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Repeated exposure to MDMA provides neuroprotection against subsequent MDMA-induced serotonin depletion in brain

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Abstract

Repeated exposure to sub-lethal insults has been reported to result in neuroprotection against a subsequent deleterious insult. The purpose of this study was to evaluate whether repeated exposure (preconditioning) to a non-5-HT depleting dose of MDMA in adult rats provides neuroprotection against subsequent MDMA induced 5-HT depletion. Treatment of rats with MDMA (10 mg/kg, ip every 2 hrs for 4 injections) resulted in a 50-65% depletion of 5-HT in the striatum, hippocampus and cortex, and these depletions were significantly attenuated in rats that received a preconditioning regimen of MDMA (10 mg/kg, ip daily for 4 days). The 5-HT depleting regimen of MDMA also resulted in a 40-80% reduction in 5-HT transporter immunoreactivity (SERTir), and the reduction in SERTir also was completely attenuated in MDMA preconditioned animals. Preconditioning with MDMA (10 mg/kg, i.p.) daily for 4 days provided neuroprotection against methamphetamine-induced 5-HT depletion, but not DA depletion, in the striatum. Additional studies were conducted to exclude the possibility that alterations in MDMA pharmacokinetics or MDMA induced hyperthermia in rats previously exposed to MDMA contributes towards neuroprotection. During the administration of the 5-HT depleting regimen of MDMA, there was no difference in the extracellular concentration of the drug in the striatum of rats that had received 4 prior, daily injections of vehicle or MDMA. Moreover, there was no difference in the hyperthermic response to the 5-HT depleting regimen of MDMA in rats that had earlier received 4 daily injections of vehicle or MDMA. Furthermore, hyperthermia induced by MDMA during preconditioning appears not to contribute toward neuroprotection, inasmuch as preconditioning with MDMA at a low ambient temperature at which hyperthermia was absent did not alter the neuroprotection provided by the preconditioning regimen. Thus, prior exposure to MDMA affords protection against the long-term depletion of brain 5-HT produced by subsequent MDMA administration. The mechanisms underlying preconditioning-induced neuroprotection for MDMA remain to be determined.

1. Introduction

3,4-Methylenedioxymethamphetamine (MDMA, 'ecstasy'), an amphetamine derivative, is a psychomotor stimulant and a popular drug of abuse. MDMA is considered to be selectively neurotoxic to serotonergic nerve terminals in rats, guinea pigs and non-human primates [21, 24]. MDMA administered to adult animals causes a decrease in brain 5-HT and its metabolite

5-HIAA, a decrease in the activity of tryptophan hydroxylase, the rate limiting enzyme in the synthesis of 5-HT and in the density of 5-HT uptake sites labeled by [³H]-paroxetine [15,26]. Although the mechanisms through which MDMA produces 5-HT neurotoxicity are not completely elucidated, oxidative stress, bioenergetic stress and/or mitochondrial dysfunction may contribute to the mechanism of MDMA induced 5-HT neurotoxicity [5,6,20].

Recently Piper et al. [19] reported that the intermittent administration of MDMA (10 mg/kg, b.i.d.) to adolescent rats resulted in tolerance to subsequent MDMA-induced 5-HT neurotoxicity in adulthood. The authors suggested that alterations in MDMA pharmacokinetics or attenuation of MDMA induced hyperthermia could contribute towards the neuroprotection. In addition, intermittent exposure to MDMA during adolescence resulted in tolerance to temperature dysregulation and the 5-HT syndrome responses to the drug [18]. Similar studies with methamphetamine (METH) have demonstrated that biweekly METH pretreatments starting on postnatal day 40 are neuroprotective against a neurotoxic regimen of METH administered on postnatal day 90 [22].

This phenomenon of amphetamine-induced neuroprotection, in which repeated exposure to sub-toxic insults results in protection against a subsequent neurotoxic insult, is similar to ischemic preconditioning [17]. Possible mechanisms of neuroprotection in ischemic preconditioning have been suggested to include inactivation of ion channels, decreased neurotransmitter release, increased activity of anti-oxidant or anti-apoptotic enzymes, increased expression of heat shock proteins or altered cerebral metabolism [23].

The aim of the present study was to investigate whether repeated exposure of adult animals to non-5-HT depleting dose of MDMA provides neuroprotection against a subsequent 5-HT depleting regimen of MDMA and to investigate potential mechanisms involved in the neuroprotection. The potential for MDMA preconditioning to provide cross-tolerance to methamphetamine (METH)-induced long-term dopamine and 5-HT depletion also was examined.

2. Results

Effect of MDMA preconditioning on subsequent MDMA reductions in brain 5-HT and SERT

The repeated, daily exposure of rats to MDMA (10 mg/kg, ip) for 4 days (preconditioning) provided significant neuroprotection against the long-term depletion of 5-HT produced by subsequent MDMA treatment (Figure 1). Administration of the neurotoxic regimen of MDMA (10 mg/kg, ip every 2 hrs for a total of 4 injections) induced significant depletions of approximately 50-65% of striatal, cortical and hippocampal 5-HT concentrations 7 days after drug administration when compared to the values for vehicle-treated controls. However, MDMA was ineffective in reducing 5-HT concentrations in any of the brain regions of rats previously exposed daily to MDMA (10 mg/kg, ip) for 4 days. The 2-way ANOVA indicated a significant interaction between prior exposure and treatment in the striatum [$F(1,17)=11.65$, $P<0.05$], cortex [$F(1,19)=12.00$, $P<0.05$] and hippocampus [$F(1,19)=6.24$, $P<0.05$]. The preconditioning regimen of MDMA alone did not result in any significant long-term depletion in the tissue concentration of 5-HT in the striatum, cortex or hippocampus.

The administration of a 5-HT depleting regimen of MDMA (10 mg/kg, ip every 2 hrs for a total of 4 injections) also resulted in significant ($P<0.05$) reductions in SERT immunoreactivity in the striatum, cortex and hippocampus (Figure 2). SERT immunoreactivity was reduced by approximately 40-80% 7 days following MDMA administration. However, the 5-HT depleting regimen of MDMA produced no significant reduction in SERT immunoreactivity in any brain region of rats exposed to the preconditioning regimen of MDMA. The 2-way ANOVA indicated a significant interaction between prior exposure and treatment in the cortex [$F(1, 18)$

=6.40, $P=0.02$] and hippocampus [$F(1, 18)=12.18$, $P=.003$], but not in the striatum ($P=0.14$). The preconditioning regimen of MDMA (10 mg/kg, ip daily for 4 days) alone did not significantly affect SERT immunoreactivity in the striatum or cortex, although there was a trend ($P=0.053$) for a reduction in the hippocampus. A representative western blot for SERT protein in the striatum of control and MDMA-preconditioned rats given the 5-HT depleting regimen of MDMA is shown in Figure 3.

Effect of a single MDMA exposure on the subsequent MDMA reductions in brain 5-HT

In order to investigate the number of daily exposures to MDMA necessary to provide neuroprotection against subsequent MDMA-induced 5-HT depletion, a 5-HT depleting regimen of MDMA was administered to rats 24 hrs following a single preconditioning injection of MDMA (rather than 4 daily treatments). As depicted in Figure 4, the magnitude of MDMA-induced 5-HT depletion in the striatum was not significantly different between animals previously exposed to a single daily injection of vehicle or MDMA. In contrast, partial neuroprotection was observed in the cortex and hippocampus. Although cortical and hippocampal concentrations of 5-HT were significantly ($P<0.05$) reduced by the 5-HT depleting regimen of MDMA in MDMA preconditioned rats, the extent of 5-HT depletion was significantly ($P<0.05$) less than that in the cortex and hippocampus of vehicle preconditioned animals (Figure 4). Two way ANOVA revealed that there was no significant interaction between prior exposure and treatment in the striatum or cortex, whereas the interaction was significant in the hippocampus [$F(1,21)=5.36$, $P<0.05$].

Effect of interval between MDMA preconditioning and subsequent MDMA treatment on neuroprotection

In order to determine the persistence of the neuroprotection afforded by prior exposure to MDMA against subsequent MDMA-induced 5-HT depletion, the interval between preconditioning and treatment with the neurotoxic regimen of MDMA was extended to 4 days (rather than 1 day) (Figure 5). Treatment with MDMA (10 mg/kg, ip every 2 hrs for a total of 4 injections) produced similar reductions in striatal 5-HT concentrations in control rats and rats previously exposed daily for 4 days to MDMA and in whom the post-preconditioning interval was extended to 4 days. Two way ANOVA revealed a significant effect of treatment [$F(1,15)=20.0$, $P<0.05$], but no significant interaction between prior exposure and treatment [$F(1,15)=0.03$, $P=0.86$]. Similarly, the 5-HT depleting regimen of MDMA resulted in reductions in 5-HT concentrations in the cortex and hippocampus that were no different in control and MDMA-preconditioned rats when the interval between the preconditioning regimen and the 5-HT depleting regimen was 4 days rather than 1 day (data not shown).

Effect of MDMA preconditioning on MDMA-induced hyperthermia

In as much as hyperthermia has been viewed as an important determinant of the extent of MDMA-induced 5-HT depletion, the pattern of hyperthermia during the administration of the 5-HT depleting regimen of MDMA was determined in vehicle and MDMA preconditioned animals. As indicated in Figure 6, body temperatures increased significantly during the course of the administration of MDMA (10 mg/kg, ip, every 2 hrs for a total of 4 injections). Repeated measures ANOVA revealed a significant effect of time [$F(7,168)=17.81$, $P<0.001$], but no significant effect of treatment [$F(1,168)=1.16$, $P>0.20$]. The extent of MDMA-induced hyperthermia was similar in rats previously exposed to daily vehicle or MDMA treatments.

Effect of MDMA preconditioning on brain MDMA concentrations

In order to exclude the possibility that altered brain pharmacokinetics of MDMA contributed to the neuroprotective effect of MDMA preconditioning, extracellular concentrations of MDMA were determined in the brain during the administration of the 5-HT depleting regimen

of MDMA to rats previously exposed to daily vehicle or MDMA treatment for 4 days (Figure 7). Extracellular concentrations of MDMA continued to increase during the treatments with MDMA (10 mg/kg, ip at 2 hr intervals for a total of 4 injections). Maximal extracellular concentrations of MDMA were approximately 800 pg/ μ L (uncorrected for recovery). Repeated measures ANOVA indicated no significant differences in the extracellular concentrations of MDMA in the striatum of rats previously exposed to vehicle or MDMA. Similarly, no significant difference was noted in the extracellular concentration of the MDMA metabolite, MDA, in the striatum of rats previously exposed to vehicle or MDMA (data not shown).

Effect of ambient temperature on MDMA preconditioning

Recently, it has been reported that the repeated elevation of body temperature (i.e., heat shock) provides neuroprotection against MDMA-induced 5-HT neurotoxicity [8]. Thus, consideration was given to the possibility that the neuroprotective effect of MDMA preconditioning resulted from the repeated hyperthermic episodes produced by daily MDMA treatments. To this end, rats were exposed for 4 days to MDMA administration at 19°C, a temperature at which MDMA treatment did not produce hyperthermia (data not shown), prior to the subsequent administration of the 5-HT depleting regimen of MDMA at 23°C. Despite the lack of daily drug-induced hyperthermia, daily exposure to MDMA at 19°C still afforded significant protection against subsequent MDMA-induced 5-HT depletion (Figure. 8). The administration of MDMA (10 mg/kg, ip every 2 hrs for a total of 4 injections) did not result in a significant reduction of tissue concentrations of 5-HT in the striatum, cortex or hippocampus of rats previously exposed to daily MDMA treatment at 19°C, in contrast to the 50-65% depletion of 5-HT observed in rats previously exposed to vehicle. ANOVA revealed a significant interaction between prior exposure and treatment for the cortex [$F(1,16)=10.43$, $P<0.005$] and hippocampus [$F(1,16)=10.34$, $P<0.005$] but not for the striatum [$F(1,12)=1.80$, $P=0.20$].

Effect of MDMA preconditioning on METH-induced reductions in 5-HT and dopamine

Finally, the long-term depletion of dopamine and 5-HT produced by METH administration was determined in rats exposed previously to daily MDMA treatment (Figure 9). Concentrations of 5-HT in the striatum, cortex and hippocampus were significantly ($P<0.05$) reduced by 40-50% 7 days following the administration of METH (10 mg/kg, sc every 2 hrs for a total of 4 injections) to control animals. In contrast, METH administration produced no significant reductions in striatal, cortical or hippocampal concentrations of 5-HT in rats previously exposed to daily MDMA treatments. Two way ANOVA indicated a significant interaction between prior exposure and treatment in the striatum [$F(1,35)=5.67$, $P=0.023$], cortex [$F(1,35)=37.30$, $P<0.001$] and hippocampus [$F(1,35)=10.36$, $P=0.003$].

The monoamine depleting regimen of METH also resulted in a 70% reduction in striatal dopamine concentrations ($P<0.05$) in control animals. Daily exposure to MDMA did not significantly alter the extent of striatal dopamine depletion produced by the neurotoxic regimen of METH (Table 1).

3. Discussion

The significant findings of this study are as follows: 1) repeated exposure (preconditioning) of adult rats to MDMA provides neuroprotection against a subsequent 5-HT depleting regimen of the drug, as well as to the long-term reduction in SERT immunoreactivity that accompanies depletion of brain 5-HT, 2) preconditioning with MDMA diminishes the extent of the METH induced depletion of 5-HT, but not dopamine, in the striatum 3) the protective effect of prior MDMA exposure depends on the duration of the preconditioning regimen and is relatively transient in nature, 4) alterations in MDMA pharmacokinetics or MDMA-induced hyperthermia do not appear to contribute to the neuroprotection provided by preconditioning.

The present finding that prior treatment with MDMA attenuates the long-term depletion of brain 5-HT produced by subsequent exposure to MDMA is in accord with a previous report by Piper et al [19]. These investigators demonstrated that exposure of adolescent rats to intermittent MDMA treatment provided subsequent neuroprotection in adult rats to MDMA-induced reductions in SERT binding. The repeated exposure of adolescent or adult rats to another amphetamine derivative, METH, also has been shown to confer protection against the subsequent depletion of dopamine and 5-HT induced by high dose METH administration [4, 10,22].

In the present study, as well as that of Piper et al [19], the preconditioning regimen of MDMA for 4 days alone did not result in a significant long-term reduction in 5-HT concentrations in brain. However, the preconditioning regimen did result in a modest reduction in SERT immunoreactivity in the hippocampus (but not in the striatum or cortex). Piper et al [19] also noted a slight, but significant, reduction in hippocampal (but not striatal or cortical) SERT binding following repeated exposure of adolescent rats to MDMA. Thus, the preconditioning regimen per se may produce a limited insult to serotonergic axon terminals.

Baumann et al [1] and Shankaran and Gudelsky [25] also have demonstrated a type of tolerance to the effects of MDMA following repeated administration that differs from that reported by Piper et al [19] and that demonstrated in the present study. In the aforementioned studies, a neurotoxic regimen of MDMA resulted in tolerance to the acute neuroendocrine, behavioral and thermal effects of subsequent, low-dose MDMA administration. This tolerance is considered to be the result of the depletion of the endogenous mediator, i.e., 5-HT, of these effects. Inasmuch as the preconditioning regimens employed in the study by Piper et al [19], as well as in the present study, are not neurotoxic in nature, mechanisms other than 5-HT depletion are most likely involved in the protective effects of preconditioning.

It is noteworthy that in the present study prior exposure to MDMA resulted in neuroprotection against depletions of striatal 5-HT, but not dopamine, produced by subsequent METH administration. Although it has been suggested that similar processes of oxidative and/or bioenergetic stress underlie MDMA and METH induced 5-HT and/or dopamine neurotoxicity, repeated exposure to MDMA exerts selectivity in its neuroprotective effects to 5-HT axon terminals.

In the present study, the neuroprotective effect of MDMA preconditioning was relatively transient in nature. Attenuation of MDMA-induced reductions in 5-HT concentrations or SERT immunoreactivity was noted when an interval of 1 day, but not 4 days, lapsed prior to the administration of the neurotoxic regimen of MDMA. In contrast, Piper et al [19] observed that protection against MDMA-induced 5-HT neurotoxicity was still evident 7 days following repeated exposure to the drug. Differences in the dosing regimen of MDMA during preconditioning or the age of the animals at the time of preconditioning may account for this difference.

Previous studies suggest that hyperthermia produced by MDMA contributes to the mechanism of MDMA-induced 5-HT neurotoxicity. Maintenance of rats at a cool ambient temperature [2,14] or administration of drugs that prevent MDMA-induced hyperthermia [9,13] attenuate MDMA-induced 5-HT depletion. The neuroprotection afforded by prior exposure to MDMA [19] or METH [22] was shown to be accompanied by a reduction in the hyperthermic response elicited by the subsequent neurotoxic regimen of the drug, and it was suggested that the blunted hyperthermia could contribute to the neuroprotective effects of prior exposure to these amphetamines. However, Danaceau et al [4] concluded that a diminished METH-induced hyperthermic response is not required for the development of tolerance to the dopamine and 5-HT depleting effects of the drug. In the present study, prior exposure to MDMA provided

subsequent neuroprotection against 5-HT depletion in the absence of any significant alteration in MDMA-induced hyperthermia. Thus, with regard to the regimen of MDMA employed herein, diminished MDMA induced hyperthermia during the neurotoxic regimen is not evident and most likely does not account for the neuroprotective effects of preconditioning.

An attempt was made to exclude the possibility that differences in the pharmacokinetics of MDMA in the brains of control animals and those previously exposed to MDMA may have contributed to the differences in the subsequent vulnerability to MDMA-induced 5-HT neurotoxicity. Using *in vivo* microdialysis, it was determined that the acute administration of MDMA resulted in similar extracellular concentrations of MDMA, as well as MDMA metabolite MDA (data not shown), in the striatum of drug naïve rats and rats exposed previously to 4 daily injections of MDMA. These results are suggestive that the neuroprotection afforded by prior exposure to MDMA is not the result of a diminished accumulation of MDMA in the brain.

It should be noted that work by Monks and colleagues [7,11] supports the view that thioether metabolites of MDMA, and not MDMA itself, are the reactive neurotoxicants responsible for MDMA-induced 5-HT neurotoxicity. The present study does not exclude the possibility that prior exposure to MDMA alters the subsequent accumulation of the thioether metabolites of MDMA in brain tissue.

Recently, Escobedo et al [8] have reported that protection against MDMA-induced 5-HT neurotoxicity can be achieved by repeated exposure to heat shock (i.e., elevated body temperature) and may be mediated by heat shock proteins. Inasmuch as repeated daily exposure to MDMA is accompanied by daily hyperthermic episodes, consideration was given to the possibility that repeated hyperthermia produced by MDMA contributes to the mechanism of neuroprotection provided by drug preconditioning. To address this issue, the preconditioning regimen of MDMA was administered at an ambient temperature of 19°C at which MDMA-induced hyperthermia was absent. Despite the absence of MDMA-induced hyperthermia during the preconditioning, prior exposure to MDMA still attenuated the depletion of brain 5-HT produced by the subsequent neurotoxic regimen of the drug. Thus, repeated MDMA-induced hyperthermia during the preconditioning regimen does not appear to contribute to the neuroprotection provided by repeated exposure to MDMA.

The phenomenon of neuroprotection provided by preconditioning with MDMA is similar to neuroprotection provided by ischemic preconditioning in which exposure to sub-toxic ischemic insults results in tolerance to subsequent, lethal ischemic episode [12]. Mechanisms of neuroprotection afforded by ischemic preconditioning may include inactivation of ion channels, increased activity of antioxidant or anti-apoptotic enzymes, altered cerebral metabolism or increased expression of heat shock proteins. An additional, potential mechanism for the neuroprotection afforded by stimulant preconditioning may be the down-regulation of SERT. In view of the necessity of SERT function for MDMA-induced 5-HT neurotoxicity, such a MDMA-induced down-regulation of SERT could afford neuroprotection. The relative selectivity of MDMA for SERT versus the dopamine transporter also might account for the present finding that MDMA preconditioning prevents methamphetamine-induced 5-HT, but not dopamine, depletion. Nevertheless, further studies are necessary to determine whether any of these factors contribute to the neuroprotective effects of MDMA preconditioning.

In summary, it has been established that prior exposure of adult rats to MDMA provides protection against subsequent MDMA-induced 5-HT depletion in the brain. The mechanisms underlying this neuroprotection appear not to involve the induction of hyperthermia per se, attenuation of the hyperthermic response occurring during the MDMA neurotoxic regimen or alterations in brain MDMA pharmacokinetics. Additional studies are necessary to further

elucidate the mechanisms that contribute towards neuroprotection afforded by MDMA preconditioning.

4. Materials and Methods

4.1 Animal Procedures

Adult male rats (250–275 g) of the Sprague-Dawley strain (Harlan Laboratories, Indianapolis, IN) were used in the studies. The animals were housed three per cage in a temperature- and humidity-controlled room with a 12-h light/dark cycle and allowed food and water ad libitum. Animals undergoing surgery were housed one per cage postoperatively. All procedures were in strict adherence to the National Institutes of Health guidelines and approved by the institutional animal care and use committee.

4.2 Drugs and Drug Treatment

MDMA was provided by the National Institute on Drug Abuse (Bethesda, MA) and METH was purchased from Sigma Chemical Co. (St. Louis, MO). Both drugs were dissolved in 0.15 M NaCl and administered intraperitoneally. The preconditioning regimen consisted of a single, daily injection of MDMA (10mg/kg i.p.) or vehicle for four days. The day after the last preconditioning injection rats received a 5-HT depleting regimen of MDMA (10 mg/kg, ip at 2 hr intervals for a total of 4 injections). In other experiments, the number of preconditioning injections was varied, as was the interval between the administration of the preconditioning and 5-HT depleting regimens of MDMA. In one experiment, METH (10 mg/kg, sc, every 2 hrs for a total of 4 injections) was administered 24 hrs following the last preconditioning injection of MDMA or vehicle.

4.3 In Vivo Microdialysis Procedures

Rats were implanted with a stainless steel guide canula under ketamine/xylazine (70/6 mg/kg i.p.) anesthesia 48 to 72 h before the insertion of the dialysis probe. On the day of the dialysis experiment, a concentric style dialysis probe was inserted through the guide canula into the striatum. The coordinates for the tip of the probe were A, 1.3 mm; L, 3.1 mm; and V, -7 mm from bregma, according to the stereotaxic atlas of Paxinos and Watson (17). The active portion of the membrane for the striatum was 4.5 mm. The probes were connected to an infusion pump set to deliver modified Dulbecco's phosphate buffered saline containing 1.2 mM CaCl_2 and 5 mM glucose at a constant rate of 2.0 $\mu\text{L}/\text{min}$. After an equilibration period of 1.5 hr, dialysis samples were collected every 30 min. At least 3 baseline samples were obtained prior to drug treatment.

4.4 Assay of tissue 5-HT and dopamine

Rats were killed by decapitation 7 days following treatment with the 5-HT and/or dopamine depleting regimens of MDMA or METH, and the striatum, hippocampus and cortex were dissected from 1 mm coronal sections of the brain and stored at -80°C . Tissue samples were homogenized in ice-cold 0.2 N perchloric acid, and the homogenates were then centrifuged at 14,000 rpm for 5 min. Aliquots of the supernatant (20 μL) were injected onto a C-18 reverse phase column connected to a LC-4B amperometric detector (Bioanalytical Systems, West Lafayette, IN) equipped with a glassy carbon target electrode. The mobile phase for the separation of 5-HT and dopamine consisted of 100 mM citric acid, 75 mM sodium phosphate, 50 mg/l disodium ethylenediamine tetraacetate, 176 mg/l octane sulfonic acid sodium salt, 15% methanol, pH 4.2, pumped at a flow rate of 0.8 ml/min. Peak heights were recorded with an integrator, and quantities of 5-HT and DA were calculated on the basis of known standards. Tissue 5-HT and dopamine concentrations are reported as ng/mg tissue.

4.5 Body temperature measurements

On the day of the experiment, the rats were allowed to acclimatize at 24 °C for two hours before body temperatures were measured. Measurements of rectal temperature were made using a telethermometer and a thermister probe. The probe was lubricated with a small amount of petroleum jelly and inserted 5 cm into the rectum of each rat for at least 30 seconds, until a stable temperature was obtained. Measurements were taken every 30 min for a one-hour period prior to administration of MDMA and until 2 hours after the fourth and final injection of MDMA.

4.6 Western blot analysis for 5-HT transporter (SERT) expression

Hippocampal tissue (left or right side of whole hippocampus) was homogenized in 0.5mL of ice-cold 0.32M sucrose and centrifuged at low speed ($800 \times g$, 17min, 4°C). The supernatant (S1) was removed and centrifuged at high speed ($22,000 \times g$, 17min, 4°C) to yield pelleted (P2) synaptosomes. The pellet was resuspended in 150µL of ice-cold dH2O and an aliquot (75µL) was centrifuged at high speed ($22,000 \times g$, 17min, 4°C). The supernatant (S3) was removed and the pellet was resuspended in ice-cold dH2O to form the membrane fraction. Protein values of the hippocampal preparation were determined via the Bradford method. Protein samples were then mixed with 2× Tris-glycine- loading buffer (Invitrogen, Carlsbad, CA, USA), heated at 85°C for 5 min and stored at -80°C until assayed. Western Blot analysis was performed using Tris-glycine 10% 10 lane gels in a Novex mini cell apparatus (Invitrogen, Carlsbad, CA, USA). Protein (30 µg) was loaded into each lane and the gel run at 150V for 90min (running buffer: 25mM TRIS; 192mM Glycine; 0.02% SDS). Gels were transferred at 120V for 2h to a polyvinylidene difluoride (PVDF) membrane (transfer buffer: 25mM TRIS; 192mM Glycine; 20% Methanol; 0.02% SDS). The membranes were then blocked for 1h at room temperature (RT) in blocking buffer (10mM TRIS; 150mM NaCl; pH 8.0; 5% non-fat dry milk and 0.05% Tween-20) and probed with SERT primary antibody (dilution 1:1250; Santa Cruz, CA, USA) overnight at 4°C. The following day, membranes were rinsed 3× in TBST, probed with HRP-conjugated anti-goat secondary antibody (dilution 1:2500, Santa Cruz, CA, USA) for 1h at RT and visualized via enhanced chemiluminescence (ECL) (Amersham Biosciences, NJ, USA). The net band intensity was measured using a Kodak Gel Logic 100 imaging system (Rochester, NY, USA) and presented as relative optical density units on each gel normalized to saline controls.

4.7 Assay for MDMA

The concentration of MDMA in dialysis samples was quantified by HPLC with UV detection. Aliquots (20 µl) of the dialysis samples were injected on a C-18 column connected to a Waters 2996 Photodiode Array Detector monitoring all wavelengths from 190 nm to 300nm. The mobile phase consisted of 7.8 % acetonitrile solution in water containing 0.29% hexylamine (v/v) and 5% of 85% phosphoric acid (v/v), pH 2.1 pumped at a flow rate of 0.8 ml/min. The column temperature was set at 40° C, and the samples were maintained at 4° C. The identification of MDMA within each sample was confirmed by its spectral profile matched against standards, as well as its retention time. The quantification of unknown samples was performed against a seven-point standard curve. Peak area at a wavelength of 199 nm (the peak absorbency for both MDMA and MDA) was chosen as the method of integration using the Waters Empower Pro (v. 5.0) software. Quality control samples were interspersed with each run to ensure HPLC calibration (3).

4.8 Statistical Analysis

The effects of preconditioning with MDMA on 5-HT and/or dopamine depletions induced by MDMA or METH were analyzed by two-way analysis of variance (ANOVA). The effect of preconditioning with MDMA on brain MDMA concentrations and MDMA induced

hyperthermia was measured by two-way repeated measures ANOVA. SERT immunoreactivity was evaluated with a 2-way ANOVA. Multiple pair wise comparisons were performed using Student-Newman-Keuls test. Treatment differences for all the data were considered statistically significant at $p < 0.05$.

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Figure 1. Effect of repeated exposure to MDMA for 4 days on the subsequent MDMA-induced depletion of 5-HT in the striatum, hippocampus and cortex

Rats were treated with vehicle or MDMA (10 mg/kg, ip) daily for 4 days. On the following day, the animals received MDMA (10 mg/kg, ip, every 2 hr for a total of 4 injections) or vehicle and were sacrificed by decapitation 7 days later. The values represent the mean $\pm$ S.E of 5-6 rats. * represents $p < 0.05$ when treatment groups are compared within the factor of prior exposure and # represents $p < 0.05$ when treatment groups are compared across the factor of prior exposure.

Figure 2. Effect of repeated exposure to MDMA on the reduction in SERT immunoreactivity ($SERT_{ir}$) in the striatum, cortex and hippocampus produced by a subsequent neurotoxic regimen of MDMA

Rats were treated as described in the legend to figure 1. Values represent the mean $\pm$ SE of 6 rats. *Indicates $P < 0.05$ when treatment groups are compared within the factor of prior exposure, and # represents $P < 0.05$ when treatment groups are compared across the factor of prior exposure.

Figure 3. Representative western blot for SERT in the striatum

Rats were treated according to the drug regimens described in the legend to figure 1. Actin served as the loading control. Lane 1: Vehicle/Vehicle; Lanes 2, 5, 8: Vehicle/MDMA; Lanes 3, 6, 9: MDMA/Vehicle; Lanes 4, 7: MDMA/MDMA.

Figure 4. Effect of single exposure to MDMA on the subsequent MDMA-induced depletion of 5-HT in the striatum, hippocampus and cortex

Rats were treated with a single injection or vehicle of MDMA (10 mg/kg, ip) for 1 day. On the following day, the animals received MDMA (10 mg/kg, ip, every 2 hr for a total of 4 injections) or vehicle and were sacrificed by decapitation 7 days later. The values represent the mean $\pm$ S.E of 5-6 rats. * represents $p < 0.05$ when treatment groups are compared within the factor of prior exposure and # represents $p < 0.05$ when treatment groups are compared across the factor of prior exposure.

Figure 5. Effect of lengthened interval between prior exposure and the 5-HT depleting regimen of MDMA on the neuroprotection afforded in the striatum by preconditioning

Rats were treated with vehicle or MDMA (10 mg/kg, ip) daily for 4 days. Four days later, the animals received MDMA (10 mg/kg, ip, every 2 hr for a total of 4 injections) or vehicle and were sacrificed by decapitation 7 days later for striatal 5-HT determinations. The values represent the mean $\pm$ S.E of 5-6 rats. * represents $p < 0.05$ when treatment groups are compared within the factor of prior exposure.

Figure 6. Effect of prior exposure to MDMA on subsequent MDMA-induced hyperthermia
Rats were treated with vehicle or MDMA (10 mg/kg, ip) daily for 4 days. On the following day, the animals received MDMA (10 mg/kg, ip, every 2 hr for a total of 4 injections) or vehicle, as indicated by the arrows. Values represent the mean $\pm$ S.E of 6 rats.

Figure 7. Effect of repeated exposure to MDMA for 4 days on the extracellular concentration of MDMA in the striatum during the administration of the 5-HT depleting regimen

Rats were treated with vehicle or MDMA (10 mg/kg, ip) daily for 4 days. On the following day, microdialysis samples of the striatum were obtained from the animals during the 5-HT depleting regimen of MDMA (10 mg/kg, ip, every two hrs for a total of 4 injections) beginning at time 0. Values represent the mean $\pm$ S.E of 5 rats.

Figure 8. Effect of cool ambient temperature on the neuroprotection afforded by prior exposure to MDMA

Rats were treated with vehicle or MDMA (10 mg/kg, ip) daily for 4 days at an ambient temperature of 19°C. On the following day, the animals received MDMA (10 mg/kg, ip, every 2 hr for a total of 4 injections) or vehicle at an ambient temperature of 23°C and were sacrificed by decapitation 7 days later. The values represent the mean $\pm$ S.E of 5-6 rats. * represents $p < 0.05$ when treatment groups are compared within the factor of prior exposure and # represents $p < 0.05$ when treatment groups are compared across the factor of prior exposure.

Figure 9. Effect of repeated exposure to MDMA for 4 days on the subsequent METH-induced depletion of 5-HT in the striatum, cortex and hippocampus

Rats were treated with vehicle or MDMA (10 mg/kg, ip) daily for 4 days. On the following day, the animals received METH (10 mg/kg, sc, every 2 hr for a total of 4 injections) or vehicle and were sacrificed by decapitation 7 days later. The values represent the mean $\pm$ S.E of 5-6 rats. * represents $p < 0.05$ when treatment groups are compared within the factor of prior exposure and # represents $p < 0.05$ when treatment groups are compared across the factor of prior exposure.

Table 1

Effect of prior exposure to MDMA on the depletion of striatal dopamine produced by subsequent METH administration.

Prior Exposure	Treatment	
	DA (ng/mg tissue)	
	VEH	METH
Vehicle	13.4 ± 0.6	4.3 ± 0.5 *
MDMA	12.4 ± 0.6	5.5 ± 0.9 *

Rats received vehicle or MDMA (10 mg/kg, ip) daily for 4 days prior to the administration of vehicle or METH (10 mg/kg, sc, every 2 hr for a total of 4 injections) on day 5. Striatal concentrations of dopamine were determined in rats 7 days following METH treatment. N=5-6 animals.

* Indicates P<.05 compared to the value for the vehicle treated animals within the same prior exposure treatment.