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Research report

Restraint as a stressor in mice: Against the dopaminergic neurotoxicity of D-MDMA, low body weight mitigates restraint-induced hypothermia and consequent neuroprotection☆

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Abstract

In experimental studies of stress, restraint of laboratory rodents, perceived as easy to apply and believed to be reproducible, is a commonly used manipulation. The restraint manipulation is utilized as a technique to characterize the physiological, cellular and molecular consequences of stress as well as a tool to understand the ways in which stress may interact with toxic substances. In previous work, we utilized restraint in an examination of the effect of stress on the striatal dopaminergic neurotoxicity engendered by a series of substituted amphetamines. Contrary to our expectations, and most likely due to its body temperature-reducing properties in the mouse, restraint provided total or near total protection against the neurotoxicity of these agents. During subsequent studies utilizing C57Bl6/J female mice of varying weights and ages the degree of temperature reduction and the associated ability to block (20–100%) the dopamine depletion associated with the neurotoxic amphetamine 3,4-methylendioxyamphetamine (D-MDMA, 20 mg/kg of mouse body weight, every 2 h, s.c., total of four doses) were found to vary considerably more than had been previously observed. An in-depth analysis of the role mouse weight plays in the temperature reduction induced by restraint indicates mouse weight is a primary determinant of hypothermia and subsequent neuroprotection. It suggests the induction of stress in rodents by restraint is a complex effect that may lead to unanticipated results. The restraint manipulation is not as straight-forward a procedure as is commonly believed. Our data indicate that consistent application of restraint may require an adjustment of the restrainer device to mouse body weight.

Theme: Disorders of the nervous system

Topic: Neurotoxicity

Keywords: Restraint-induced hypothermia; Restraint-induced neuroprotection; D-MDMA-induced neurotoxicity; Restraint; D-MDMA; Stress; Hypothermia; Neurotoxicity; Neuroprotection

Abbreviations: ANOVA, analysis of variance; ANCOVA, analysis of co-variance; CMTR, composite measure of temperature response; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; GFAP, glial fibrillary acidic protein; HPLC, high-performance liquid chromatography; HVA, homovanillic acid; 5-HIAA, 5-hydroxyindoleacetic acid; MHPG, 3-methoxy, 4-hydroxyphenylethyleneglycol; D-MDMA, methylenedioxymethamphetamine; NE, norepinephrine; 5-HT, serotonin

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1. Introduction

In research projects aimed at understanding the impact stress can have on various body systems and disease models, the perceived ease of application, wide involvement of higher and lower brain centers and activation of both the HPA-axis (release of CRH, ACTH and glucocorticoids) as well as the sympathetic nervous system make restraint a widely used manipulation [8,15,23]. In previous work we chose to use restraint to examine how

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alterations in homeostasis (i.e., stress) would affect the dopaminergic striatal neurotoxicity induced by the amphetamines just as others have shown that a variety of stressful manipulations (e.g, electric shock, alterations in caging, etc.) including restraint can alter the general lethality and toxicity of these agents as well as their activating and sensitizing properties [16,30,2,3,29,6,7].

Amphetamine, as well as its derivatives D-methamphetamine, D-methylenedioxyamphetamine (D-MDA) and D-methylenedioxymethamphetamine (D-MDMA), are striatal neurotoxicants as evidenced by marked increases in glial fibrillary acidic protein (GFAP), a biochemical measure of injury induced gliosis, as well as the presence of argyrophilia and long-term decrements in dopamine (DA), its metabolites and tyrosine hydroxylase (TH) protein. The decrements in DA-associated parameters indicate dopaminergic projections to the striatum appear to be the likely target [16,21].

The actions of the amphetamines have long been linked to their thermoregulatory actions and at neurotoxic dosages elevated core body temperatures are observed. The degree of dopamine depletion and other indices of neural damage can be blocked or reduced by manipulations that will lower body temperature during the course of amphetamine administration. These observations confirm that body temperature plays some as yet undetermined role in their neurotoxic capabilities [16]. Restraint of the mouse results in body temperature reductions through factors that are not yet fully elucidated but include reduced motor activity [26]. In previous studies, restraint-induced hypothermia during the course of D-MDMA administration resulted in nearly complete protection from the dopamine-depleting effects of the drug [16].

In recent work examining the contribution of mouse strain, we included C57Bl6/J mice as a comparison strain in each experiment as this strain has been used extensively in our work documenting the D-MDMA neurotoxicity and the neuroprotection of restraint. Over the course of these experiments, we noted a wide range in the degree to which restraint could protect against the striatal dopamine depletions induced by a body weight-adjusted dose of D-MDMA, despite the same neurotoxic regimen, a consistent application of the restraint procedure and the utilization of the same restraint devices from our previous work. Mouse age and weight were the only two factors varied among the set of ten experiments, though held consistent within a given experiment. We hypothesized that one or both of these variables makes a marked contribution to the effects observed when utilizing restraint. The following report describes an in-depth analysis of the ways in which each affects restraint-induced body temperature reduction and consequent neuroprotection. Mouse weight appears to be the dominant factor in the neuroprotective effects induced by restraint of the C57Bl/6 female mouse against a body-weight adjusted neurotoxic dose of D-MDMA.

2. Materials and methods

2.1. Materials

The following drugs and chemicals were kindly provided by or obtained from the sources indicated: high-performance liquid chromatography (HPLC) standards (Sigma Chemical, St. Louis, MO, USA); D-MDMA (Research Technology Branch, National Institute on Drug Abuse, Rockville, MD, USA). Reagents used for HPLC were of HPLC grade (ESA, Chelmsford, MA, USA).

2.2. 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' (National Academy Press, 1996). Female C57BL/6J mice 5 to 10 weeks of age were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and maintained in a colony certified by the American Association for Accreditation of Laboratory Animal Care. This strain and sex of mice were chosen because of our previous experiments which demonstrated that C57BL/6J female mice were susceptible to D-MDMA-induced decreases in striatal dopamine [16,21]. Upon receipt, the mice were housed in groups of six to eight in a temperature-controlled ($21\pm1^{\circ}C$) and humidity controlled ($50\pm10\%$) colony room maintained under filtered positive pressure ventilation on a 12-h light/12-h dark cycle beginning at 0600 EDT. The plastic tub cages were $30.5\times30.5\times15$ cm in height; cage bedding consisted of heat-treated pine shavings spread at a depth of approximately 4 cm. Food (ProLab ISOPRO RMH 3000, irradiated food containing 22% crude protein, 5% crude fat, 5% crude fiber, 6% ash and 2.5% added minerals) and water were available ad libitum.

2.3. Group assignment

On the day of dosing, mice were moved from the animal quarters about 1 h prior to being randomly assigned to new cages for the duration of the experiment. Reassignment occurred approximately 30 to 45 min prior to initiation of restraint and 60 to 75 min prior to the first injection. All mice were placed in a large tub cage (47 cm L×20 cm W×20 cm H). Mice were then rapidly removed and randomly assigned to new cages/groups (saline/unrestrained, saline/restrained, D-MDMA/unrestrained, D-MDMA/restrained) and remained in these same groups until brain tissue was collected. Once assigned to new cages mice were weighed and their number within the group marked on their tails with a laboratory marker (Sharpie non-toxic permanent marker) so that individual mice could be followed throughout the experiment. Within

each experiment mouse age was held constant but among experiments, mice ranged from 5 to 21 weeks of age on the day of drug administration/restraint. Mouse weight correlated with age ($R=0.6$).

2.4. Drug or vehicle administration

D-MDMA (20 mg/kg of mouse body weight, calculated as base) or saline vehicle (0.9%) was administered s.c. in a volume of 1 ml/100 g body weight, every 2 h, beginning at 30 min after initiation of restraint for a total of four injections. To minimize circadian influences on toxicity, the first injection was always given between 9:00 and 10:00 AM. This dosage regimen causes reproducible decrements in striatal dopamine and other markers of neural damage in C57BL/6 mice [16,21]. Female mice were used throughout these experiments as the original characterization of the neurotoxicity of D-MDMA and the neuroprotection afforded by restraint were conducted in this sex.

2.5. Restraint

Thirty min before the first dose of saline or D-MDMA, restrained mice were secured into a 35-ml centrifuge tube (Nalgene Ultraplus thin wall 25-mm inner diameter×89 mm length, Nalge Nunc International, Rochester, NY, USA) which had been perforated in the round end to provide airflow from the front to the back of the tube. Animals were prevented from backing out of the tube by a small binder clip (0.75 inches wide) which was held in place by a 20 g 1-inch needle penetrating through the tube walls and the center of the clip. Restraint of the mice in this fashion 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 [10]. Restrainers were placed on toweling to facilitate absorption of urine and feces. A baseline rectal temperature measurement was taken immediately prior to application of restraint and each time an animal was removed from the device for an injection of D-MDMA or saline. Mice remained in the restraint for 2 h after the final injection resulting in a total restraint period of approximately 8.5 h. A temperature measurement was also made following removal from the restrainer and mice were housed six–eight per cage) until collection of tissue for assay; all cage-mates had received the same treatment. A final rectal temperature measurement was made approximately 24 h after the first injection. During restraint and dosing, room temperature averaged 22°C.

2.6. Temperature measurement

Rectal temperature was recorded with a Bat-10 thermometer coupled to a RET-3 mouse rectal probe (Physitemp, Clifton, NJ, 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. Mice were held by the base of the tail while the temperature probe was inserted to a premarked depth of 2.8 cm. This method minimized handling, and in conjunction with the use of a fast-rise time of the rectal probe made it possible to obtain reliable measurements of rectal temperature in less than 60 s per mouse. Temperature sampling times were 30 min before first injection, then immediately before each injection, 2 h after last injection and 24 h after first injection.

2.7. Brain dissection and tissue preparation

All tissue was obtained 72 h after the initial D-MDMA injection, a time point at which striatal dopaminergic neurotoxicity is evident as indicated by markers of neural injury and decreases in DA content (see Ref. [21] for a complete time course). Immediately after decapitation, whole brains were removed from the skull with the aid of blunt curved forceps. Striatum was dissected free-hand on a thermoelectric cold plate (Model TCP-2, Aldrich Chemical, Milwaukee, SI, USA) using a pair of fine curved forceps (Roboz, Washington, DC, USA). Striatum from the left side of the brain was weighed, frozen on dry ice and stored at −70°C for subsequent analysis of DA, serotonin (5-HT), norepinephrine (NE) and metabolites [homovanillic acid (HVA); 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindoleacetic acid (5-HIAA), 3-methoxy, 4-hydroxyphenylethyleneglycol (MHPG)].

2.8. Dopamine analysis

DA, 3,4-dihydryoxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and other neurotransmitter substances were analyzed by HPLC with electrochemical detection using the following system: Tissue homogenates were prepared by sonication (Kontes Micro ultrasonicator/cell disruptor) on ice using a 30-s pulse in 0.2 N perchloric acid, containing 3,4-dihydroxybenzylamine 1 μM as internal standard. The homogenate is centrifuged at 10 000×*g* for 15 min, and resulting supernatant immediately injected using the autosampler described below. Each brain area was prepared in a standard volume (striatum 0.3 ml, hippocampus 0.2 ml, cortex 0.5 ml) then results were expressed as μg/g original tissue weight. Sample (10 ml) was injected using a temperature-controlled (4°C) Waters 717Plus Autosampler (Waters Corporation, Milford, MA, USA) connected to a Waters 515 HPLC pump. Sample was passed over a reversed-phase C_{18} column (Waters SYMMETRY, 4.6×250 mm, 5 micron, 100 A). Samples were detected using the Waters 464 pulsed electrochemical

detector (range 10 nA, potential 700 mV) connected by means of the Waters bus SAT/IN module to a computer using Millenium Software 32. The mobile phase consisted of sodium dihydrogen phosphate 75 mM, 1-octanesulfonic acid 1.7 mM, ethylendiaminetetraacetic acid 25 micoMolar, acetonitrile 10% v/v, all adjusted to a pH of 3.0 with phosphoric acid, pumped at a flow-rate of 1 ml per min. Under these conditions the average run time is 30 min with representative retention times (in min) for norepinephrine (5.99), epinephrine (7.19), 3,4-dihydoxybenzylamine (internal standard, 8.24), DOPAC (8.93), dopamine (11.28), 5-hydroxyindoleacetic acid (13.57), HVA (19.77), 5-hydroxytryptamine (26.1). Quantitation was accomplished by the use of the internal standard (10 pmol DHBA per injection) method using daily standard curves of each analyte (0.5–25 pmol per injection). The limit of detection is 0.5 pmol per injection, inter-assay variation is ±3%.

2.9. Statistical analysis

The SAS statistical analysis software package (SAS Institute, Cary, NC, USA) was used for all data analysis. The PROC UNIVARIATE procedure was used to assess the distribution characteristics of the measures of brain tissue dopamine concentration, a composite measure of temperature response (CMTR) to experimental conditions, and observed mouse characteristics. Using PROC GLM, Analysis of Variance (ANOVA) and Analysis of Covariance (ANCOVA) methods were employed to assess joint relations among the experimental design variables of treatment status and of restraint status as well as the observed variables of mouse weight and age (independent variables) in the prediction of brain tissue concentrations of dopamine (dependent variable). These same methods were utilized to assess relations among these independent variables in the prediction of the composite measure of temperature response (CMTR).

The CMTR was calculated for each mouse by the application of Simpson's Rule to temperatures measured at times 0, 2, 4, 6, and 8 h. This measure represents the area under the curve of a plot of temperature (°C) versus time (h), and has units of °C–h. The temperature composite variable reflects the impact of experimental conditions and the interaction of mouse characteristics with those conditions in the generation of heat. A composite variable was chosen after examining a series of ANCOVA statistical models predicting brain dopamine concentration from treatment status and the composite temperature measurement. The proportion of variance (R^2) explained in the distribution of brain dopamine concentrations progressively increased from 39 to 46% with the progressive inclusion of one, two, three, four, and five measures of temperature into the creation of the composite variable.

A square root transformation of brain dopamine concentration was used in all statistical models. This transformation was used after demonstrating that: (1) it produced a less skewed distribution of the residual error in statistical models; and (2) in models with the same independent variables and of the same form, notably more variation in the dopamine distribution is explained using the transformation compared to no transformation (e.g., 48% vs. 42%). This latter observation underscores a natural consequence of the experimental design. The effect of experimentally (i.e., D-MDMA) induced perturbations and interactions with mouse characteristics (i.e., weight) produces systematic decreases in brain dopamine concentration and elevations in the temperature composite measure primarily in groups treated with D-MDMA. Control (i.e., saline treated) animals do not demonstrate such decreases. Thus, the square root transformation emphasizes the range of dopamine concentrations in which D-MDMA effects are occurring — in which measures of association are 'created' by the experiment — and de-emphasizes the range of dopamine concentrations characteristic of measurements in control animals. All results, however, are reported in terms of the original dopamine concentration unit of μg/g tissue with the exception of slope estimates.

Estimates of average values within experimental design groupings and estimates of measures of association such as regression coefficients are reported with 95% confidence limits. In the case of models assessing the presence of statistical interaction a *P*-value is reported.

3. Results

3.1. Effects of D-MDMA, restraint and the combination thereof, on striatal dopamine in C57Bl6/J mice

D-MDMA administered to unrestrained female C57Bl6/J mice causes a reproducible decline in striatal dopamine. Over a series of ten individual experiments, with an accumulated sample size of 61 animals that received D-MDMA 20 mg/kg of mouse body weight, s.c. every 2 h for a total of four injections, the average dopamine decrement was 82%. That is, D-MDMA treated unrestrained mice exhibited striatal dopamine concentrations that were 18% of controls (saline, unrestrained) Table 1. The dopamine metabolites DOPAC and HVA exhibited similar decrements in D-MDMA treated animals (data not shown). The decrements in dopamine metabolites is further evidence of dopaminergic nerve terminal injury since a D-MDMA-induced change in dopamine turnover would have been indicated by unchanged or elevated levels of metabolites.

Restraint alone does not alter striatal dopamine concentrations. Striatal dopamine concentrations in mice that were restrained for 8.5 h but received saline 200 ml every 2 h for a total of four injections were not different from the dopamine concentrations of unrestrained animals that received saline (Table 1).

Restraint accompanied by D-MDMA administration

Table 1
Striatal dopamine concentrations in C57Bl/6J female mice treated with saline or D-MDMA; unrestrained or restrained[a]

	Saline, unrestrained, $n=62$	D-MDMA, unrestrained, $n=61$	Saline, restrained, $n=62$	D-MDMA, restrained, $n=47$
Mouse body weight (average across all experiments) (g)±S.E.M (S.D.)	19.24±0.18 (1.42)	19.22±0.23 (1.8)	19.25±0.20 (1.57)	19.11±0.22 (1.51)
Striatal dopamine (μg/g striatal tissue)±S.E.M (S.D.)	12.38±0.81 (6.38)	2.27±0.30 (2.34)	12.40±0.83 (6.54)	8.48±0.79 (5.42)
Dopamine as percent of saline injected control (%)		18		68
Percent dopamine depletion compared to saline injected control (%)		82		32

[a] Treatment regimens were as follows: saline group received sterile saline 200 μl, s.c. every 2 h, for a total of four injections; D-MDMA group received D-MDMA 20 mg/kg s.c. every 2 h, for a total of four injections; restrained animals were assessed for rectal temperature then secured into restrainer 30 min before receiving their first injection. At each injection, animal was removed from restrainer, rectal temperature was obtained, then injection was made and animal was returned to the restrainer.

partially blocks depletion of striatal dopamine induced by D-MDMA. Mice administered D-MDMA 20 mg/kg of mouse body weight, s.c. every 2 h for a total of four injections while being restrained exhibited striatal dopamine concentrations that averaged 68% of saline/restrained dopamine concentrations. This compares to the 18% of saline controls in unrestrained D-MDMA treated mice (Table 1). The data in Table 1 contains means calculated from the raw dopamine values for all 232 mice included in the data set without regard to square root transformation of dopamine calculations. During the course of analysis of the data it was shown that the distribution of values was not normal, thus a square root transformation of the data was performed in order to compensate. This is analogous to performing a log transform to meet the assumptions necessary to the correct use of the statistical models discussed in the Methods section. In this case the transformation was to use the square root of the striatal dopamine value. Subsequent to this transformation, descriptive statistics for data for each of the groups was tabulated in Table 2. Relationships between the groups are the same; however, the mean values calculated by squaring the estimated mean of the square root transformed DA distribution are now slightly lower.

3.2. Inter-experimental variation of restraint-induced neuroprotection

In contrast to the relatively constant degree of dopamine depletion caused by the body weight adjusted dose regimen of D-MDMA in unrestrained mice (80.9±2%, Table 1), restraint-induced protection of striatal dopamine varied significantly from experiment to experiment as did average weight of animals (Fig. 1). Over the ten individual experiments, restraint-induced protection, defined as the percent control dopamine in the 'protected' or restrained/D-MDMA treated group minus the percent control dopamine in the unrestrained/D-MDMA treated group, varied

Table 2
Estimates and 95% confidence intervals (95% C.I.) or standard deviations (S.D.) of the average values of brain dopamine concentration[a], mouse weight, and composite temperature measurement, and of the simple regression relation of the square root of brain dopamine concentration versus mouse weight, among joint levels of saline/D-MDMA treatment and unrestrained/restrained conditions

Measure	Saline unrestrained	D-MDMA unrestrained	Saline restrained	D-MDMA restrained
Sample size (N)	62	61	62	47
Dopamine[a] (μg/mg tissue)	11.4	1.8	11.3	7.6
95% C.I.	[9.8, 13.0]	[1.2, 2.4]	[9.8, 13.0]	[6.2, 9.1]
Weight (g)	19.2	19.2	19.3	19.1
S.D.	1.5	1.8	1.6	1.5
Temperature composite (°C–h)	296.4	301.8	287.2	285.6
95% C.I.	[295.5, 297.3]	[301.1, 302.5]	[286.1, 288.2]	[283.8, 287.3]
$[\text{Dopamine}]^{1/2}$ vs. weight	−0.10	0.09	0.06	0.25
95% C.I.	[−0.26, 0.05]	[−0.04, 0.22]	[−0.09, 0.20]	[0.07, 0.43]

[a] Estimates and 95% C.I. of average brain dopamine concentration based upon squaring of parameters after square root transformation of primary data.

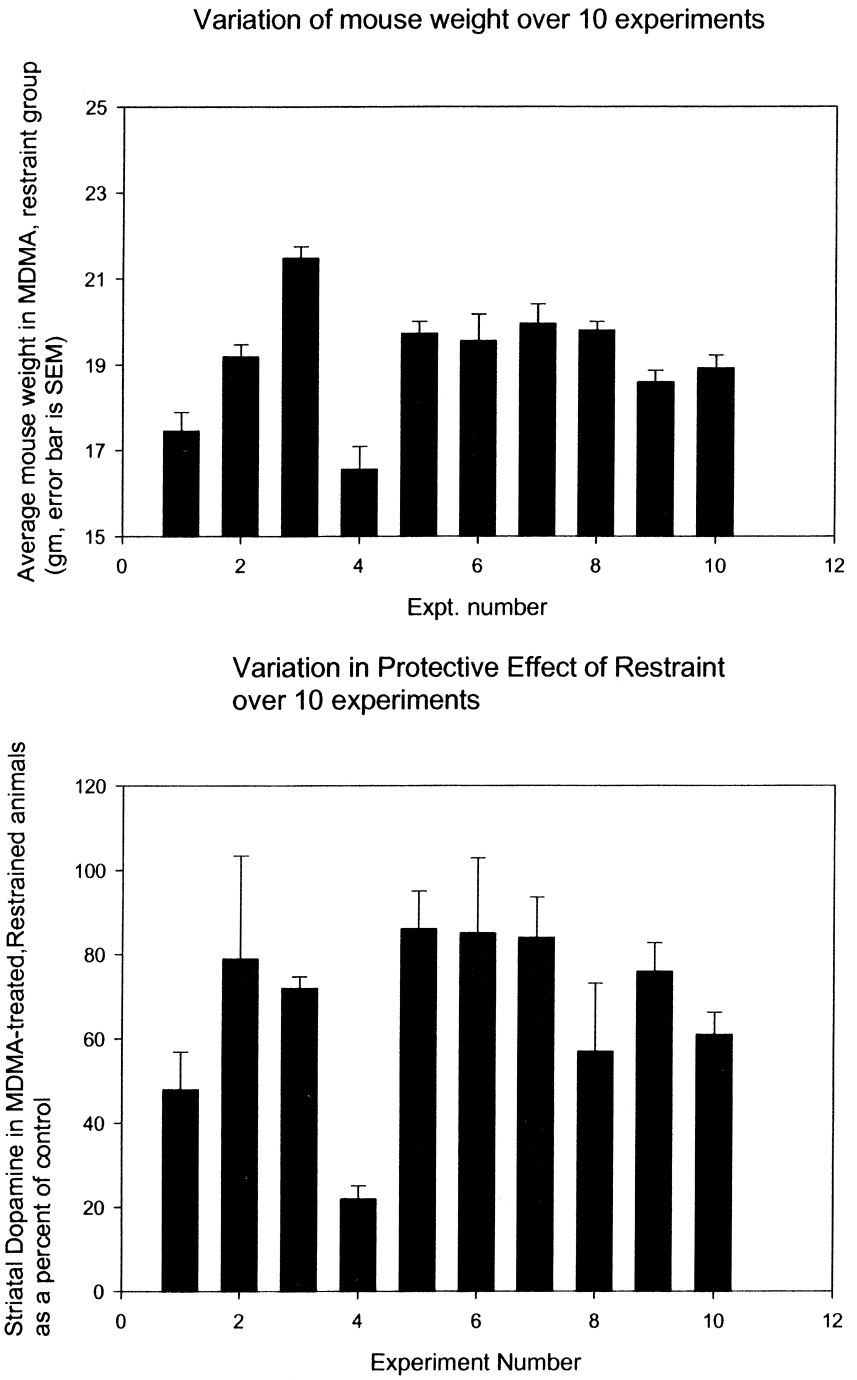

Fig. 1. Correlation of average mouse weight with striatal dopamine retained in brains of protected or restrained, D-MDMA treated mice. D-MDMA administered as 20 mg/kg mouse body weight every 2 h for a total of four doses. Percent control is calculated as raw dopamine level of D-MDMA treated group divided by raw dopamine level of saline treated group. The appropriate control for unrestrained/D-MDMA treated is unrestrained/saline treated; the control for restrained/D-MDMA is restrained/saline. Percent protection is calculated as percent control striatal dopamine in restrained condition minus percent control striatal dopamine in unrestrained condition. This reflects the increased retention of striatal dopamine caused by the restraint condition.

from a low of 10% to a high of 69% relative to striatal dopamine levels for the saline/unrestrained group of each individual experiment. Over the ten experiments, the average weight of the restrained/D-MDMA group varied from a low of 16.57 g to a high of 21.48 g body weight. Fig. 1 illustrates a rough correlation of weight of the animal to percent control dopamine in the restrained/D-MDMA treated group. Age was kept constant within each

experiment but over the ten experiments varied from 5 to 21 weeks with the average age being 12 weeks.

3.3. Effect of D-MDMA treatment and restraint on mouse rectal temperature

D-MDMA treatment elevates rectal temperature, by contrast restraint causes declines in rectal temperature. The rectal temperature was monitored over the course of the 8.5-h dosing period in all mice. Fig. 2 shows the pattern of temperatures observed in each treatment/restraint group. In order to make the temperature information more amenable to analysis, a composite measure of temperature response variable (CMTR) was constructed by integration of temperature over time using Simpson's Rule (see Methods). The average CMTR was most elevated by D-MDMA treatment without restraint but most reduced with restraint. Average CMTR values for each treatment group are shown in Table 3.

3.4. Effect of mouse weight on restraint modulated neurotoxicity

The weight of the animal has direct impact on the ability of restraint to modulate dopamine depletions induced by treatment with D-MDMA. An ANOVA was carried out to find the best and most parsimonious model to accurately describe the data obtained in the 232 mouse set. The best and most parsimonious statistical model among the independent variables of treatment status, restraint status, and mouse weight was afforded by all main effects and all two-way interactions ($P<0.05$ for each two-way interaction; global $F_{6,225}=35.2$, $P<0.001$). A completely specified model revealed that the three-way interaction accounted for no additional variance in the distribution of dopamine concentration ($P=0.99$). The nature of relations underlying this statistical model is illustrated by the last row of entries in Table 2 and by Fig. 3.

Although weight is constant among joint treatment/restraint groupings, the relation of dopamine concentration

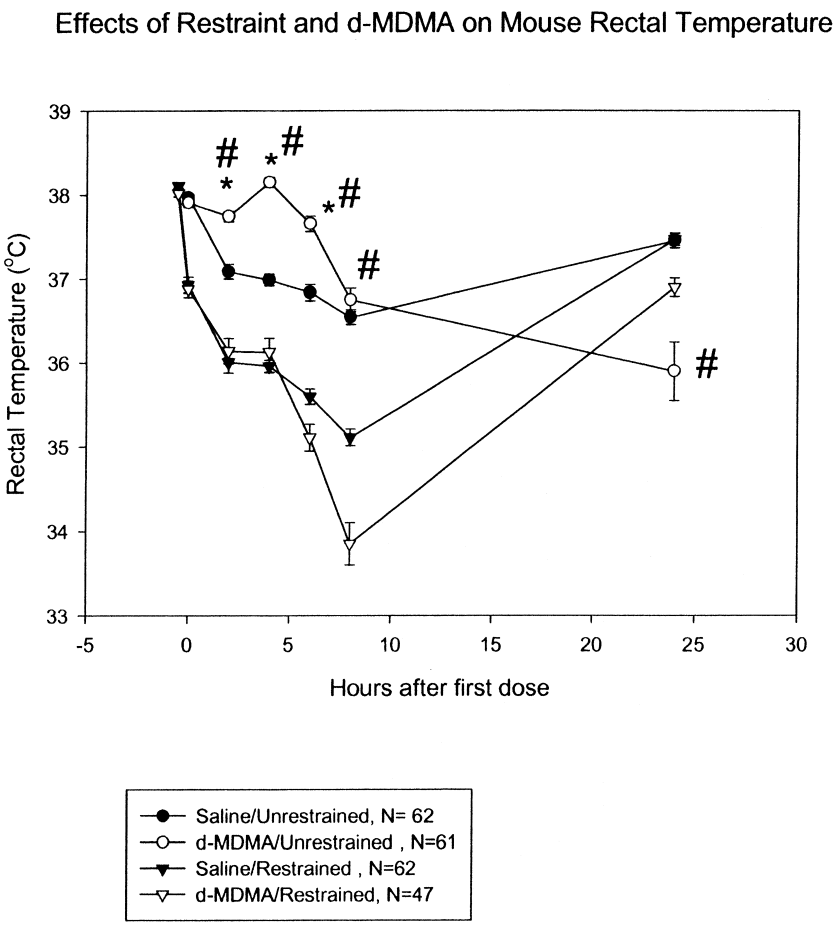

Fig. 2. Effects of restraint and D-MDMA on mouse rectal temperature. This figure illustrates the average temperatures at each time point of all animals in each treatment group as follows: filled circle=saline/unrestrained ($N=62$); open circle=D-MDMA/unrestrained ($N=61$); filled triangle=saline/restrained ($N=62$); open triangle=D-MDMA/restrained ($N=47$). The pound sign indicates a temperature that is significantly different from the D-MDMA/restrained group, $P<0.05$.

Table 3
Average composite measure of temperature response in C57Bl/6J female mice treated with D-MDMA or saline, restrained or unrestrained[a]

Treatment group	Average composite measure of temperature response (CMTR) (°C–h)±S.D.	Percent of control CMTR (%)	Percent elevation or reduction compared to control CMTR (%)
Saline, unrestrained, $n=62$	296.36±3.7		
D-MDMA, unrestrained, $n=61$	301.8±2.64	102 of unrestrained saline	Elevated 2
Saline, restrained, $n=62$	287.17±4.26	97 of unrestrained	Reduced 3
D-MDMA, restrained, $n=47$	285.57±6.16	86 of unrestrained D-MDMA	Reduced 15

[a] Treatment regimens were as follows: saline group received sterile saline 200 μl, s.c. every 2 h, for a total of four injections; D-MDMA group received D-MDMA 20 mg/kg s.c. every 2 h, for a total of four injections; restrained animals were assessed for rectal temperature then secured into restrainer 30 min before receiving their first injection. At each injection, animal was removed from restrainer, rectal temperature was obtained, then injection was made and animal was returned to the restrainer. CMTR were calculated according to Simpson's rule using rectal temperatures at 0, 2, 4, 6, and 8 h after the initial dose of saline or D-MDMA.

to weight is notably different (Table 2). In the D-MDMA/restrained grouping, higher concentrations of dopamine are noted among heavier mice (on average, 0.25[μg/g tissue]$^{1/2}$ per g). No relations are suggested among any other joint grouping.

Using a multivariate extension of the methods of [28], the impact of mouse weight on average brain dopamine concentration in the D-MDMA/restrained grouping reveals that for mice weighing 20.8 g or more, the concentrations become indistinguishable from saline controls (Fig. 3c; 10.1 versus 11.1 μg/g tissue, respectively; $P=0.49$). However, this difference becomes progressively larger, and statistically significant ($P<0.001$), as mouse weight decreases. This progressively larger difference is driven by progressively smaller dopamine concentrations among mice with lower weights in the D-MDMA/restrained grouping (Fig. 3b and a: 95% C.I. 6.3–9.3 μg/mg tissue at 19.2 g, 95% C.I. 4.1 – 7.4 μg/g tissue at 17.6 gm, respectively).

3.5. The effect of animal weight on restraint induced temperature reduction

The weight of the animal has direct impact on ability of restraint to cause temperature decrease reflected as the temperature composite variable. An analysis of variance model predicting the temperature composite measure from treatment, restraint, and weight variables revealed that a completely specified statistical model characterizes the relations between dependent variable and independent variables ($P<0.01$ for all three two-way interactions; $P<0.001$ for the three way interaction; global $F_{7,225}=118$, $P<0.001$). This model accounts for 79% of the variance of the temperature composite variable, suggesting these factors are primary causes or markers of causal processes for temperature elevation sustained over the 8-h time period. An illustration of the effect of weight on temperature regulation is observed in the D-MDMA/restrained group where the step size differential between weights is large (Fig. 4)

3.6. The effect of age on restraint-induced neuroprotection

Given the correlation of mouse weight and age described above, a question arose whether age-related changes in mouse physiology accounted for the effects of weight on restraint-induced temperature reductions and associated neuroprotection. To test this hypothesis we examined the ability of age to substitute for weight in prediction of mouse brain dopamine. An analysis of variance model was constructed similar to that of weight (see Section 3.4) using the effects of treatment, restraint, age and their second-order interactions. The ability of this model to predict brain dopamine was not improved relative to the model based on weight (global $F_{6,225}=32.2$ versus $F_{6,225}=35.2$ for age versus weight, respectively). Furthermore, within this model, the factor of age and the interactions of age with treatment and with restraint failed to demonstrate any degree of statistical significance ($P>0.02$ for all age-related variables). This observation, coupled with the observations that: (1) the ANCOVA parameter coefficients for treatment, restraint, and their interaction remain effectively unchanged upon addition of age variables, and (2) these parameters are notably altered for the main effects of treatment and restraint upon addition of weight variables indicates that age is effectively a nuisance variable while weight is a relevant modulating variable.

3.7. Sustained temperature perturbation mediates and/or modulates relations of treatment, restraint and weight with brain dopamine concentrations

Without regard to either the experimental design factors of treatment and restraint or the mouse characteristic of weight, a cubic polynomial model of the CMTR accounted for 24% of the variance of [dopamine]$^{1/2}$. By taking into

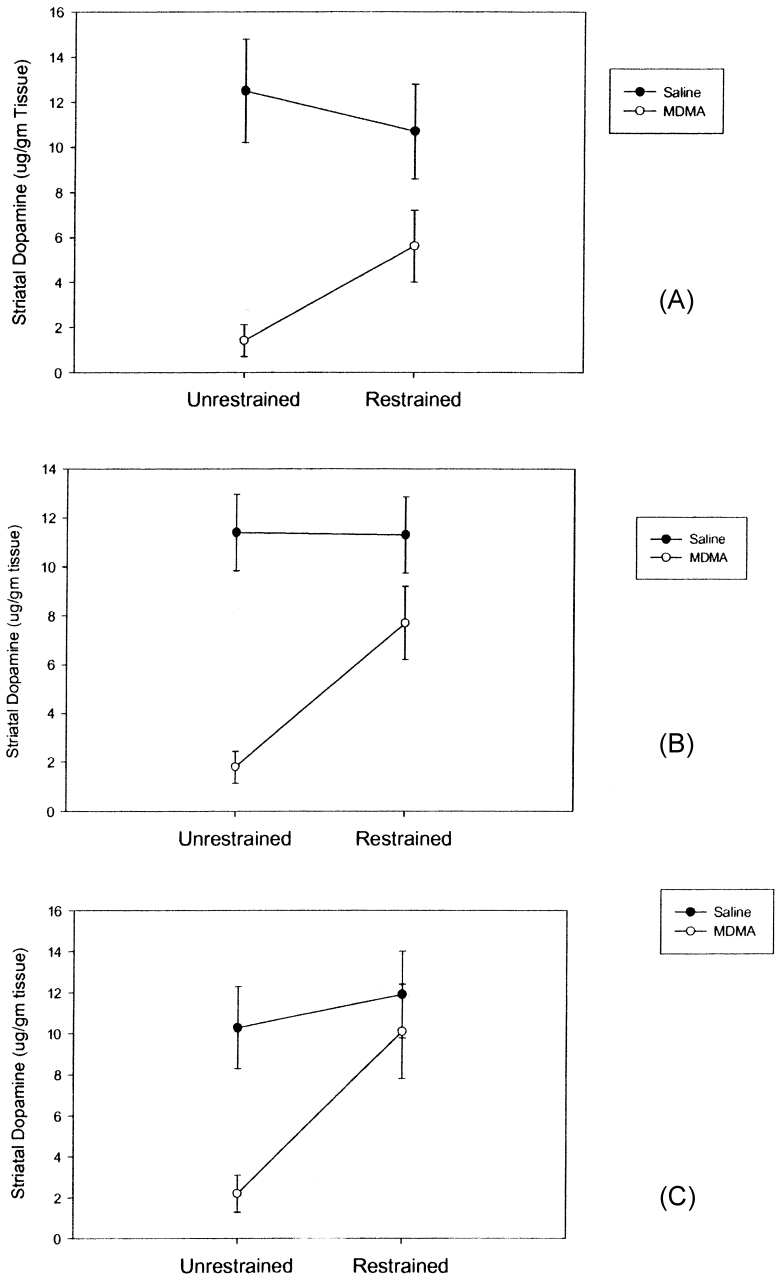

Fig. 3. Effects of weight on the ability of restraint to protect brain dopamine. Fig. 3A indicates the effect of restraint to protect striatal dopamine in mice of 17.6 g body weight. At this weight D-MDMA treatment caused significant depletion of brain dopamine regardless of restraint status. Fig. 3B indicates that at a weight of 19.2 g (the mean body weight), restrained animals were protected from the dopamine depleting effects of D-MDMA treatment. Fig. 3C shows nearly complete protection of striatal dopamine in restrained animals with a body weight of 20.8 g that were treated with D-MDMA. By contrast, restraint caused insignificant changes in striatal dopamine in saline-treated animals, regardless of weight. Filled circle indicates saline, open circle indicates D-MDMA.

account joint relations with treatment, restraint, and weight (statistical model of third section above but using CMTR as the dependent variable) an adjusted temperature composite variable was then created from the residual variation. A cubic model of this residual temperature composite variable accounted for none of the variance of $[\text{dopamine}]^{1/2}$ (0.4%).

A residual variable of $[\text{dopamine}]^{1/2}$ was then created by removing the relations with a cubic polynomial model of CMTR (same model as described in the first sentence of the preceding paragraph). Then a statistical model was created based upon the independent variable specifications described in the third section above for treatment, restraint and weight, but utilizing as dependent variable this residual $[\text{dopamine}]^{1/2}$ variable. The proportion of accounted variance in this residual $[\text{dopamine}]^{1/2}$ variable dropped

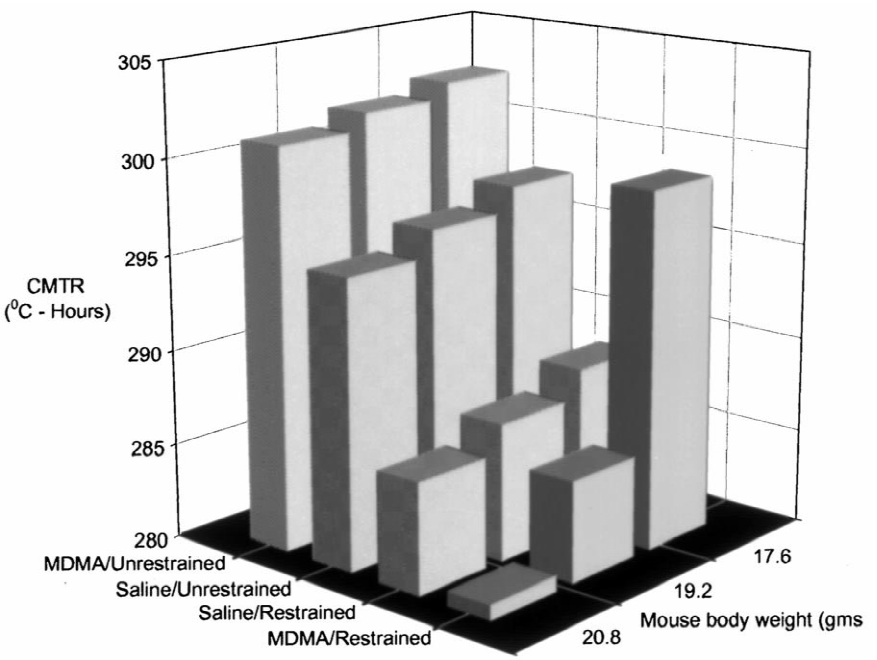

Fig. 4. Effects of weight, restraint and D-MDMA treatment on rectal body temperature, represented over time as CMTR. Compared to the saline/unrestrained control group ($N=62$), the average CMTR at each weight range is highest for D-MDMA/unrestrained group ($N=61$). The differential between each weight class is similar among the three groups including D-MDMA/unrestrained, saline/unrestrained and saline/restrained. However, the step size differential is quite different between weight classes in the D-MDMA/restrained group in which the lowest CMTR is observed in the largest mice. The CMTR also correlates inversely with striatal dopamine concentrations.

from 48 to 23%. In conclusion, these observations suggest that sustained temperature elevation mediates and/or modulates various aspects of relations of treatment, restraint, and weight with brain dopamine concentration.

4. Discussion

Our data replicate our previous work and show that restraint of the C57Bl/6J female mouse in 35-ml centrifuge tubes will reduce body temperature relative to baseline temperature and, provided sufficient reduction is achieved, will protect against the striatal dopamine depletion induced by the neurotoxic amphetamine D-MDMA [16,17]. Our data also demonstrate that both the temperature reduction and hence the neuroprotection are markedly dependent on body weight despite the use of a body-weight adjusted dose of D-MDMA. Mice at 17.6 g or lower display little or no protection in restraint while those above 20.8 g show almost total protection. Many investigators utilize 50-ml centrifuge tubes, which vary in internal diameter from 2.5 to 3.0 cm, as restraint devices for mice. However our results suggest that the ability to apply restraint in a uniform fashion with these devices may be more complex than appreciated. For example, restraint devices may cause more uniformly reproducible effects if the device could be adjusted according to some subject specific parameter such as body volume or body girth. Our future experiments will attempt to define such a parameter. An awareness of the physiological changes that may be affected by variables such as body weight may aid investigators in applying restraint in a more consistent fashion and would be expected to reduce or explain unexpected variations in experimental results as it did in our studies.

The restraint-induced reduction in body temperature observed by us and others [14,16,17] appears to have received little attention, although the use of restraint as a stressor is common in studies of rats and mice. In particular, the use of 50-ml centrifuge tubes to restrain mice is quite common across a variety of disciplines (e.g., neuroscience, immunology, pharmacology, etc.) interested in examining the consequences of stress in their respective areas [23,9,13,22,25]. Restraint of rodents is used most notably as a general stressor with measurement of corticosterone levels being most often used as a gage of the degree of stress imposed (e.g. [22]).

Despite the use of restraint as a common stressor for mice, body temperature is rarely measured in this context and many investigators assume this manipulation will elevate body temperature (e.g. [24]). Our work shows that a reduction in body temperature can be associated with this manipulation and is sufficient to alter toxicity of exogenously administered substances. The assumption of an association between restraint and elevations in body temperature may be related to reports of hyperthermia in restrained rats (e.g. [27,19,20]) although complete immobilization of this species can cause a reduction in body

temperature as well [11]. Clarification of the exact differences in the temperature response to restraint between the rat and mouse is important and will become more so because of the increasing availability and use of genetically altered mice. Whether all strains of mice, especially those utilized in the production of targeted genetic manipulations, respond to restraint by a reduction in body temperature is unknown. In the limited number of strains we have tested to date, restraint causes a reduction in body temperature provided mice are of a sufficient weight. A more extensive and direct comparison of mouse strains may reveal differences in this response as it has for other endpoints [5,4].

The particular aspect of our restraint procedure that induces the decrease in body temperature is unknown but may be related to the amount of movement possible. We and others have inferred from visual inspection that all mice had the same degree of mobility. That is, the movement of the limbs and the tail of the mouse was restricted but still possible; a restrained mouse was able to rotate from a supine to a prone position but could not rotate through the rostral–caudal axis. We used the visual determination as a rough indication of the degree of restraint each mouse experienced. However, it is obvious from our data that heavier mice displayed a greater decrease in body temperature implying a difference in the actions of the restraint technique despite a visual appearance of uniformity. If restraint and immobilization are considered to be on a continuum, then as the weight of the mouse increases the degree of movement would be increasingly restricted. Greater limitations in movement (i.e., immobilization) may trigger release of endogenous substances or activation of pathways different from those associated with restraint. Other factors such as the rigidity of the restraint device and the degree of compression induced in heavier mice may also be a significant factor. It is well known that activation of brain pathways and centers controlling the response to a stressor vary with the stressor [12] but direct comparisons of restraint versus immobilization have not been conducted. Such direct comparisons would facilitate an understanding of the controlling factors operating in each of these stressors. Utilization of mapping strategies able to identify pathways or brain areas activated in response to immobilization versus restraint would be useful (see Ref. [1] or [23] for an application of such techniques).

Many manipulations that serve as stressors, including restraint, cause an activation of specific neural pathways through the synthesis or release of endogenous substances. Modulation of the actions of these substances (e.g., serotonin, dopamine, endogenous endorphins, etc.) whose effects can be manipulated by exogenously administered agents can cause a decrease in body temperature. Restraint is also known to elevate glucocorticoid levels and in fact their measurement is often used as an index of the stress induced by this manipulation (e.g. Ref. [22]) restraint-induced elevations in corticosterone could be hypothesized to play a role in the neuroprotection observed in our studies. However, this is unlikely as administration of a dosage of corticosterone in the drinking water incapable of involuting the thymus does not impact on D-MDMA neurotoxicity [18]. Further, a supraphysiological level of corticosterone achieved through pellet implant and capable of producing thymic involution does not affect D-MDMA-induced damage (Johnson and Miller, unpublished observations). Given that temperature is an integrative response involving a multitude of neurotransmitters and hormones it is difficult to do more than speculate as to the exact biochemical basis of our findings without further study.

In summary, restraint is often chosen as a stressor in the laboratory mouse because of perceived ease of application and because it can activate a full spectrum of endocrine and neurological pathways. However, the consistent application of this technique may be more difficult than appreciated. Further, restraint is used in a variety of applications for the assessment of toxicity (e.g., nose-only inhalation devices). Restraint of the mouse can decrease body temperature to a degree sufficient to alter the neurotoxicity of agents effecting damage through mechanisms linked in some unknown way to body temperature elevations. This neuroprotective aspect of restraint appears to be directly determined by mouse weight. As body temperature may play an important role in the toxicity of chemicals to other organ systems, our data illustrate how underestimation of the importance and impact of common experimental variables can influence the assessment of toxicity.

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