striatal concentrations of d-MDMA. Restraint stress, 15°C housing, ethanol co-administration or corticosterone co-administration were each conducted as described in the methods. Striatal DA, DOPAC, HVA, TH, and GFAP were determined 72 h after the first dose of d-MDMA as described in Materials and methods. Absolute values for striatal DA, DOPAC, HVA, TH and GFAP for group housed saline controls were reported in legend for Fig. 1. Absolute values for other controls (restraint, 15°C single housed, 22°C single housed saline treated, ethanol pretreated and corticosterone pretreated were not significantly different from group housed saline values. Absolute values for group housed d-MDMA treated animals are as follows: striatal values for DA 2.32 µg/g tissue (±0.12), DOPAC 0.32 µg/g tissue (±0.10), HVA 0.46 µg/g tissue (±0.17), GFAP 0.790 µg/mg total protein (±0.089); for single housed 22°C d-MDMA treated; striatal values for DA 7.79 µg/g tissue (±0.99), DOPAC 0.83 µg/g tissue (±0.11), HVA 1.07 µg/g tissue (±0.11), GFAP 0.666 µg/mg total protein (±0.051). Striatal d-MDMA was determined 2 h after the last dose of d-MDMA as described in methods. Striatal d-MDMA absolute values for group housed and single housed d-MDMA injected control animals were 6.95 (±0.44) and 5.78 (±1.22) µg d-MDMA/g brain tissue, respectively. The number of animals in each group was as follows: restraint stress *n*=14, 15°C *n*=34, ethanol co-administration *n*=14, corticosterone *n*=12. Each *bar* indicates mean±SEM. *Asterisks* indicate a significant difference from saline injected control, (ANOVA followed by Tukey's HSD, *P*<0.05). The *dagger* (†) indicates a significant difference from animals injected with d-MDMA alone, (ANOVA followed by Tukey's HSD, *P*<0.05)

of d-MDMA treatment (data not shown). Note that animals single housed at 15°C were compared to animals single housed at 22°C given either saline or d-MDMA 15 mg/kg s.c. every 2 h for a total of four injections. d-MDMA administered to single housed animals at 22°C caused a neurotoxic lesion similar to group housed d-MDMA treated animals consisting of DA, DOPAC and TH depletion as well as GFAP elevation in striatum (see figure legend for absolute values) and replicates our previous work with higher dosages (Miller and O'Callaghan 1995).

Effect of stress manipulations on mouse striatal d-MDMA concentration

In an unstressed mouse the average concentration of d-MDMA was 6.95±0.44 µg/g striatum at 2 h after the last injection. Restraint stress, single housing at 15°C or co-administration of ethanol each raised the concentration of d-MDMA in mouse striatum by 4- to 7-fold (Fig. 2F). In contrast, corticosterone had no effect on the concentration of d-MDMA in striatum (Fig. 2F)

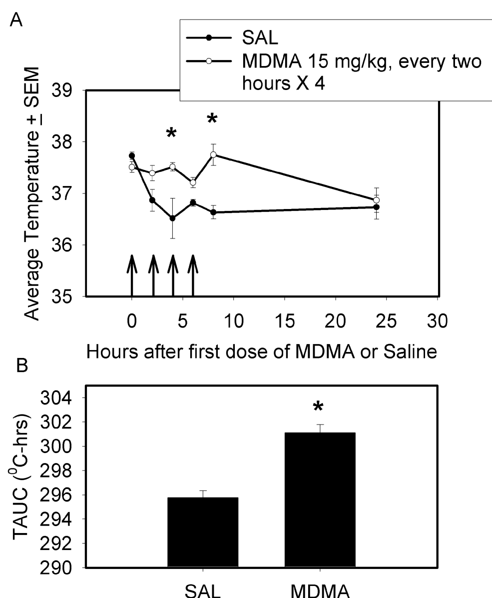

Fig. 3 Effect of d-MDMA or saline on rectal temperature in mice maintained at 22°C ambient temperature. d-MDMA or saline were administered as described in methods, arrows indicate injection times. Temperatures were obtained as described in methods. Graph **A** presents the average rectal temperature versus time curve for d-MDMA and saline treated animals. The number of animals in each group was as follows: saline *n*= 40, d-MDMA *n*=70. *Error bars* indicate SEM, the SEM is not shown if smaller than the radius of the point. Graph **B** presents the mean±SEM of TAUC for 0–8 h, which is an integration of the temperature versus time curve over the 0–8 h period. *Asterisks* indicate a significant difference from saline injected control (ANOVA followed by Tukey's HSD, *P*<0.05)

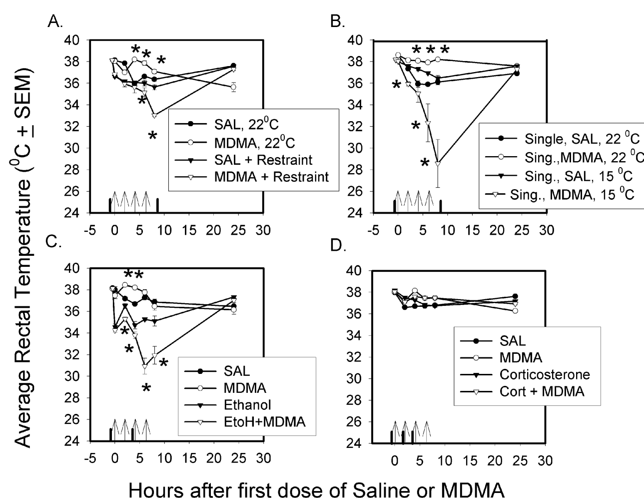

Fig. 4 Effect of stress conditions and d-MDMA or saline injection on mouse rectal temperature. d-MDMA or saline were administered and temperatures were obtained as described in methods. The number of animals in each group was as follows: restraint stress *n*=14, single housed at 15°C *n*=34, single housed at 22°C *n*=7, ethanol co-administration *n*=14, corticosterone *n*=12. Each point indicates the average rectal temperature. *Error bars* indicate SEM and are not shown if smaller than the radius of the point. *Asterisks* indicate a significant difference from saline injected control, (ANOVA followed by Tukey's HSD, *P*<0.05). In all parts, arrows indicate times of saline or d-MDMA injection. Graph **A** presents the average rectal temperature versus time curve for d-MDMA and saline treated animals with or without restraint. *Solid bars* represent start and end of restraint. Graph **B** depicts the effect of saline, or d-MDMA injection combined with single housing at 22°C versus single housing at 15°C on mouse rectal temperature. *Solid bars* indicate start and end of single housing at 22 or 15°C. Graph **C** depicts the effect of ethanol (3 g/kg s.c.) co-administration with saline or d-MDMA on rectal temperature. *Solid bars* represent times of ethanol injection. Graph **D** depicts the effect of corticosterone co-administration with saline or d-MDMA on rectal temperature. *Solid bars* indicate times of corticosterone (20 mg/kg, i.p.) injection. *Asterisks* indicate a significant difference from saline injected control animals (ANOVA followed by Tukey's HSD, *P*<0.05

Effects of d-MDMA and stress manipulations on mouse rectal temperature

Multiple injections of d-MDMA can alter rodent rectal temperature (Miller and O'Callaghan 1994; Dafters and Lynch 1998; Johnson et al. 2000; O'Loinsigh et al. 2001). The thermal effects of d-MDMA are dependent on ambient temperature and dose. Here, using an ambient temperature of 22°C and a dose of 15 mg/kg every 2 h×4, we observed a slight but reliable d-MDMA-induced temperature elevation (an increase or hyperthermia relative to saline injected animals). By contrast with rats, mice do not appear to require temperatures of 39–41°C to exhibit neurotoxicity with d-MDMA treatment (see O'Callaghan and Miller 2002 for a discussion of this issue). Over 13 experiments conducted at 22°C, mice treated with d-MDMA (15 mg/kg every 2 h×4 s.c.), were always slightly hyperthermic (~1°C) compared to saline injected controls and the expression of the data as an AUC makes this hyperthermia quite clear (Fig. 3). Alone, the stress manipulations caused only a slight reduction in body temperature (Fig. 4A–C, Fig. 5) but when administered in combination with d-MDMA treatment, each of those stressors caused body temperature reductions as indicated by the significant decrease in TAUC (Fig. 5). By contrast, corticosterone used as a stress mimic, did not cause hypothermia either by itself or in combination with d-MDMA (Figs 4D, 5).

Effects of pretreatment with MK-801 on d-MDMA-induced neurotoxicity, brain concentrations of d-MDMA and rectal temperature

Commensurate with our previous work (Miller and O'Callaghan 1994, 1995), MK-801 pretreatment completely blocked the neurotoxic effect of d-MDMA, preventing the DA and TH depletion as well as the elevation of striatal GFAP caused by d-MDMA (Fig. 6A,B,C). This protection was accompanied by a small and non-significant elevation of striatal d-MDMA concentration compared to animals treated with d-MDMA alone (Fig. 6D). The small MK-801-induced elevation of striatal d-MDMA concentration is in great contrast to the 4–7-fold elevations caused by the stressors (Fig. 2F). MK-

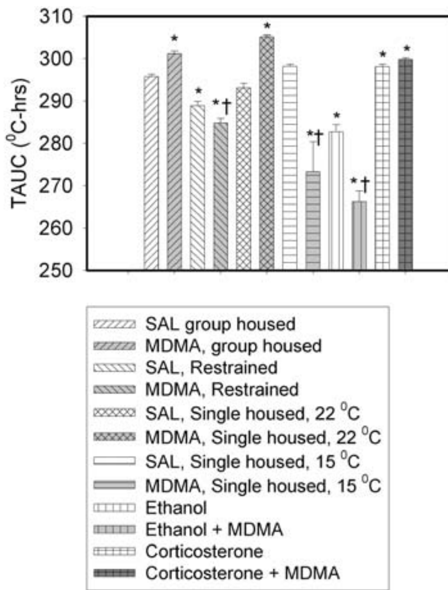

Fig. 5 Effects of stress manipulations on TAUC of mice treated with saline or d-MDMA. Graph presents the TAUC, which is an integration of the temperature versus time curve over the 0–8 h period. *Asterisks* indicate a significant difference from saline injected control (ANOVA followed by Tukey's HSD, $P<0.05$). *Dagger sign* (†) indicates a significant hypothermia compared to d-MDMA alone (ANOVA followed by Tukey's HSD, $P<0.05$)

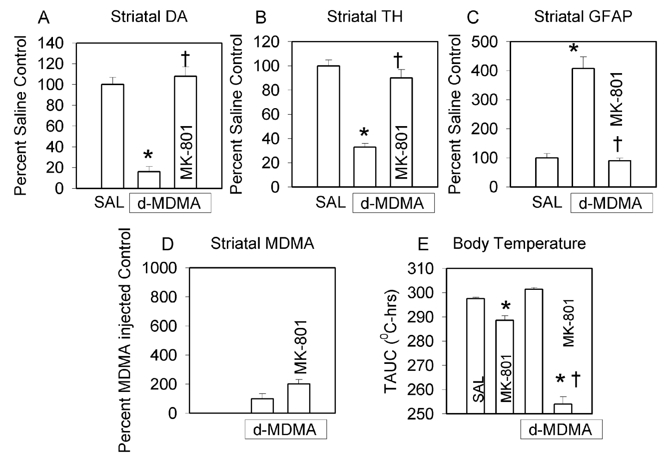

Fig. 6 Effects of pretreatment with the glutamate receptor antagonist MK-801 on d-MDMA-induced dopaminergic neurotoxicity, striatal concentrations of d-MDMA and rectal temperature. Pretreatments were conducted as described in methods. Briefly, MK-801 (1 mg/kg s.c.) was given 30 min before the first and third injections of saline or d-MDMA. Animals received either saline (200 µl every 2 h×4) or d-MDMA (15 mg/kg s.c. every 2 h×4). The number of animals in each group was as follows: MK-801 n=10, saline n=10). Rectal temperatures were obtained 30 min before the first and immediately before each d-MDMA or saline injection. TAUC was calculated as described in methods. Tissue was harvested either 2 h after the last injection of saline or d-MDMA for determination of striatal d-MDMA concentration or at 72 h after the first injection of saline or d-MDMA for determination of striatal DA, TH and GFAP. Pretreatment with MK-801 alone had no significant effect on striatal DA, TH or GFAP as values were not significantly different from those reported for saline controls (see legend for Fig. 1). In the graph, each bar represents the mean±SEM. *Asterisks* indicate a significant difference from saline injected control (ANOVA followed by Tukey's HSD, $P<0.05$). The *dagger sign* (†) indicates a significant difference from animals injected with d-MDMA alone, (ANOVA followed by Tukey's HSD, $P<0.05$)

801 alone caused a slight lowering of rectal temperature compared with saline treatment (Fig. 6E) but a profound hypothermia when combined with d-MDMA.

Correlation of neuroprotection with striatal d-MDMA concentration or rectal temperature

Our data indicated that both reductions in rectal temperature and elevations in brain d-MDMA concentration were linked to a neuroprotective effect in the mouse. To determine which contributed the most to neuroprotection, linear regressions were conducted using the various measures of neuroprotection as dependent variables and either TAUC or fold elevation of striatal d-MDMA concentration as the independent variable. Using TAUC to predict percent control DA, percent control GFAP and percent control TH produced significant R^2 values ranging from 0.67 to 0.83 (Fig. 7A–C). In contrast, using fold elevation of striatal d-MDMA concentration to predict neuroprotection produced low non-significant R^2 values ranging from 0.33 to 0.59 (Fig. 7D–F). The use of more complex regressions including both temperature and fold elevations of striatal d-MDMA along with an interaction term were only slightly better predictors (data not presented). Within the multivariable models, the TAUC parameter was always the significant variable. Thus, although stress manipulations combined with d-MDMA administration altered both striatal d-MDMA concentration and TAUC, the decrease or hypothermia reflected in TAUC more accurately predicts neuroprotection.

Discussion

The level of a toxic compound at the target site can be related to its toxicity. Conditions able to reduce target site concentrations may lower toxicity and d-MDMA toxicity is reduced by several stressful manipulations. Here we report these manipulations raise rather than lower striatal d-MDMA by 4- to 7-fold. Stress manipulations, including restraint stress, low temperature housing (15°C) and ethanol co-administration protect mice against the dopaminergic neurotoxicity induced by repeated d-MDMA treatment (Miller and O'Callaghan 1994). These stressful, yet neuroprotective treatments are known to cause hypothermia when administered with d-MDMA treatment. Here, we examined a possible specific mechanism of the protection, i.e. that stress-induced hypothermia might reduce brain concentration of d-MDMA. Instead, stress manipulations elevated striatal d-MDMA concen-

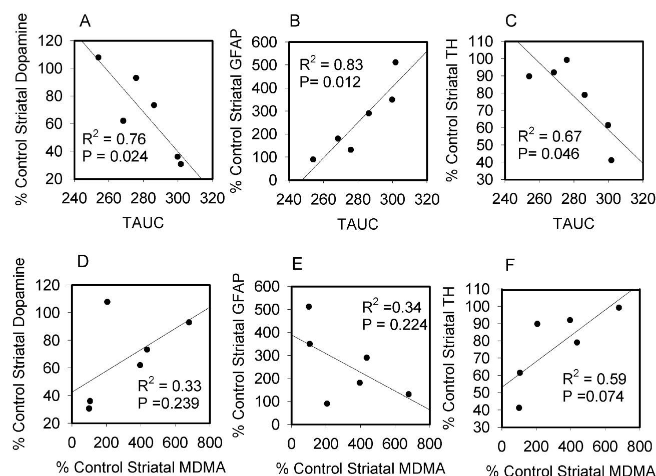

Fig. 7 Correlation between neuroprotection and striatal concentrations of d-MDMA or temperature (*TAUC*). Least squares regressions were performed on data from six different manipulations including d-MDMA alone, d-MDMA plus restraint, d-MDMA plus ethanol, d-MDMA plus 15°C single housing, d-MDMA plus corticosterone and d-MDMA plus MK-801. R^2 and P values are indicated on each section. Correlations were significant in **A–C** and not significant in **D–F**. **A** Least square regression using percent control dopamine as an indicator of neuroprotection (*y*-value) for each condition versus the TAUC value (*x*-value) for each condition. **B** Least square regression using percent control GFAP (*y*-value) versus TAUC (*x*-value). **C** Least square regression using percent control TH (*y*-value) versus TAUC (*x*-value). **D–F** Least squares regressions of percent control DA, percent control GFAP and percent control TH versus percent control striatal d-MDMA

tration 4- to 7-fold. Consistent with our own data, Clausing et al. (1995) found low-ambient temperature stress elevated brain amphetamine concentration in the rat. Hence, our data extend Clausing's finding to the mouse and to other stressors (restraint stress and ethanol co-administration) using the related amphetamine derivative d-MDMA.

Corticosterone release is a commonality of the protective stress treatments that might play a role in elevating striatal d-MDMA and in neuroprotection. Interestingly, corticosterone also has been reported to inhibit the activity of several isoforms of the P450 superfamily (Lampen et al. 1995; Christians et al. 1996, Iber et al. 1997). d-MDMA is known to be metabolized by cytochromes P450, including the rat/human isoforms CYP2D1/6, CYP3A2/4 (Cho et al. 1990; Tucker et al. 1994; Lin et al. 1997; Wu et al. 1997; Kreth et al. 2000; Maurer et al. 2000). The mouse isoforms responsible for metabolism of d-MDMA have not been completely elucidated but based on genetic homology and substrate specificity may include Cyp2d22 (the putative mouse ortholog of CYP2D6; Blume et al. 2000) and Cyp3a13 (a putative mouse ortholog of CYP3A4; Anakk et al. 2003). Thus, stress-induced corticosterone release might be inhibiting d-MDMA metabolism, a possible explanation for the elevated brain levels of d-MDMA observed in stressed animals. We directly tested the ability of corticosterone to mediate the neuroprotection by administering it at doses sufficient to produce blood levels similar to those produce by restraint. However, using the corticosterone co-treatment we found no neuroprotection and observed no evidence of CYP450 inhibition, i.e. brain levels of d-MDMA were not elevated. Furthermore, we did not observe a hypothermic response to corticosterone. These results suggest that the elevation of striatal d-MDMA concentration, neuroprotection and hypothermia associated with stress manipulations is not directly mediated by stress-induced corticosterone release.

The possibility exists that the elevated brain levels of d-MDMA observed in stress exposed animals may be a result of reduced excretion of d-MDMA. We did not measure excretion of d-MDMA and metabolic products, therefore we cannot exclude this possibility. An additional possible explanation for the elevated brain d-MDMA concentration in association with neuroprotection could be that metabolism of d-MDMA to neurotoxic products is required for full expression of d-MDMA induced neurotoxicity and that stress by a mechanism other than corticosterone release is inhibiting d-MDMA metabolism. Indeed, previously, in rat, several groups of investigators have administered d-MDMA into brain by microinjection or by microdialysis, bypassing potential peripheral metabolism of the drug and have shown that neurotoxicity is not observed (Molliver et al. 1986; Paris and Cunningham 1992; Esteban et al. 2001; Nixdorf et al. 2001). Such data could be interpreted as support of the notion that metabolic transformation of d-MDMA is required for neurotoxicity. We have, however, conducted preliminary experiments with irreversible (1-aminobenzotriazole) and competitive (SKF525A) cytochromes P450 inhibitors. We found that striatal levels of both d-MDMA and the d-MDMA metabolite 3,4-methylenedioxymethamphetamine (MDA) can be elevated to a moderate extent with these treatments but no neuroprotection is observed (Johnson and Miller, unpublished data). Thus, our current data on the one hand support the notion that metabolism is required for neurotoxicity (i.e. we observed elevated brain levels of unchanged d-MDMA in protected animals) but on the other hand refute the same notion (i.e. metabolism inhibitors do not protect against neurotoxicity). However, we were not able to completely inhibit the metabolism of d-MDMA in these preliminary experiments as at least one metabolic product, MDA, was still formed. Further experiments will be required to determine whether complete inhibition of d-MDMA metabolism can block d-MDMA neurotoxicity in the mouse.

Pharmacological pretreatments known to lower core temperature in the presence of d-MDMA but which are not considered to be stress manipulations, have been reported to protect animals from the neurotoxic effects of d-MDMA (Miller and O'Callaghan 1994, 1995). Therefore, we determined whether the protective manipulation of pretreatment with MK-801 would alter brain concentrations of d-MDMA to the same extent as other protective stress manipulations. MK-801 caused significant hypothermia, but only insignificantly elevated striatal levels of d-MDMA. Clearly, these data show that not

all protective manipulations are associated with altered striatal d-MDMA levels. Furthermore, hypothermia itself does not appear to be causative of high striatal d-MDMA levels.

Since both temperature reduction and elevation of brain d-MDMA appeared to be implicated in neuroprotection, we constructed mathematical models to predict which factor was most important. We found that body temperature reduction (lowered TAUC) was clearly correlated with all indices of neuroprotection. Correlations of striatal concentration of d-MDMA with neuroprotection were generally insignificant

Our data serve to reinforce the fact that hypothermia is a major factor in stress mediated neuroprotection against the dopaminergic neurotoxicity caused by d-MDMA in the mouse. Although altered distribution of d-MDMA offers a possible explanation for temperature-related neuroprotection, our findings argue to the contrary, because striatal d-MDMA concentration was significantly elevated by stress manipulations.

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