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Effects of 3,4-methylenedioxymethamphetamine (MDMA) on serotonin transporter and vesicular monoamine transporter 2 protein and gene expression in rats: implications for MDMA neurotoxicity

Dominik K. Biezonski and Jerrold S. Meyer

Neuroscience and Behavior Program, University of Massachusetts, Amherst, Massachusetts, USA

Abstract

3,4-Methylenedioxymethamphetamine (MDMA; 'Ecstasy') is a popular recreational drug used worldwide. This study aimed to determine the effects of this compound on the expression of nerve terminal serotonergic markers in rats. Experiment 1 investigated MDMA-induced changes in levels of the serotonin transporter (SERT) and the vesicular monoamine transporter 2 (VMAT-2) in the hippocampus, a region with sparse dopaminergic innervation, after lesioning noradrenergic input with *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP-4). Adult male Sprague–Dawley rats were administered 100 mg/kg DSP-4 or saline 1 week prior to either an MDMA (10 mg/kg × 4) or saline binge. Two weeks following the binge treatment, the DSP-4/MDMA group unexpectedly showed little change in hippocampal VMAT-2 protein expression compared with DSP-4/Saline controls, despite large reductions in SERT levels in all regions examined in the MDMA-treated animals. Furthermore, animals treated with binge MDMA (Experiment 2) showed a striking decrease in SERT gene expression (and a lesser effect on VMAT-2) measured by quantitative RT-PCR in pooled dorsal and median raphe tissue punches, when compared with saline-treated controls. These results demonstrate that MDMA causes substantial regulatory changes in the expression of serotonergic markers, thus questioning the need to invoke distal axotomy as an explanation of MDMA-related serotonergic deficits.

Keywords: 3,4-methylenedioxymethamphetamine, neurodegeneration, neurotoxicity, serotonin, serotonin transporter, vesicular monoamine transporter 2.

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The entactogen 3,4-methylenedioxymethamphetamine (MDMA; 'Ecstasy') is a ring-substituted amphetamine derivative, the use of which is prevalent among adolescents and adults (Banken 2004; UNODCC, 2008). High doses of this compound in rats lead to significant decrements in markers of serotonergic axons and terminals while sparing cells of the raphe nuclei (O'Hearn *et al.* 1988; Battaglia *et al.* 1991). More specifically, MDMA causes depletions in serotonin (5-hydroxytryptamine; 5-HT) and the major 5-HT metabolite 5-hydroxyindoleacetic acid, decreases in tryptophan hydroxylase (TPH) activity, and reduced levels of the serotonin transporter (SERT) as measured either by membrane binding or autoradiography (Green *et al.* 2003). Several studies have also found reductions in 5-HT-, TPH-, or SERT-immunoreactive fiber density (Green *et al.* 2003; Xie *et al.* 2006), which has been interpreted as MDMA-induced degeneration of distal serotonergic axons and nerve terminals.

The significance of MDMA effects on levels of these axon/terminal markers has been debated for over two decades (see Molliver *et al.* 1990; Ricaurte *et al.* 2000; Baumann *et al.* 2007). Although it is certainly possible that the observed changes result from neurotoxic damage (i.e.

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Address correspondence and reprint requests to Jerrold Meyer, PhD, Department of Psychology, Tobin Hall, 135 Hicks Way, University of Massachusetts, Amherst, MA 01003-7710, USA.
E-mail: jmeyer@psych.umass.edu

Abbreviations used: 5,7-DHT, 5,7-dihydroxytryptamine; 5-HT, 5-hydroxytryptamine or serotonin; DA, dopamine; DAT, dopamine transporter; DSP-4, *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine; GFAP, glial fibrillary acidic protein; MDMA, 3,4-methylenedioxymethamphetamine; METH, methamphetamine; NE, norepinephrine; NET, norepinephrine transporter; PAGE, polyacrylamide gel electrophoresis; PD, postnatal day; qRT-PCR, quantitative RT-PCR; SDS, sodium dodecyl sulfate; SERT, serotonin transporter; SERT-KO, serotonin transporter knockout; TBS, Tris-buffered saline; TBS-T, Tris-buffered saline containing Tween 20; TPH, tryptophan hydroxylase; VMAT-2, vesicular monoamine transporter 2.

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'terminal pruning'), it should also be recognized that these measures are all indirect and subject to regulation. For example, it is well known that MDMA acutely inhibits TPH (Schmidt and Taylor 1987) and, in the case of multiple doses, causes long-term reductions in TPH activity (Stone *et al.* 1987). Because TPH is rate-limiting for 5-HT biosynthesis, it is likely that decreases in this enzyme restrict the extent of 5-HT production, thus reducing the levels of this neurotransmitter regardless of whether or not axonal damage has occurred. Moreover, MDMA has been reported to alter SERT gene expression (Kovács *et al.* 2007; Kirilly *et al.* 2008), which could lead to changes in SERT protein levels independent of axotomy. Finally, this transporter is subject to endosomal trafficking (Ramamoorthy and Blakely 1999; Carneiro and Blakely 2006), bringing into question the significance of MDMA-induced reductions in SERT binding when such assays involve the use of plasma membrane preparations. Consequently, it is difficult to distinguish whether diminution in expression of various serotonergic markers following MDMA exposure results from actual damage, as a consequence of selective depletion or down-regulation of each marker in the absence of axotomy, or both.

To help resolve this controversy, this study provides new evidence concerning the effects of MDMA on protein and gene expression of two different markers of serotonergic nerve terminals. Two weeks following binge administration of MDMA in rats, we used validated immunoblotting methods as well as quantitative RT-PCR (qRT-PCR) analysis to measure drug-induced changes in the protein (Experiment 1) and gene expression (Experiment 2) of SERT and the vesicular monoamine transporter 2 (VMAT-2). The differential pattern of MDMA effects on these markers suggests that this compound may adversely affect serotonergic neurons without necessarily inducing neurodegeneration.

Materials and methods

Drugs and antibodies

MDMA HCl was generously provided by the National Institute on Drug Abuse Drug Supply Program (RTI International, Research Triangle Park, NC, USA). 5,7-dihydroxytryptamine (5,7-DHT) creatinine sulfate, methamphetamine (METH) HCl, and *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP-4) were obtained from Sigma Chemical Company (St Louis, MO, USA). Anti-SERT (PC177L) and anti-VMAT-2 (AB1767) antibodies were obtained from Calbiochem (San Diego, CA, USA). Anti-β-actin antibodies were purchased from Sigma Chemical Company.

Vertebrate animals

Adult male Sprague–Dawley rats (Experiments 1 and 2) or pregnant dams (Experiment 1) were obtained from Charles River Laboratories (Kingston, NY, USA). Male wild-type and SERT-knockout (KO) BJ.6 mice (Experiment 1) were obtained from Taconic Farms (Hudson, NY, USA). All animals were housed in standard plastic tubs with a bedding of wood shavings, with food and water available *ad lib*. Pregnant dams were singly housed under a standard 12 : 12 h light–dark cycle, while all other animals were pair-housed under a reverse 12 : 12 h light–dark cycle (lights on at 7 PM). All male rats and mice were habituated to the experimenters by gentle handling for ~1 min each day for at least 3 days prior to the beginning of drug administration or killing. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996), and the experimental protocols were approved by the University of Massachusetts-Amherst Institutional Animal Care and Use Committee.

Animal killing and tissue dissection

In Experiment 1, on the day of killing, animals were lightly anesthetized with CO_2 and decapitated. Brains were rapidly removed, chilled by immersion in ice-cold 0.9% NaCl for 1 min, and then placed into an acrylic brain block (Braintree Scientific, Braintree, MA, USA). A 2-mm thick slice beginning 4 mm from the anterior pole was removed, and the parietal cortex was separated from the underlying striatum. Where applicable, the hippocampus, occipital cortex, and cerebellum were dissected free hand from remaining tissue. All brain regions were frozen on dry ice and stored at −70°C until time of analysis. The hippocampus and striatum were chosen based on their extensive serotonergic innervation and their vulnerability to MDMA-induced serotonergic deficits (O'Hearn *et al.* 1988; Green *et al.* 2003). Similarly the parietal cortex, though less extensively innervated than the frontal cortex, shows a greater MDMA-induced loss of 5-HT-immunoreactive fibers than the frontal cortex (O'Hearn *et al.* 1988). In addition, the occipital cortex and cerebellum were selected for analysis of regional SERT levels as discussed in Experiment 1. In Experiment 2, on the day of killing, all animals were anesthetized and decapitated as described. Whole brains were removed and immediately frozen in 2-methyl-butane previously cooled in powdered dry ice. Frozen brains were then stored at −70°C until the day of processing.

5,7-Dihydroxytryptamine administration

As part of the initial validation of our SERT immunoblotting procedures in Experiment 1, neonatal rats were administered the 5-HT neurotoxin 5,7-DHT to lesion serotonergic fibers. This compound was given to neonatal animals because they lack an efficient blood–brain barrier, thereby allowing peripheral administration (Towle *et al.* 1984; Pranzatelli and Martens 1992). On the day of parturition (postnatal day 0; PD0), litters were culled to eight male pups, and each litter was randomly assigned to the treatment or control condition ($n = 1$ litter per group). On PD2, pups in the treatment group were administered 20 mg/kg desipramine i.p. 60 min prior to a 100 mg/kg i.p. administration of 5,7-DHT dissolved in 0.9% NaCl vehicle containing 0.1% ascorbic acid. This treatment was repeated on PD 5. Pups in the control group were given i.p. injections of saline vehicle on both days. All pups were housed with their respective dams until weaning at PD 25, after which they were pair-housed until PD 35 when they were killed and brain tissues were collected as described in the section on Animal killing and tissue dissection.

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Methamphetamine administration

The VMAT-2 immunoblotting procedures in Experiment 1 were validated, in part, by means of METH lesioning of striatal dopamine (DA) terminals. Adult rats (n = 5 per group) were administered s.c. injections of either (±)-METH HCl (4 × 10 mg/kg, 2-h interdose interval) or 0.9% saline vehicle. One week after the treatment, all animals were killed and striatal samples were obtained for later analysis.

Administration of MDMA and *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine

In both experiments, young adult male rats (PD 72) were subjected to an MDMA binge dosing paradigm previously used in our laboratory (Piper *et al.* 2006) and similar to that reported in many other studies of MDMA neurotoxicity (Green *et al.* 2003). The dosing regimen consisted of four s.c. injections of 10 mg/kg (±)-MDMA HCl in 0.9% NaCl, with an interdose interval of 1 h. Control animals received the same regimen of saline vehicle only (n = 10 per group in Experiment 2). Rectal temperatures during MDMA dosing were monitored as described in Piper *et al.* (2005). In Experiment 1, we used the selective noradrenergic neurotoxin DSP-4 to lesion ascending noradrenergic projections from the locus coeruleus (Fritschy *et al.* 1990) prior to MDMA administration. Rats in this experiment were given i.p. injections of either 100 mg/kg DSP-4 or saline 1 week prior to either the binge dose of MDMA or saline, thus forming four groups: saline/saline (n = 8), DSP-4/saline (n = 6), saline/MDMA (n = 8), and DSP-4/MDMA (n = 6). In both experiments, animals were killed 2 weeks following MDMA treatment for analysis. The same post-drug period has been used in many previous studies to document significant decrements in serotonergic axon and terminal markers (Green *et al.* 2003).

Serotonin transporter binding

Serotonin transporter binding analysis was performed as described in Piper *et al.* (2005). Briefly, washed membrane preparations were assayed in triplicate using a 1.0 nM concentration of [^{3}H]citalopram (84.2 Ci/mmol; PerkinElmer, Waltham, MA, USA). Non-specific binding was determined in parallel incubations containing 10 μM unlabeled fluoxetine. Protein levels in each sample were determined by means of the Bio-Rad© DC protein assay (Hercules, CA, USA) using bovine gamma globulin as the standard.

Norepinephrine transporter binding

Norepinephrine (NE) transporter (NET) binding was conducted as described by Tejani-Butt (1992), with minor modifications. Samples were weighed and homogenized with a Polytron in 30 vols of ice-cold assay buffer containing 50 mM Tris, 120 mM NaCl, and 5 mM KCl, pH 7.4. Homogenates were then centrifuged at 20 000 *g* for 20 min at 4°C and resuspended, followed by three additional homogenizations and washes. Membrane preparations were finally homogenized in ice-cold buffer containing 50 mM Tris, 300 mM NaCl, and 5 mM KCl, pH 7.4, as the increased concentration of NaCl enhances binding of nisoxetine to NET (Tejani-Butt 1992). The binding reaction was carried out by incubating the washed membranes in triplicate with 2.0 nM [^{3}H]nisoxetine (87.2 Ci/mmol; PerkinElmer) for 4 h at 4°C. Non-specific binding was defined using 10 μM desipramine. The reaction was terminated by addition of 5 mL ice-cold buffer and filtration through GF/B filters presoaked in 0.05% polyethyleneimine. Filters were washed twice more with buffer and then counted in a Packard 1900CA liquid scintillation analyzer (PerkinElmer). The protein concentration of each sample was determined as described in the section on Serotonin transporter binding.

Preparation of whole-cell lysates/protein extraction for immunoblotting

Tissue preparation conformed to the basic methodology of Wang *et al.* (2004, 2005) with minor modifications. Frozen tissues were weighed and homogenized using a Teflon-glass motorized pestle in 30 vols of ice-cold radioimmunoprecipitation assay buffer, pH 7.4, containing 0.05 M Tris, 1% Igepal CA-630, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 1 mM sodium *o*-vanadate, and 1% of a commercially available protease inhibitor cocktail (P8340; Sigma Chemical Company). The use of a detergent-containing lysis buffer allowed solubilization of all membrane-bound (both plasmalemmal and endosomal) proteins for subsequent analysis. Following homogenization, samples were agitated on a rocker for 30 min at 4°C, and then centrifuged at the same temperature for 15 min at 22 000 *g*. The resulting supernatant was collected and frozen at −70°C for subsequent polyacrylamide gel electrophoresis (PAGE) under denaturing conditions (SDS–PAGE). Protein concentration of samples was determined as described in the section on Serotonin transporter binding.

Preparation of synaptosomes/protein extraction for immunoblotting

Frozen tissues were weighed and homogenized in 30 vols of ice-cold 0.32 M sucrose using a Teflon-glass motorized pestle as before. Although it is preferable to use fresh tissue when preparing synaptosomes for metabolic studies, frozen samples can readily be used for synaptosomal preparations when the goal is to determine protein expression in the sample (e.g. see Eravci *et al.* 2008; Hallett *et al.* 2008). The homogenate was centrifuged at 1000 *g* to remove nuclei and cellular debris, and the resulting supernatant was then centrifuged at 16 000 *g* to yield a crude synaptosomal fraction. Synaptosomes were resuspended and homogenized in radioimmunoprecipitation assay buffer (30 vols for striatal samples and 5 vols for hippocampal samples) to solubilize the synaptosomal membrane-bound proteins as mentioned in the section on Preparation of whole-cell lysates/protein extraction for immunoblotting. After a final centrifugation at 22 000 *g*, the resulting supernatant was collected and frozen at −70°C for later SDS–PAGE.

Immunoblotting

On the day of analysis, loading buffer (Thermo Scientific, Waltham, MA, USA) was added to a volume of sample calculated to contain 30–60 μg of protein, and the resulting mixture was boiled for 5 min using a dry bath. Molecular weight ladders (MAGICMARK™) were prepared according to manufacturer's recommendations (Invitrogen, Carlsbad, CA, USA). All samples were run on 10% precast polyacrylamide gels (Pierce, Rockford, IL, USA) at 100 V for ~60 min. Proteins were electrotransferred at 100 V onto Immobilon™ polyvinylidine difluoride membranes (Millipore, Billerica, MA, USA) for 80 min at 4°C. Following transfer, polyvinylidine difluoride membranes were washed in Tris-buffered saline (TBS) for 10 min and blocked in 5% non-fat milk buffer in TBS for 1 h at 22°C. Membranes were then probed with primary antibodies against

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SERT or VMAT-2 overnight at 4°C, with the exception of membranes containing striatal protein, which were incubated with VMAT-2 antibody for 1 h at 22°C. Membranes were rinsed twice with TBS containing 0.2% Tween 20 (TBS-T), and then washed in TBS-T for 10 min followed by three 5-min washes. Membranes were then incubated with secondary antibodies labeled with horseradish peroxidase at 22°C for 2 h, washed for 10 min followed by three 5-min washes in TBS-T, and finally immersed in enhanced chemiluminescent reagent (ECL-Plus; Amersham Biosciences, Piscataway, NJ, USA) for 1 min. The resulting chemiluminescent reaction was exposed on Hyperfilm ECL (GE Healthcare, Piscataway, NJ, USA), after which membranes were stripped and reprobed with an antibody against β-actin to verify equal protein loading. As an additional control, an identical 'standard sample' was run on all gels to normalize variability in band density across blots in subsequent analyses. Protein bands were quantified using Scion Image™ densitometry software (Scion Corporation, Frederick, MD, USA), and the optical density of bands was expressed as percent of control.

Dorsal/median raphe tissue punches for real-time PCR

Whole brains were cut coronally using a cryostat set at −10°C. Approaching the midbrain, the anterior boundaries of the dorsal and median raphe (same plane) were identified using the following two landmarks: (i) complete lateralization of the hippocampus concomitant with (ii) rapid enlargement of the cerebral aqueduct (Paxinos and Watson 1998). A 1-mm section encompassing the entire rostral-caudal extent of both nuclei was then taken and placed on an ionized glass slide (Thermo Electron Corporation, Waltham, MA, USA). The slide was chilled on dry ice for 1 min, after which punches of both nuclei were taken using a sterile needle/expeller with an internal diameter of 1 mm. Tissue punches from each animal were pooled and stored at −70°C until RNA extraction.

RNA extraction and real-time PCR analysis

Total RNA was extracted from combined dorsal/median raphe punches using an RNeasy Micro Kit (Qiagen, Germantown, MD, USA). Briefly, punches were disrupted and homogenized in Qiazol reagent, after which chloroform was added, the samples were centrifuged at 12 000 *g*, and the clear, upper phase containing total RNA was carefully collected. Samples were then mixed with 70% ethanol and loaded onto microcentrifuge columns. Before final RNA extraction using Rnase-free water, columns were subject to multiple washes using proprietary buffers, Dnase I treatment, and at least two additional washes in 80% ethanol. The RNA eluate was analyzed for purity ($260_{OD}/280_{OD}$ ratio of 1.9–2.1 in conjunction with curve analysis of $220_{OD} \rightarrow 320_{OD}$ scan) and concentration using a ND-1000 NanoDrop spectrophotometer (Thermo Scientific). Extracted RNA was diluted in Rnase-free water as needed and stored at −70°C until real-time PCR analysis.

Quantitative, real-time PCR was performed using a one-step RT-PCR Quantitect™ kit from Qiagen, with SYBR green as the amplicon fluorescence marker. Briefly, template RNA (~10 ng/reaction) was added to a master mix containing random primers (RT step), Sensiscript™ and Omniscript™ (RT step), and for the amplification step, gene-specific forward and reverse primers (Quantitect Primer Assays, Qiagen; sequences are proprietary), Hot-Start™ Taq polymerase (Qiagen), deoxynucleotidetriphosphates (dNTPs), and magnesium buffer. All thermocycling steps, as well as detection of SYBR green fluorescence intensity, were performed in triplicate on an MX3000p PCR instrument (Stratagene, La Jolla, CA, USA). Relative mRNA abundance between treatment groups was calculated by means of the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase to normalize RNA loading in each reaction. All data are expressed as fold-difference in gene expression.

Statistical analysis

For validation of SERT westerns (Experiment 1), Pearson's product–moment correlation analysis was used to determine correspondence between immunoblot and radioligand binding analyses of regional SERT. All statistical comparisons between saline and MDMA groups in Experiments 1 and 2 were performed by means of Student's *t*-tests. In Experiment 2, one outlier in the saline/SERT group was detected using the extreme Studentized deviation (Grubb's) method and was removed from all subsequent analyses. A value of $p < 0.05$ was used to determine statistical significance.

Results

Validation experiments: Control procedures validated our immunoblotting methods for analyzing SERT and VMAT-2 protein expression

As described in Xie *et al.* (2006), it is crucial when using immunoblotting to validate the choice of band(s) selected for measurement to ensure that it corresponds to the authentic protein of interest. In this study, we performed a series of validation procedures for both SERT and VMAT-2 using tissue sources known to have differing amounts of either protein. For SERT validation, we included tissue from SERT-KO mice (no SERT), tissue from rats treated with the 5-HT neurotoxin 5,7-DHT (low SERT), and tissues from various brain regions heterogeneous for SERT expression (e.g. cortex vs. cerebellum; Kish *et al.* 2005). We also determined the correlation between regional SERT levels assessed by radioligand binding and SERT assessed by immunoblotting using the band selected according to the preceding criteria. Upon testing a panel of commercially available antibodies, only Calbiochem PC177L anti-SERT proved satisfactory in our hands (see Table S1). Using this antibody, we confirmed the identity of an authentic SERT band near the predicted molecular weight of the protein of ~76 kDa (Qian *et al.* 1995) based on the protein band patterns from whole-tissue lysates derived from SERT-KO mice (Fig. S1a), 5,7-DHT treated rats (Fig. S1a), and regional brain expression in normal rats (Fig. S1b). In some blots, the SERT band resolved into a doublet, which may represent different phosphorylation states of the protein (Jayanthi *et al.* 2005). Additionally, we found a significant correlation between SERT binding and SERT immunoblotting in their respective measurement of regional levels of the protein (Fig. S1c and d).

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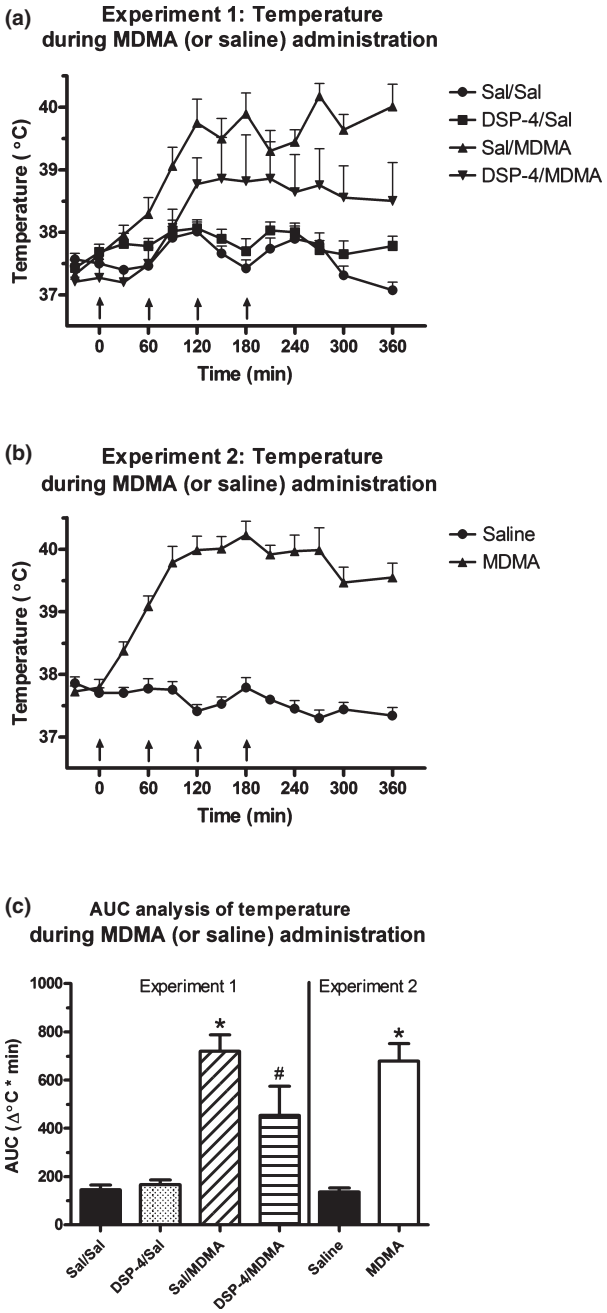

Fig. 1 Core body temperature on day of MDMA administration in Experiments 1 and 2. (a) Temperature measurements (mean ± SEM) taken prior to, during, and following MDMA or saline dosing in Experiment 1, with arrows indicating times of injection. MDMA or saline was given 1 week following DSP-4 or saline pre-treatment, yielding four treatment groups as noted. (b) Core temperature measurements obtained during MDMA or saline administration in Experiment 2 are presented as in (a). Area under the curve (AUC) analyses of temperature changes in each treatment group are represented in (c) for both experiments, expressed as mean ± SEM. MDMA-treated rats showed a significant rise in body temperature in Experiments 1 and 2 when compared with saline-treated controls, an effect that was non-significantly reduced by DSP-4 pre-treatment in the first experiment [$^{*}p < 0.0001$ vs. Sal/Sal (Exp.1) or Saline (Exp. 2); $^{\#}p = 0.0192$ vs. Sal/Sal].

For the validation of our VMAT-2 immunoblots, we analyzed striatal tissue from animals treated with a neurotoxic regimen of METH, a substance known to damage nigrostriatal dopaminergic projections and thus leading to significant loss of VMAT-2 protein expression in this region (Kita *et al.* 2003). Analysis of synaptosomal VMAT-2 levels in the striatum 1 week following a METH binge confirmed the identity of a single protein band corresponding to the transporter (~70 kDa) (Fig. S1e). The established SERT and VMAT-2 protein bands were selected for quantification in all subsequent analyses.

Experiment 1: MDMA reduced synaptosomal SERT but not VMAT-2 protein expression

We reasoned that if high doses of MDMA cause degeneration of distal serotonergic axons and nerve terminals as suggested by the well-established loss of SERT binding as well as SERT-immunoreactive fiber staining, then a vesicular marker such as VMAT-2 should also be significantly decreased in MDMA-treated animals. The limitation of this approach is that VMAT-2 is expressed not only by serotonergic neurons but also by neurons that use DA and NE as their neurotransmitter. Therefore, to develop a preparation in which VMAT-2 expression is limited mainly to serotonergic sources, we focused on the hippocampus because (i) this structure is only sparsely innervated by dopaminergic afferents (see Discussion) and (ii) ascending noradrenergic fibers can be selectively lesioned with the compound DSP-4 (Fritschy *et al.* 1990).

One week following DSP-4 (or saline) pre-treatment, animals were given either an MDMA binge or saline, after which they were killed 2 weeks later for analysis. During the MDMA binge, all MDMA-treated animals experienced hyperthermia compared with the saline-treated controls (Fig. 1a), which was not significantly affected by DSP-4 pre-treatment (Fig. 1c). The efficacy of DSP-4 in lesioning the noradrenergic innervation of the hippocampus was confirmed by a significant reduction in NET binding (~61–65%) in the DSP-4- pre-treated groups compared with their respective saline controls (Fig. 2). Immunoblotting revealed that hippocampal levels of synaptosomal VMAT-2 remained relatively unchanged in the DSP-4/MDMA group (Fig. 3a and b) compared with the DSP-4/Saline controls, despite a large reduction in regional SERT levels in all groups given MDMA (Fig. 4a and b).

Experiment 2: MDMA produced a striking reduction in raphe SERT gene expression with a lesser effect on VMAT-2

To examine the possible role of altered gene expression in MDMA-related effects on SERT and VMAT-2 protein levels,

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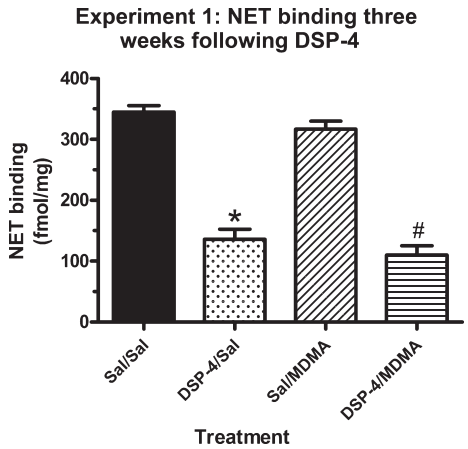

Fig. 2 Binding of [^{3}H]nisoxetine (mean ± SEM) to the norepinephrine transporter (NET) 3 weeks following DSP-4 pre-treatment in Experiment 1. DSP-4 reduced NET binding by 61% in the DSP-4/Saline group and by 65% in the DSP-4/MDMA group when compared with the respective controls (*$p < 0.001$ vs. Sal/Sal; #$p < 0.0001$ vs. Sal/MDMA).

we measured transcript levels of both markers 2 weeks following an MDMA binge. Real-time PCR methods were used to quantify SERT and VMAT-2 mRNA in combined dorsal and median raphe nuclei, both of which contain serotonergic perikarya that project extensively to the hippocampus (Piñeyro and Blier 1999). Importantly, these nuclei contain very few other monoaminergic neurons with the exception of a small population of DA-containing cells (Descarries *et al.* 1986), thus ensuring that VMAT-2 gene expression in the tissue punches would almost exclusively reflect activity in serotonergic neurons (Piñeyro and Blier 1999; Vertesa and Linley 2007). During treatment, animals given MDMA again experienced significant elevations in body temperature compared with saline-treated controls (Fig. 1b and c). Two weeks following drug administration, animals in the MDMA group exhibited a tremendous reduction (~50-fold) in SERT transcript levels with a smaller (~10-fold) but still significant reduction in VMAT-2 compared with saline-treated controls (Fig. 5).

Discussion

In this study, we examined the influence of MDMA on two different markers of 5-HT fibers and terminals to test the possibility that drug-induced serotonergic deficits may result from reduced cellular expression rather than neurodegeneration. To that end, we compared relative changes in the protein (Experiment 1) and transcript (Experiment 2) levels of SERT and VMAT-2 at 2 weeks following a binge regimen of MDMA. Given the sparse DA innervation of the hippocampus (Ciliax *et al.* 1995; Kitahama *et al.* 2007), Experiment 1 aimed to limit VMAT-2 expression in this area

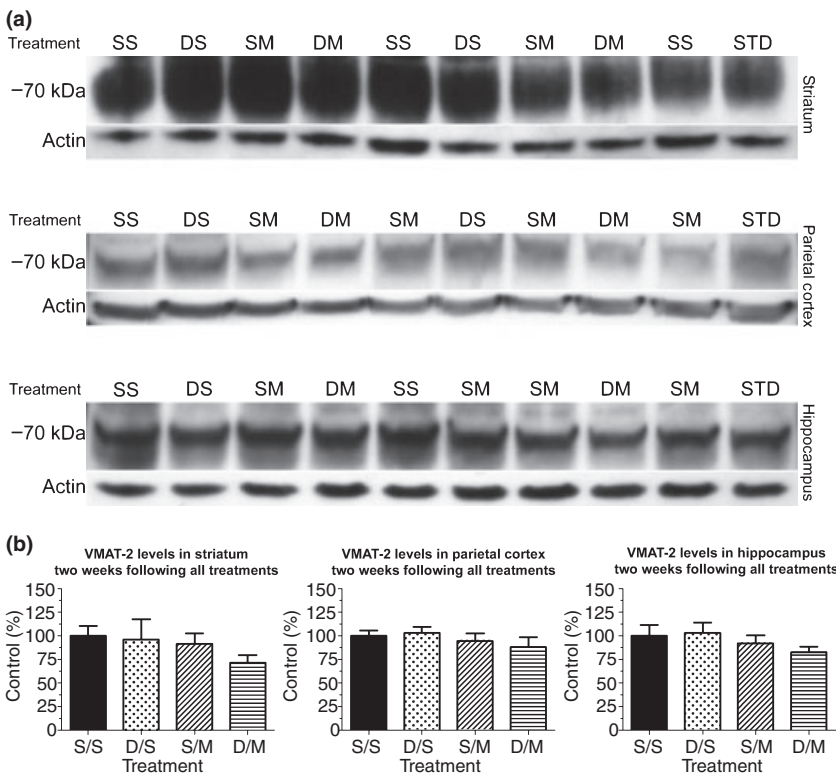

Fig. 3 Immunoblot analysis of synaptosomal VMAT-2 protein expression 2 weeks following all treatments in Experiment 1. (a) Representative VMAT-2 blots from striatum, parietal cortex, and hippocampus of animals pre-treated with DSP-4 or saline and then given MDMA or saline 1 week later. The four treatment groups are designated as follows: SS, Saline/Saline; DS, DSP-4/Saline; SM, Saline/MDMA; DM, DSP-4/MDMA. The protein band labeled 'STD' corresponds to a 'standard sample' used to normalize variability across blots in our analyses. Only the ~70 kDa protein band was used to measure VMAT-2, as initially validated by a positive control (see Fig. S1). Note the similarity of VMAT-2 expression irrespective of treatment group. (b) Quantitative comparison of VMAT-2 levels between treatment groups, expressed as mean percent SS control ± SEM. VMAT-2 expression was not significantly altered in any group in any brain region examined.

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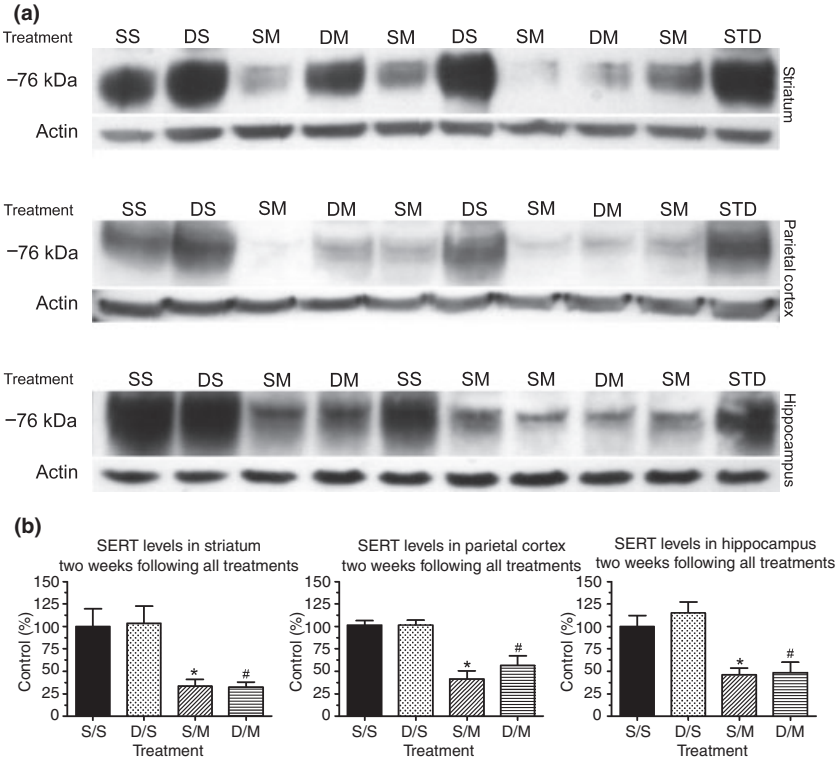

Fig. 4 Immunoblot analysis of synaptosomal SERT protein expression 2 weeks following all treatments in Experiment 1. (a) Representative SERT blots from striatum, parietal cortex, and hippocampus of animals pre-treated with DSP-4 or saline and then given MDMA or saline 1 week later. The four treatment groups are depicted as in Fig. 3. Only the ~76 kDa protein band(s) was used to measure SERT, as previously validated by several control experiments (see Fig. S1). Note the degree of SERT reduction in all MDMA-treated groups in each brain region examined. (b) Quantitative comparison of SERT levels between treatment groups, expressed as mean percent SS control ± SEM. SERT expression was significantly reduced in all MDMA-treated groups in all regions examined (striatum: $^{*}p = 0.0067$ vs. SS, $^{\#}p = 0.005$ vs. DS; parietal cortex: $^{*}p < 0.0001$ vs. SS, $^{\#}p = 0.003$ vs. DS; hippocampus: $^{*}p = 0.0019$ vs. SS, $^{\#}p = 0.027$ vs. DS).

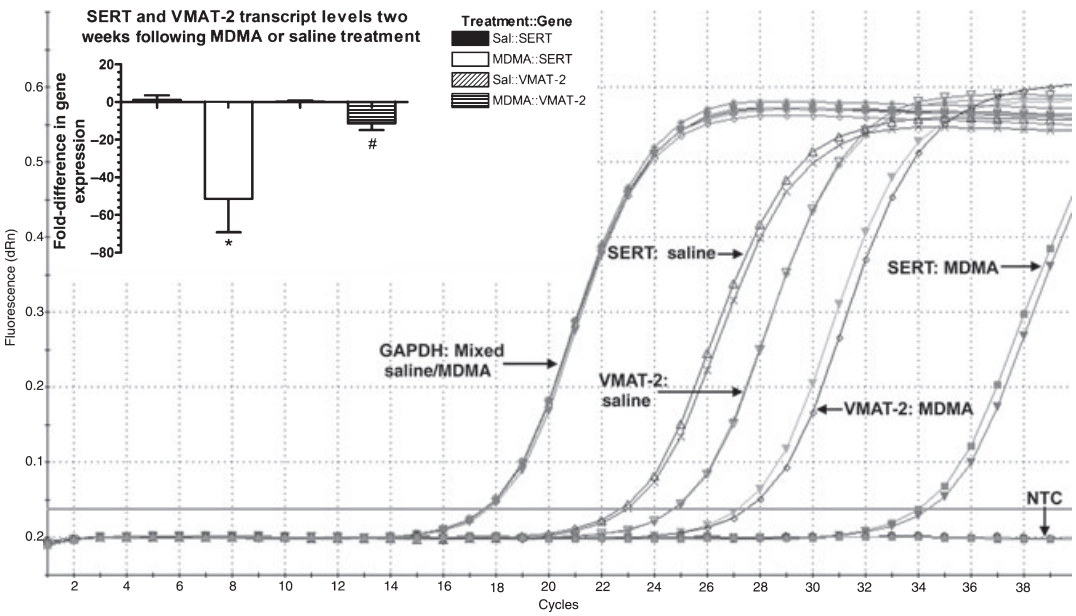

Fig. 5 Quantitative RT-PCR analysis of SERT and VMAT-2 transcript levels 2 weeks following MDMA treatment in Experiment 2. The plot shows representative amplification curves ($n = 2$ per group) for SERT and VMAT-2 cDNA as a function of treatment, as well as curves for housekeeping gene loading controls (glyceraldehyde-3-phosphate dehydrogenase; GAPDH) and no template controls (NTC). MDMA caused a rightward shift in the cycle number at which SERT and VMAT-2 cDNA was amplified beyond threshold for analysis, indicating decreased expression of both genes. (Inset) Quantitative comparison of SERT and VMAT-2 gene expression in each treatment group, expressed as fold-difference in mean expression ± SEM compared with respective controls. Groups are denoted by treatment: gene as indicated in the graph legend. MDMA exposure reduced SERT and VMAT-2 gene expression ~50- and 10-fold, respectively ($^{*}p = 0.0186$, $^{\#}p = 0.0091$ vs. respective control).

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mainly to serotonergic sources by initially lesioning NE input with the known noradrenergic neurotoxin DSP-4 (Jaim-Etcheverry and Zieher 1980). In the hippocampus of DSP-4/MDMA-treated animals, immunoblot analysis revealed only a small, non-significant effect of MDMA on VMAT-2 protein expression compared with the expression seen in DSP-4/saline controls. In contrast, levels of SERT were substantially reduced in all MDMA-treated groups, suggesting that MDMA may reduce the amount of SERT protein without inducing terminal loss. In Experiment 2, we explored the contribution of altered gene expression to MDMA-induced changes in the levels of SERT and VMAT-2 protein by measuring transcript levels of both markers in combined dorsal/median raphe punches 2 weeks following MDMA or saline administration. Gene expression of both proteins was severely reduced by MDMA treatment, again raising the possibility that this compound may regulate markers of serotonergic neurons without inducing distal axotomy.

3,4-Methylenedioxymethamphetamine is a substrate for VMAT-2 (Partilla *et al.* 2006), and administration of MDMA to rats transiently reduces vesicular DA uptake and [^{3}H]dihydrotetrabenazine binding to VMAT-2 in the striatum (where the large majority of this protein is found in DA nerve terminals) (Hansen *et al.* 2002). The DA system has also been the focus of research using VMAT-2 as a marker for nerve terminal integrity. For example, postmortem studies have revealed significant reductions in both VMAT-2 protein expression and VMAT-2-immunoreactive fiber density in the caudate and putamen of Parkinson's disease patients compared with age-matched controls (Miller *et al.* 1999). Furthermore, the extent of these depletions was shown to parallel reductions in the levels of the DA transporter in the same regions, another measure of DA-terminal integrity (Miller *et al.* 1997). Non-human primates treated with the selective nigrostriatal DA neurotoxin MPTP have shown similar decreases in both VMAT-2 and DA transporter (Miller *et al.* 1999). Interestingly, Reveron *et al.* (2005) found long-lasting reductions in striatal VMAT-2 protein expression in 10-week-old mice given an MDMA binge, which is consistent with the well-known DA neurotoxicity of MDMA in mice compared with rats. Ricaurte *et al.* (2000) also reported 35–40% decreases in [^{3}H]dihydrotetrabenazine binding in the caudate and putamen 2 weeks following repeated MDMA administration to baboons, and even larger effects were observed in the frontal cortex. As non-human primates, like rats, display mainly serotonergic neurotoxicity to MDMA, the authors interpreted their results as a sign of 5-HT terminal loss in those brain areas. On the other hand, there is reason to question the baboon findings on the basis that even a total serotonergic denervation of the caudate and putamen seems unlikely to produce 35–40% reductions in VMAT-2 because of the much greater dopaminergic compared with serotonergic innervation of those structures. More importantly, the present results showed little change in VMAT-2 protein expression under conditions that attempted to maximize the serotonergic contributions to the overall VMAT-2 synaptosomal content (particularly in the hippocampus). We considered the possibility that hippocampal VMAT-2 in the DSP-4/MDMA group might be relatively preserved even in the face of a partial axotomy if the serotonergic neurons compensatorily up-regulated VMAT-2 gene expression and vesicle recycling to enhance 5-HT sequestration in the remaining terminals. Such a scenario could potentially have normalized the VMAT-2 signal in our immunoblots. However, the opposite finding, namely a significant decrease in VMAT-2 gene expression, argues against this interpretation.

Several studies have recently investigated the effects of MDMA administration on SERT protein expression in various forebrain regions as determined by immunoblotting. The results have been mixed, with two studies finding reduced SERT expression (Bhide *et al.* 2009; Xie *et al.* 2006) but others reporting no effect (de Silva *et al.* 2005; Wang *et al.* 2004, 2005). Given the ample evidence for MDMA-induced SERT reductions measured either by radioligand binding (reviewed by Green *et al.* 2003) or immunohistochemistry (Xie *et al.* 2006), we suggest that the above mentioned negative results are most likely because of a lack of adequate validation of the antibodies used in the immunoblotting procedures (see Supplementary Table S1). Importantly, the decrease in synaptosomal SERT immunoreactivity found in this study was accompanied by only a minor decrease in VMAT-2 protein levels, suggesting that MDMA-induced reductions in SERT may be related to a down-regulation phenomenon and not necessarily dependent on neurodegeneration.

Additional evidence against a neurodegenerative effect of MDMA is supported by research showing a failure of MDMA to provoke glial responses thought to occur in reaction to neural damage. Importantly, several studies (O'Callaghan and Miller 1993; Pubill *et al.* 2003; Wang *et al.* 2004, 2005) found no MDMA-induced increases in glial fibrillary acidic protein (GFAP), an astroglial marker that is elevated following treatment with METH, MPTP, 5,7-DHT, and other recognized neurotoxins (O'Callaghan and Miller 1993). Another measure of astrogliosis, the expression of heat-shock protein 27, followed the same trend. Finally, MDMA similarly failed to produce evidence for microglial activation as measured by OX-6 immunoreactivity or [^{3}H]PK-11195 binding to the peripheral benzodiazepine receptor (Pubill *et al.* 2003). It should be noted, however, that at least two studies have documented increases in GFAP expression in the hippocampus (but not other brain regions) following MDMA treatment, suggesting that this region may be particularly vulnerable to MDMA-induced neural damage (Aguirre *et al.* 1999; Adori *et al.* 2006). Another study showed post-MDMA increases in GFAP immunoreactivity in the hippocampus, cortex, and cerebellum in response to a

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very high dose (40 mg/kg) of the drug administered i.p. (Sharma and Ali 2008). Given these latter findings, it would be of interest for future studies to investigate whether MDMA affects glial responses in the context of our present effort as an absence of MDMA-induced gliosis would further support our contention that this compound reduces SERT expression in the absence of terminal loss. On the other hand, if such responses were indeed evidenced, it would be critical to assess the neurochemical phenotype of the affected terminals as this would clarify whether glial reactivity following MDMA was occurring in response to serotonergic or non-serotonergic damage.

Despite much focus on the neurochemical effects of MDMA on serotonergic neurons, little research exists investigating changes in the gene expression of 5-HT neuronal markers following exposure to this compound. Regarding the effects of MDMA on SERT gene expression, *in situ* hybridization studies found decreases in SERT gene expression 2 weeks (Kirilly *et al.* 2008) and up to several months following MDMA (Li *et al.* 2006). This study is the first to quantify MDMA-related changes in midbrain raphe SERT gene expression by qRT-PCR, an approach that permits more precise quantification than *in situ* hybridization. It is noteworthy that despite an average 50-fold reduction in SERT gene expression 2 weeks after an MDMA binge, similarly treated animals exhibited only a 50% reduction in synaptosomal SERT protein levels. This apparent disconnect has previously been documented for this protein (Rattray *et al.* 1996), and may reflect post-treatment increases in SERT mRNA translation efficiency, alterations in post-translational modification of SERT, or inherently slow turnover rates of the SERT protein. Likewise, these possibilities may also in part explain our finding of only marginal decreases in VMAT-2 protein levels despite a 10-fold reduction in VMAT-2 gene expression in MDMA-treated animals. Future studies should aim to determine the effects of MDMA on the molecular machinery governing production of proteins used to identify monoaminergic and other neural subtypes.

Importantly, our interpretation of the results of Experiment 1 relies, in part, on the success of DSP-4 lesioning of hippocampal NE fibers. As documented by Jaim-Etcheverry and Zieher (1980) and Fritschy and Grzanna (1992), DSP-4 is an alkylating agent that targets NET as well as structures within NE terminals, presumably causing a pruning of locus coeruleus afferents throughout the brain that peaks at 2–4 weeks following administration. Interestingly, the notion that DSP-4 induces neurodegeneration of NE fibers is based on indirect measures similar to those typically used to assess MDMA neurotoxicity. Such measures include severe depletions in NE, inhibition of radiolabeled NE uptake *in vivo*, and most convincingly, long-lasting reductions in DA β-hydroxylase immunoreactivity (Landa *et al.* 1984; Fritschy *et al.* 1990). Using NET as an alternative marker for NE axons and terminals, we achieved a significant reduction in hippocampal levels of this protein following DSP-4 pre-treatment, implying success in lesioning NE input to this region. However, as is the case with SERT and DA β-hydroxylase, NET is yet another marker liable to regulation, bringing into question the validity of considering reductions in these proteins as evidence for neurodegeneration (see Booze *et al.* 1988). Notably, we found no change in regional VMAT-2 expression in the DSP-4/saline group, despite the fact that this protein is abundant in noradrenergic fibers. Given the presence of significant numbers of DA and 5-HT nerve terminals in the striatum and parietal cortex, respectively (Reader *et al.* 1989; Kabani *et al.* 1990), the relative contribution of VMAT-2 from noradrenergic sources in those regions may have been insufficient to allow detection of loss following DSP-4 treatment. However, this explanation is less clear for the hippocampus, as the number of noradrenergic and serotonergic varicosities bearing VMAT-2 may be similar (Oleskevich *et al.* 1989; Oleskevich and Descarries1990) (though this has never been directly examined). Consequently, we cannot exclude the possibility that a failure of DSP-4 to produce noradrenergic terminal degeneration in the hippocampus contributed to our inability to find an effect of MDMA on VMAT-2 protein expression. However, even if DSP-4 failed to lesion NE fibers in the hippocampus, it is important to point out that VMAT-2 levels were almost completely unchanged in the Sal/MDMA group, where modest reductions (∼30% assuming equal 5-HT : NE contribution to overall VMAT-2 content) would have been expected if serotonergic terminals were damaged by an order of magnitude relative to the extent of SERT depletions in this region (60%). In further support of this argument, we have recently found a lack of effect of MDMA on TPH protein expression (another marker of 5-HT terminals) in synaptosomes when measured by validated immunoblotting (manuscript in preparation). Together, these findings reinforce the notion that the majority of serotonergic nerve terminals may indeed be spared following MDMA exposure, though additional experiments are necessary to confirm our findings by use of more direct measures not dependent on marker staining. Such experiments should investigate ways to directly highlight and subsequently measure changes in 5-HT fiber density in response to MDMA, such as through the use of anterograde or retrograde tracers, or by using reporter genes (e.g. green fluorescent protein) driven by 5-HT neuron-specific promoters. Lastly, it bears mention that DSP-4 is a commonly used tool in neuropharmacology research, and thus future studies should more carefully address the neurotoxic capacity of this compound. This will enable greater confidence in use of DSP-4 to lesion NE fibers in other studies, as well as potentially clarify the nature of MDMA effects on serotonergic neurons in the context of the present findings.

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Despite many years of intensive research, the exact nature of MDMA neurotoxicity remains controversial. Substantial evidence has been offered both for (Molliver *et al.* 1990; Ricaurte *et al.* 2000; Green *et al.* 2003) and against (O'Callaghan and Miller 1993; Pubill *et al.* 2003; Baumann *et al.* 2007; Wang *et al.* 2007) the neurodegeneration hypothesis. Wang *et al.* (2007) have proposed three possible models of MDMA neurotoxicity: (i) neurodegeneration, (ii) neuroadaptation, and (iii) a mixed model involving both kinds of changes (i.e. significant loss of serotonergic nerve terminals along with adaptive changes in the functioning of the remaining terminals). The present results demonstrating a profound down-regulation of SERT gene expression accompanied by a significant reduction in expression of the VMAT-2 gene certainly confirms that the serotonergic neurons of the dorsal and median raphe nuclei undergo major adaptive changes following a high-dose MDMA treatment regimen. Moreover, even with the caveats expressed earlier, the lack of a significant decrease in synaptosomal VMAT-2 protein expression suggests at the very least that MDMA does not produce a massive loss of serotonergic nerve terminals. Therefore, our findings are consistent either with the neuroadaptation or mixed model of Wang *et al.* (2007), as we cannot exclude the possibility of a partial degenerative response in the MDMA-treated animals.

Finally, a general implication of our findings is that drug-induced depletions in protein markers liable to regulation may not necessarily reflect neurodegeneration. Other factors, such as changes in gene expression of these proteins, must be addressed to determine the nature of adverse effects of any purported neurotoxin.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Table S1 Panel of anti-SERT antibodies used in validating the immunoblotting procedure for measuring SERT protein expression

Fig. S1 Immunoblot validation results for identification of authentic SERT and VMAT-2 protein bands.

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