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RESTORATION OF 3,4-METHYLENEDIOXYMETHAMPHETAMINE-INDUCED 5-HT DEPLETION BY THE ADMINISTRATION OF L-5-HYDROXYTRYPTOPHAN

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Abstract—Background: 3,4-Methylenedioxymethamphetamine (MDMA) causes persistent decreases in brain 5-HT content and 5-HT transporter (SERT) binding, with no detectable changes in SERT protein. Such data suggest that MDMA impairs 5-HT transmission but leaves 5-HT nerve terminals intact. To further test this hypothesis, we carried out two types of experiments in rats exposed to high-dose MDMA. First, we examined the effects of MDMA on SERT binding and function using different *in vitro* assay conditions. Next, we treated rats with the 5-HT precursor, L-5-hydroxytryptophan (5-HTP), in an attempt to restore MDMA-induced depletions of 5-HT.

Methods: Rats received three i.p. injections of saline or MDMA (7.5 mg/kg), one injection every 2 h. Rats in one group were decapitated, and brain tissue was assayed for SERT binding and [^{3}H]5-HT uptake under conditions of normal (100 or 126 mM) and low (20 mM) NaCl concentration. Rats from another group received saline or 5-hydroxytryptophan/benserazide (5-HTP-B), each drug at 50 mg/kg i.p., and were killed 2 h later.

Results: MDMA reduced SERT binding to 10% of control when assayed in 100 mM NaCl, but this reduction was only 55% of control in 20 mM NaCl. MDMA decreased immunoreactive 5-HT in caudate and hippocampus to about 35% of control. Administration of 5-HTP-B to MDMA-pretreated rats significantly increased the 5-HT signal toward normal levels in caudate (85% of control) and hippocampus (66% of control).

Conclusion: 1) Following high-dose MDMA treatment sufficient to reduce SERT binding by 90%, a significant number of functionally intact 5-HT nerve terminals survive. 2) The degree of MDMA-induced decreases in SERT binding depends on the *in vitro* assay conditions. 3) 5-HTP-B restores brain 5-HT depleted by MDMA, suggesting that this approach might be clinically useful in abstinent MDMA users. © 2007 Published by Elsevier Ltd on behalf of IBRO.

Key words: amphetamine, ecstasy, 5-HT transporter, RTI-55, binding.

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Abbreviations: DA, dopamine; HPLC-ECD, high pressure liquid chromatography with electrochemical detection; MDMA, (±)-3,4-methylenedioxymethamphetamine; PBS, phosphate-buffered saline; SERT, 5-HT transporter; TP-2-64, 4-(2-benzhydryloxy-ethyl)-1-(4-nitro-benzyl)piperidine oxalate; $VMAT_2$, vesicular monoamine transporter 2; 5-HTP, L-5-hydroxytryptophan; 5-HTP-B, L-5-hydroxytryptophan plus benserazide; 5, 7-DHT, 5,7-dihydroxytryptamine; [^{125}I]RTI-55, 3β-(4′-^{125}iodophenyl)tropan-2β-carboxylic acid methyl ester.

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(±)-3,4-Methylenedioxymethamphetamine (MDMA, "ecstasy") is a commonly abused illicit drug (Banken, 2004). Numerous studies in rodents and nonhuman primates show that MDMA administration produces long-lasting decreases in markers of 5-HT nerve terminal integrity (for review see (Sprague et al., 1998; Green et al., 2003; Lyles and Cadet, 2003)). The MDMA-induced changes in 5-HT markers include depletion of tissue 5-HT, reduction in 5-HT transporter (SERT) binding and function (Commins et al., 1987; Schmidt, 1987), and loss of tryptophan hydroxylase activity. Immunohistochemical analysis of 5-HT shows an apparent loss of 5-HT nerve terminals throughout the forebrain. The spectrum of decrements produced by MDMA administration is typically described as 5-HT neurotoxicity or axotomy.

Recently, we reported that neurotoxic doses of MDMA which reduce brain 5-HT concentrations by about 50% do not detectably alter SERT protein expression as measured by Western blot analysis (Wang et al., 2004, 2005). In contrast, we observed that administration of the neurotoxin, 5,7-dihydroxytryptamine (5,7-DHT) decreases brain 5-HT levels and SERT protein expression in parallel. Although our findings with regard to SERT protein have been challenged (Xie et al., 2006), the results suggest that functional serotonergic nerve terminals may remain after MDMA treatment. As recently reviewed (Baumann et al., 2007), the persistence of functional 5-HT nerve terminals after MDMA or 5,7-DHT treatments is supported by the fact that extracellular 5-HT concentrations remain unchanged following severe depletion of tissue 5-HT (Kirby et al., 1995; Shankaran and Gudelsky, 1999).

The goal of the present study was to further examine the possibility that functional 5-HT nerve terminals remain after administration of MDMA. In one series of experiments we determined the magnitude of MDMA-induced decreases in SERT binding and [^{3}H]5-HT uptake under different *in vitro* assay conditions. Interestingly, MDMA treatment decreased SERT Bmax to 10% of control when assays were conducted in normal NaCl, but this decease was only to 55% of control when assay were conducted in low NaCl conditions. In the second series of experiments, rats were pretreated with 5-HT-depleting doses of MDMA or vehicle, and 2 weeks later they received saline or L-5-hydroxytryptophan plus benserazide (5-HTP-B). Serotonergic innervation was assessed using an immunoautoradiography method designed to detect brain 5-HT. We hypothesized that functional 5-HT nerve terminals would demonstrate an increased 5-HT signal when exposed to the precursor 5-HTP. As predicted, administration of 5-HTP-B to MDMA-pretreated rats significantly increased the 5-HT signal in caudate and hippocampus.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague–Dawley rats weighing 280–320 g were singly housed (lights on: 07:00–19:00 h) with food and water freely available. Rats were maintained in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and procedures were carried out in accordance with the Animal Care and Use Committee of the National Institute on Drug Abuse Intramural Research Program. All experiments conformed to international guidelines on the ethical use of animals. All efforts were made to minimize the number of animals used and their suffering.

Chemicals

[^{3}H]5-HT (specific activity=23.7 Ci/mmol) was purchased from Perkin-Elmer (Shelton, CT, USA). Dr. Tom Prizinsano kindly provided the selective dopamine (DA) uptake inhibitor, TP-2-64 (4-(2-benzhydryloxy-ethyl)-1-(4-nitro-benzyl)piperidine oxalate), synthesized as described (Greiner et al., 2003). The sources of other chemicals are published (Rothman et al., 1998).

Drug treatments

To determine the effects of MDMA treatment on SERT binding and function, rats received three i.p. injections of MDMA (7.5 mg/kg) or saline, one injection every 2 h. Rats were decapitated 2 weeks later, and whole brains without cerebellum and caudates were used immediately for the [^{3}H]5-HT uptake assays, or stored at −80 °C until SERT binding assays were carried out using 3β-(4′-^{125}iodophenyl)tropan-2β-carboxylic acid methyl ester ([^{125}I]RTI-55) as the radiolabeled ligand. As expected, the acute administration of MDMA produced elements of the 5-HT syndrome. Two weeks after MDMA administration, the rats demonstrated no gross behavioral abnormalities (Baumann et al., 2007).

For the 5-HTP-B experiments, rats received i.p. injections of MDMA or saline as above. A separate group of rats received single i.c.v. injections of 5,7-DHT (150 μg in 10 μl) or its ascorbate vehicle into the lateral ventricle; rats receiving i.c.v. injections were pretreated with nomifensine (15 mg/kg, i.p.) to block the uptake of 5,7-DHT into catecholaminergic nerves. Two weeks later, rats from all groups were treated with i.p. 5-HTP-B (50 mg/kg of each) then perfused for immunoautoradiography assay 2 h later, as described below. All rats receiving L-5-hydroxytryptophan (5-HTP) received the aromatic-L-amino-acid decarboxylase inhibitor, benserazide (B) 30 min beforehand to prevent the peripheral conversion of 5-HTP to 5-HT. Control rats received i.p. injections of saline (1 ml/kg) or i.c.v. injections of 10 μl 0.1% ascorbate in saline.

For reserpine dose-response experiments, rats received single i.p. injections of saline or reserpine (0.1, 0.4, 1.6 and 6.4 mg/kg). Rats were killed 24 h later, brain regions were dissected out (caudate, hippocampus, and prefrontal cortex), and tissue was stored at −80 °C until the day of monoamine assay. Monoamines were measured by high pressure liquid chromatography with electrochemical detection (HPLC-ECD) as described (Baumann et al., 1998). For reserpine/5-HTP-B experiments, rats were treated with either i.p. saline or 6.4 mg/kg reserpine. Twenty-four hours later, rats received i.p. saline or 5-HTP-B and were perfused 2 h later as described below for the MDMA experiments for visualization of 5-HT by immunoautoradiography.

SERT binding assays

Frozen rat brains (minus caudates) were each thawed in 20 ml of ice-cold binding buffer (BB: 55.2 mM sodium phosphate buffer, pH 7.4) for 15 min, and then homogenized with a Brinkman Polytron (setting 5 for 20 s). The homogenates were centrifuged twice at 20,000×g for 15 min, with resuspensions in equal volumes of ice-cold BB, and finally resuspended in 10 ml/brain of ice cold BB. The membranes were pooled, mixed and separated into 1 ml aliquots in polypropylene microfuge tubes. Each aliquot was centrifuged in a TOMY refrigerated microfuge (model MTX 150) at 15,000 r.p.m. for 5 min; the supernatant was discarded. The aliquots were stored at −80 °C until needed. Two different buffers were used, a “normal” sodium buffer (10 mM Hepes, pH 7.4 plus 100 mM NaCl) and a “low” sodium buffer (10 mM Hepes, pH 7.4, 20 mM NaCl, 80 mM choline chloride). On the day of an experiment, aliquots of membrane protein were diluted in 150 ml of normal sodium buffer or in 60 ml of low sodium buffer.

Transporter binding assays were carried out in 12×75 mm polystyrene tubes that were pre-filled with 100 μl of drug, 100 μl of radioligand, and 50 μl of a blocker or buffer (Rothman et al., 1998). For these SERT assays, we used 100 nM TP-2-64 to block the binding of [^{125}I]RTI-55 to DA transporters. [^{125}I]RTI-55 was made in a protease inhibitor cocktail (BB with 25 μg/ml chymostatin, 25 μg/ml leupeptin, 1 mM EGTA, and 1 mM EDTA). The drugs and blockers were made up in buffer containing 1 mg/ml bovine serum albumin (BSA) at pH 7.4. The experiment was initiated by the addition of 750 μl of membranes in appropriate buffer. Samples were incubated in a final volume of 1 ml at 4 °C for 18–24 h (steady state). After incubation, the samples were filtered with a Brandel cell harvester over Whatman GF/B filters presoaked in wash buffer (ice-cold 10 mM Tris/HCl, 150 mM NaCl, pH 7.4) containing 2% poly(ethyleneimine). Typical total and nonspecific cpms observed for the assay were 4000 cpm (total binding) and 80 cpm (nonspecific). Non-specific binding was determined using 1 μM indatraline, a nonselective monoamine transporter blocker.

[^{3}H]5-HT uptake assays

These assays were conducted as described elsewhere (Rothman et al., 2001). Freshly removed whole brain minus caudate was homogenized in 10% ice-cold sucrose with 12 strokes of a Potter-Elvehjem homogenizer, followed by centrifugation at 1000×g for

Table 1. Effect of MDMA treatment on SERT binding and function in rat brain

A. SERT binding with [^{125}I]RTI-55

Condition	Bmax (fmol/mg protein)	Kd (nM)
Control/high sodium	174±3	0.29±0.01
Control/low sodium	240±6†	2.9±0.1†
MDMA/high sodium	18±3.0*	0.30±0.05
MDMA/low sodium	132±6*†	3.0±0.1†

B. [^{3}H]5-HT uptake

Condition	Vmax (fmol/mg protein/30 min)	Km (nM)
Control/high sodium	15.2±1.1	32±2
Control/low sodium	4.2±0.7†	15±1†
MDMA/high sodium	11±0.7*	31±2
MDMA/low sodium	1.6±0.1*†	8.7±0.7*†

SERT binding and [^{3}H]5-HT uptake assays were conducted as described in Experimental Procedures under two different *in vitro* conditions: High NaCl (100 mM for binding or 126 mM for uptake) and low NaCl (20 mM). The high- and low-sodium assays were conducted in parallel and with the same tissue. The data sets derived from six rats (108 data points) were pooled and fit to the one site binding model for the best fit estimates of the binding parameters (±S.D.) reported above.

* $P<0.05$ when compared to the corresponding control.

† $P<0.05$ when compared to the corresponding high-sodium condition.

10 min. The supernatants were saved on ice and used immediately. For the "normal sodium" condition, we used a Krebs–phosphate buffer containing a final concentration of 126 mM NaCl, 2.9 mM KCl, 1.1 mM $CaCl_2$, 0.83 mM $MgCl_2$, 5 mM glucose, 1 mg/ml ascorbic acid, and 50 μM pargyline. For the "low sodium" assays, the concentration of NaCl was reduced to 20 mM and replaced by 106 mM choline chloride. Nonspecific uptake was measured by incubating in the presence 1 μM indatraline. The reactions were stopped after 30 min by filtering with a Brandel cell harvester over Whatman GF/B filters presoaked in wash buffer (10 mM Tris/HCl, pH 7.0). Retained tritium was measured with a Trilux liquid scintillation counter after overnight extraction in 0.6 ml of liquid scintillation cocktail (MP Biomedicals, Irvine, CA, USA). Typical total and nonspecific cpms observed for the [^{3}H]5-HT uptake assays were 1600 and 400, respectively.

In vitro data analysis

For SERT binding surface experiments (Rothman, 1986; Rothman et al., 1991) two different concentrations of [^{125}I]RTI-55 were each displaced by 8 to 10 concentrations of unlabeled RTI-55 (0.02 nM–11 nM for the normal sodium experiments, 0.2 nM–50 nM for the low sodium experiments). Similarly, surfaces for [^{3}H]5-HT uptake were generated by displacing two concentrations of [^{3}H]5-HT (2 nM and 22 nM) by nine concentrations of unlabeled 5-HT (2 nM–766 nM). The higher concentrations of [^{125}I]RTI-55 or [^{3}H]5-HT were obtained by adding RTI-55 or 5-HT to the radioligand. Binding surfaces were fit to a one-site binding model using MLAB-PC as described elsewhere (Rothman et al., 1991). Statistical difference between binding parameters was determined using the *F* test as described in detail elsewhere (Rothman, 1986; Rothman et al., 1991).

Immunoautoradiography

Animals were deeply anesthetized with 75 mg/kg i.p. pentobarbital, and 4% paraformaldehyde was perfused through the ascending aorta. Brains were removed and post-fixed in 4% paraformaldehyde for 2 h. Each brain was frozen and kept at −80 °C until sectioning. Sections (40 μm) were cut at −20 °C using a cryostat. After pre-incubation for 2 h in 0.1 M phosphate-buffered saline (PBS) solution containing 4% bovine serum albumin and 1% goat normal serum, sections were incubated overnight in the primary antibody diluted 1:50 for anti-5-HT or anti-rabbit IgG. Sections were subsequently rinsed in PBS and incubated in anti-rabbit [^{35}S]IgG solution at a concentration of 1:100 for 2 h. Sections were then rinsed in PBS and mounted to slides. After air-drying, sections were exposed to the super resolution Phospho screen that was scanned in the Packard Cyclone storage Phospho System. The images were digitized, and quantified using densimetric analysis (NIH IMAGE software). The "nonspecific" binding observed in the IgG control sections was subtracted from the corresponding gray value of each section. Changes were expressed relative to their corresponding control (defined as 100% value). Statistical difference between groups was determined using the Student's *t*-test.

RESULTS

The first set of experiments determined the effect of MDMA administration on SERT binding (Bmax and Kd) and [^{3}H]5-HT uptake (Vmax and K_m) under two different *in vitro* assay conditions: normal and low NaCl. We decided to explore the effect of low NaCl in our assay system based on reports that SERT function in non-lipid rafts is not sensitive to sodium

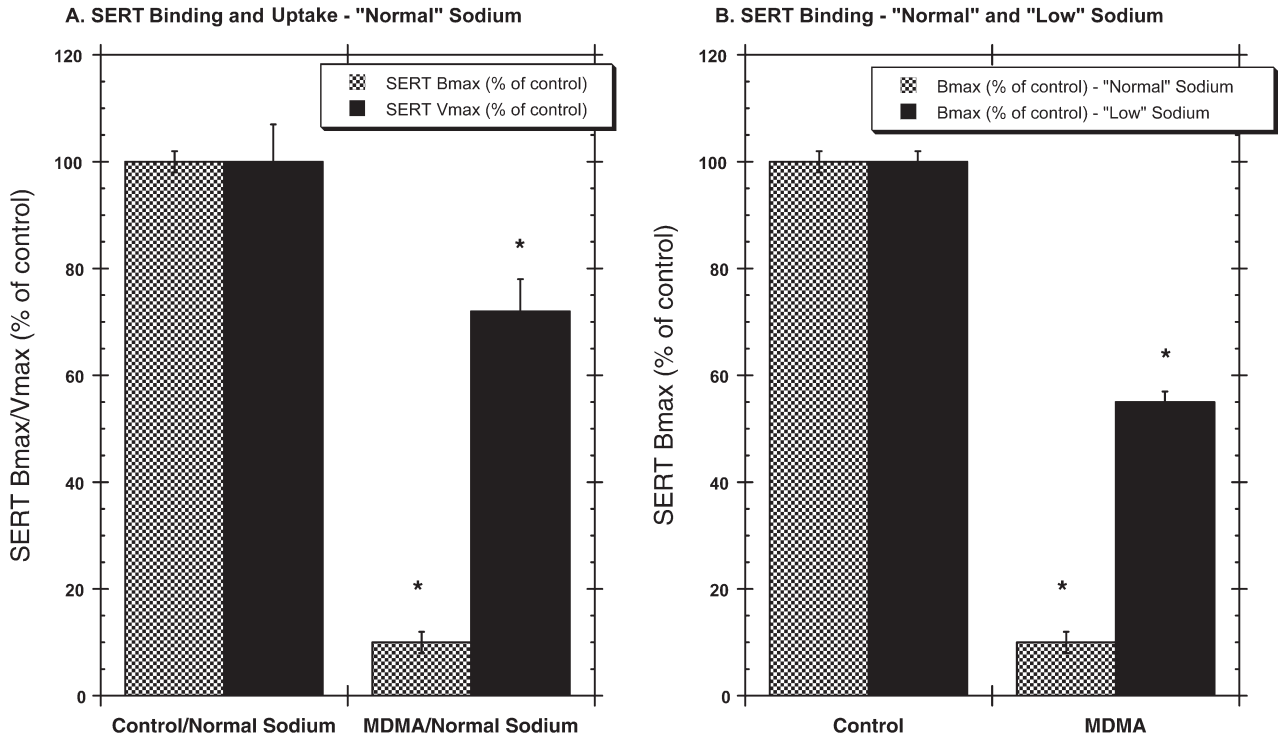


Fig. 1. Effect of MDMA pretreatment on SERT binding and [^{3}H]5-HT uptake. SERT binding and [^{3}H]5-HT uptake assays were conducted as described in Experimental Procedures under two different *in vitro* conditions: normal NaCl (100 mM for binding or 126 mM for uptake) and low NaCl (20 mM). The "normal" and "low" sodium assays were conducted in parallel and with the same tissue. The data sets derived from six rats per group were pooled (108 data points) and fit to the one site binding model for the best-fit estimates of the binding parameters (±S.D.) reported in Table 1. The data are converted to percent of control in this figure. * $P<0.01$ when compared with control (Student's *t*-test).

(Magnani et al., 2004). The results are reported in Table 1. As shown in Fig. 1A for the normal sodium assays, MDMA treatment reduced the SERT binding Bmax to 10% of control, but reduced the Vmax for [^{3}H]5-HT uptake to only 72% of control. MDMA treatment did not significantly alter the Kd for SERT binding (~0.3 nM) or the K_m for [^{3}H]5-HT uptake (~30 nM) (Table 1). Interestingly, under low sodium assay conditions, MDMA treatment reduced the SERT Bmax to 55% of control instead of the 10% of control observed under normal sodium conditions (Fig. 1B). In control preparations, lowering the sodium concentration increased the Kd of [^{125}I]RTI-55 for SERT 10-fold and increased the Bmax 1.4-fold. In the [^{3}H]5-HT uptake assays, lowering the sodium concentration decreased both the Vmax and K_m. These findings are generally in accord with earlier studies (Mann and Hrdina, 1992).

In order to validate our 5-HT immunoautoradiography method, reserpine treatment was used to produce a short-lived reduction in brain 5-HT. The HPLC-ECD data in Fig. 2 show that reserpine produced a dose-dependent reduction in 5-HT content in the prefrontal cortex 24 h later, such that a dose of 6.4 mg/kg reduced the 5-HT level to ~5% of control. Similar results were obtained for the caudate and hippocampus (data not shown). Using the immunoautoradiography method, reserpine administration (6.4 mg/kg) decreased 5-HT to 10% of control in all brain regions as depicted in Fig. 3. These data demonstrate that our 5-HT immunoautoradiography method can detect a clear loss of 5-HT signal after reserpine. Administration of 5-HTP-B did not increase 5-HT in any brain region of control rats. However, 5-HTP-B administration slightly increased 5-HT signal in the caudate and amygdala of reserpine-pretreated rats.

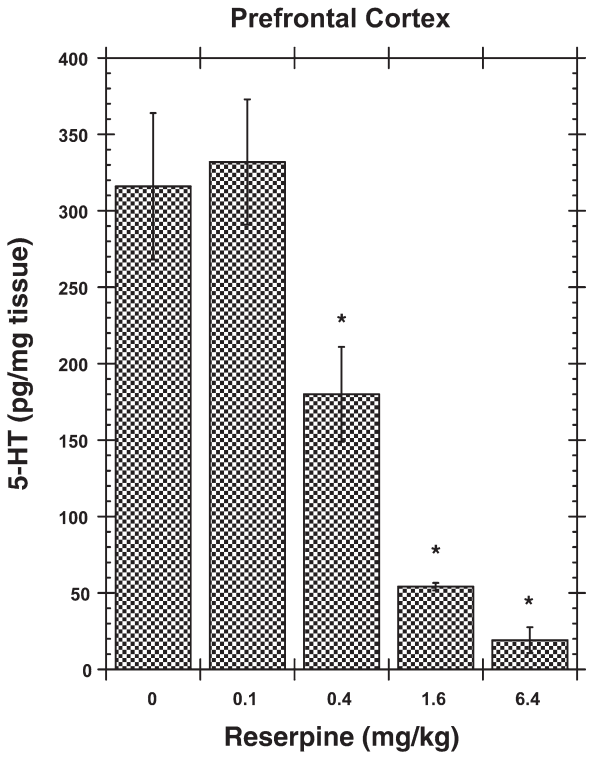


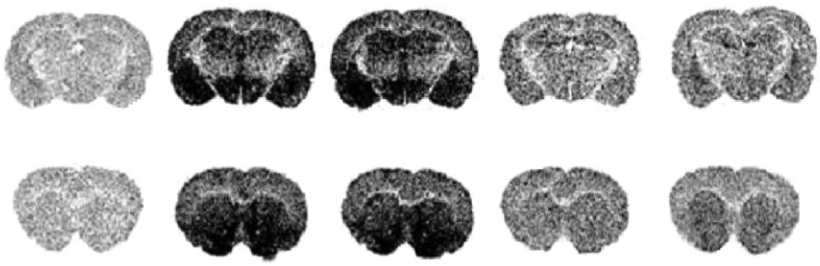
Fig. 2. Reserpine decreases brain 5-HT. Rats received the indicated doses of reserpine and were decapitated 24 h later. 5-HT levels were determined by HPLC-ECD in the frontal cortex as described in Experimental Procedures. Each value is the mean±S.D. (n=3). * P<0.01 when compared with control (Student's t-test).

Administration of MDMA decreased immunoreactive 5-HT to 25–35% of control in the five brain regions sampled (Fig. 4). Similar reductions in brain tissue 5-HT were observed using HPLC-ECD in our previous study (Wang et al., 2004). Administration of 5-HTP-B to MDMA-treated rats increased the immunoreactive 5-HT signal in brain. In the caudate, hypothalamus and amygdala, 5-HTP-B increased 5-HT almost to control levels. Treating rats with 5,7-DHT reduced brain 5-HT content to 25–40% of control (Fig. 5). Administration of 5-HTP-B to 5,7-DHT-treated rats significantly increased brain 5-HT to control levels.

DISCUSSION

The neurotoxic effects of MDMA are often demonstrated by measuring endpoints thought to be selective for the 5-HT nerve terminal: tissue 5-HT and SERT levels. The density of SERT is typically measured using radioligand binding methods, and less commonly by measuring [^{3}H]5-HT uptake into synaptosomes (Green et al., 2003) or SERT protein levels via Western blot. Few studies have concurrently measured the effect of MDMA on SERT binding and [^{3}H]5-HT uptake (Lew et al., 1996; Sabol et al., 1996), and none to our knowledge have measured SERT binding and [^{3}H]5-HT uptake concurrently under different *in vitro* assay conditions. One striking finding to emerge from our *in vitro* data is the quantitative differences in the effect of MDMA treatment on various serotonergic indices determined 2 weeks later. MDMA treatment decreased the SERT binding Bmax by ~90% ("normal" sodium assay conditions, Fig. 1A), decreased tissue 5-HT content by ~65% and decreased the Vmax for [^{3}H]5-HT uptake by 28%. Another conspicuous finding was that a simple change to low sodium buffer altered the effect of MDMA treatment on the SERT Bmax to a 45% reduction. These data illustrate the inherent difficulty in using these particular endpoints to assess the extent of MDMA-induced toxic effects, since the magnitude of change differs among them and can be manipulated by subtle alterations in assay conditions. The finding that a neurotoxic dose of MDMA does not alter *in vivo* 5-HT clearance in rat hippocampus 2 weeks after drug administration supports the idea that functional SERT sites are available despite tissue 5-HT depletion (Callaghan et al., 2007).

Another way to probe the integrity of 5-HT terminals is to determine their ability to synthesize 5-HT from a precursor, such as 5-HTP. Biogenic amine nerve terminals express the enzyme aromatic-L-amino-acid decarboxylase (EC 4.1.1.28). In serotonergic nerve terminals, this enzyme converts 5-HTP to 5-HT but is not rate-limiting. Administration of 5-HTP increases immunoreactive 5-HT in the raphe nuclei and substantia nigra pars compacta, but not the locus coeruleus, indicating that 5-HTP can produce ectopic 5-HT synthesis in dopaminergic nerves (Lynn-



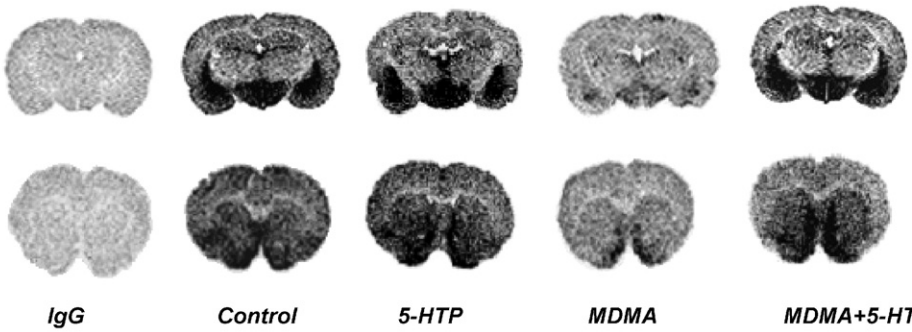
Brain region	Control	5-HTP	Reserpine	Reserpine+5-HTP
Cortex	100±9.6 %	100.8±10.1 %	10.1±0.98*%	9.9±1.0 %
Hippocampus	100±8.9 %	99.6±8.8 %	9.6±0.97* %	9.7±1.0 %
Caudate	100±10.1%	100.1±9.6 %	8.7±0.81* %	27.5±4.3† %
Hypothalamus	100±9.9 %	100.2±9.7 %	9.1±0.93* %	9.3±0.92%
Amygdala	100±9.7 %	99.4±8.9 %	9.9±0.83* %	19.1±3.2%

Fig. 3. Effect of reserpine on brain 5-HT as measured by immunoautoradiography. Rats were administered 6.4 mg/kg reserpine or saline and 24 h later received either saline or 5-HTP-B injections as described in Experimental Procedures. 5-HT levels were measured using immunoautoradiography as described in Experimental Procedures. * $P<0.01$ when compared with control (Student's *t*-test). † $P<0.01$ when compared with the reserpine group. Each value is the mean±S.D. ($n=14$ slices, 6 rats). Representative autoradiograms from each group are shown.

Bullock et al., 2004). In a recent paper, we showed that administration of 5-HTP-B to rats, at the dose used here (50 mg/kg i.p. of each drug), produced ~20-fold elevations of extracellular 5-HT in the n. accumbens as measured by *in vivo* microdialysis (Halladay et al., 2006). A similar dose of 5-HTP-B also increased postmortem striatal 5-HT content eightfold as measured by HPLC-ECD. It is of interest, therefore, that 5-HTP-B administration did not increase 5-HT in control brains when measured by the immunoautoradiography method (Fig. 3). One likely explanation for this observation is that each assay method detects different pools of brain 5-HT. *In vivo* microdialysis detects extracellular 5-HT which would be washed away by the methods used to prepare tissue sections for immunoautoradiography. HPLC analysis of postmortem tissue 5-HT detects the sum of vesicular and non-vesicular pools of transmitter, while only the vesicular pool of 5-HT would remain after preparation of tissue sections for immunoautoradiography. Reserpine binds irreversibly to vesicular monoamine transporter 2 ($VMAT_2$), leading to depletion of vesicular 5-HT (Cooper et al., 2003). The data in Fig. 3 reveal that reserpine decreased immunoreactive 5-HT signal by about ~90%. We believe that administration of 5-HTP-B failed to increase the immunoreactive 5-HT signal in reserpinized rats, despite large elevations of extracellular 5-HT (Halladay et al., 2006), because the reserpine treatment impaired the ability of the vesicles to accumulate 5-HT. Viewed collectively, these considerations suggest that our immunoautoradiography method specifically detects vesicular 5-HT.

Since 5-HTP administration can lead to the synthesis of 5-HT in dopaminergic nerves (Lynn-Bullock et al., 2004), it seems possible that increases in 5-HT produced by 5-HTP-B in rats pretreated with MDMA or 5,7-DHT are occurring in dopaminergic nerve terminals. Several factors argue against this possibility. First, 5-HTP-B administration does not increase 5-HT in the caudate of control animals that have intact DA nerve terminals. Second, after pretreatment with MDMA or 5,7-DHT, 5-HTP-B substantially increases 5-HT in hippocampus, a region with sparse dopaminergic innervation.

The rationale for the MDMA dosing regimen used in this study has been described in detail (Baumann et al., 2007). Previous work showed that this treatment regimen produces a long-term decrease in brain 5-HT and SERT binding, without detectably changing expression of the SERT protein. MDMA treatment does not decrease brain DA or NE in the rat (Green et al., 2003; Baumann et al., 2007). Using the immunoautoradiography method, we observed a brain distribution of 5-HT consistent with that observed with other methods (Baumann et al., 1998). Pretreatment with MDMA produced a fairly uniform decrease in 5-HT in the brain regions sampled, and administration of 5-HTP-B to rats pretreated with MDMA increased brain 5-HT levels toward normal levels (Fig. 4). Similarly, pretreatment with 5,7-DHT produced a fairly uniform decrease in 5-HT in the brain regions sampled and administration of 5-HTP-B to rats pretreated with 5,7-DHT increased brain 5-HT levels to normal levels (Fig. 5). Indeed, the autora-



Brain region	Control	5-HTP	MDMA	MDMA+5-HTP
Cortex	100±11.8 %	100.5±10.7 %	34.2±3.8* %	54.6±7.0† %
Hippocampus	100±10.4 %	101.8±10.4 %	35.2±3.7 * %	65.5±7.8† %
Caudate	100±11.5 %	98.6±9.5 %	33.6±4.0* %	84.7±11.2†%
Hypothalamus	100±9.4 %	100.7± 9.6 %	22.9±3.1* %	83.6±12.1† %
Amygdala	100±9.9 %	99.7±10.4 %	25.3±3.3* %	85.7±10.9†%

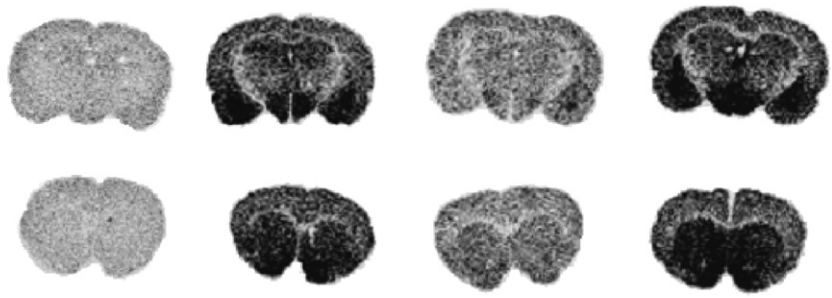
Fig. 4. Effect of MDMA on brain 5-HT as measured by immunoautoradiography. Rats were administered MDMA or saline and 2 weeks later received either saline or 5-HTP-B injections as described in Experimental Procedures. 5-HT levels were measured using immunoautoradiography as described in Experimental Procedures. * $P<0.01$ when compared with control (Student's *t*-test). † $P<0.01$ when compared with the MDMA group. Each value is the mean±S.D. ($n=14$ slices, 6 rats). Representative autoradiograms from each group are shown.

diograms obtained from the MDMA/5-HTP-B and the 5,7-DHT/5-HTP-B rats look nearly identical to control rats.

One simple interpretation of this experiment is that after neurotoxic MDMA and 5,7-DHT treatments, intact surviving 5-HT nerve terminals possess $VMAT_2$-containing vesicles that are depleted of 5-HT. This could explain the relatively uniform decrease in brain 5-HT produced by MDMA and 5,7-DHT. Administration of 5-HTP-B to the MDMA- or 5,7-DHT-pretreated rats increases the availability of cytoplasmic 5-HT, allowing the vesicles to fill to capacity, thereby producing an almost normal 5-HT immunoautoradiogram. Since the vesicles normally accumulate 5-HT to full capacity, this scenario implies that after MDMA or 5,7-DHT cytoplasmic 5-HT must drop to such a low level that its availability becomes rate limiting for the accumulation of vesicular 5-HT. The long-term inactivation of tryptophan hydroxylase produced by MDMA and 5,7-DHT is completely consistent with this hypothesis (for review see: (Bendotti et al., 1993; Baumann et al., 2007)).

Whether or not MDMA is neurotoxic to 5-HT nerve terminals remains controversial (Baumann et al., 2007). In considering the contribution of our findings to the debate on MDMA neurotoxicity, it is useful to consider three possible models for the effect of MDMA on 5-HT nerve terminals: a "neuroadaptive" model, a "neurotoxic" model, and a mixed model. The neuroadaptive model posits that the 5-HT nerve terminals are intact after MDMA administration, but vesicular 5-HT content is reduced, due to persistent inhibition of 5-HT synthesis. A neurotoxic model posits that some fraction of 5-HT nerve terminals is destroyed, while the remaining intact terminals are normal. The mixed model posits that some fraction of 5-HT nerve terminals is destroyed and the remaining 5-HT nerve terminals display reduced vesicular 5-HT as noted above. Table 2 summarizes the distinct profile of MDMA-induced alterations predicted by each of the three models. Given the general tendency for altered neuron systems to maintain homeostasis, all three models predict that extracellular 5-HT will remain unchanged. Since all three models predict a lower 5-HT content and presumably less 5-HT release, the three models similarly predict decreased SERT density in order to help maintain normal extracellular 5-HT. Only the neuroadaptive model predicts a lack of reactive gliosis and the "refill" of intact 5-HT nerve terminals after 5-HTP. Thus, the effects of high-dose MDMA on 5-HT nerve terminals are most compatible with the adaptation model. MDMA pretreatment does not alter extracellular 5-HT or cause reactive gliosis (Baumann et al., 2007), findings that are supported by a recent study where MDMA had no effect on expression of the cleaved microtubule-associated protein tau (Straiko et al., 2007).

We have asserted that MDMA-induced destruction of 5-HT nerve terminals should be accompanied by a loss of SERT protein and we failed to detect MDMA-induced decreases in SERT protein expression using two different anti-SERT antibodies (Wang et al., 2004, 2005). A recent



Brain region	Control	5,7-DHT	5,7-DHT+5-HTP
Cortex	100±10.5 %	38.6±5.8*%	103.7±10.2 %
Hippocampus	100±9.6 %	38.4±5.1*%	101.6±10.4 %
Caudate	100±10.5 %	26.8±4.0*%	116.6±13.3 %
Hypothalamus	100±8.9 %	29.7±3.6*%	109.5±11.1 %
Amygdala	100±9.3 %	27.9±3.3*%	108.8±10.7 %

Fig. 5. Effect of 5,7-DHT on brain 5-HT as measured by immunoautoradiography. Rats were administered 5,7-DHT or saline and 2 weeks later received either saline or 5-HTP-B injections as described in Experimental Procedures. 5-HT levels were measured using immunoautoradiography as described in Experimental Procedures. * $P<0.01$ when compared with control (Student's *t*-test). † $P<0.01$ when compared with the 5,7-DHT group. Each value is the mean±S.D. ($n=14$ slices, 6 rats). Representative autoradiograms from each group are shown.

paper challenged our Western blot findings (Xie et al., 2006). Although we believe the study of Xie et al. is compromised due to overloading of gels with protein, we undertook additional control studies with the anti-SERT antibody used in our 2005 paper (Wang et al., 2005). These studies revealed that our antibody does indeed cross-react to some degree with certain structural proteins that have molecular weights similar to SERT proteins (~50 and 70 kDa). It is now clear that the antibody we used in our 2005 paper is not ideal, but the fact that 5,7-DHT treatment decreased expression of bands at the expected molecular weight provides some evidence that the antibody detects SERT, despite cross-reaction with other proteins. More definitive experiments await the development of better anti-SERT antibodies.

In considering the contribution of our findings to the effects of 5,7-DHT treatment, we note that 5,7-DHT is an established 5-HT neurotoxin (Baumgarten and Lachenmayer, 2004; Bendotti et al., 1993; Semple-Rowland et al., 1996) that decreases 5-HT immunoreactivity in the 5-HT nerve terminal projection plexus (Azmitia and Whitaker-Azmitia, 1997), increases expression of markers related to gliosis (O'Callaghan and Miller, 1993; Munoz et al., 2005; Wang et al., 2005) and decreases expression of SERT protein (Wang et al., 2005). Rats pretreated with 5,7-DHT also maintain extracellular 5-HT levels at control levels (Kirby et al., 1995; Baumann et al., 2007), and *in vivo* 5-HT uptake remains normal (Montanez et al., 2003). Indeed, *in vivo* 5-HT clearance is not reduced by 5,7-DHT until there is at least 90% reduction in SERT binding (Montanez et al., 2003). Viewed collectively, the effect of 5,7-DHT is most compatible with the mixed neuroadaptive/neurotoxic model. This agent increases expression of markers related to gliosis (O'Callaghan and Miller, 1993; Munoz et al., 2005; Wang et al., 2005), decreases expression of SERT protein (Wang et al., 2005) while extracellular 5-HT levels are maintained at control levels (Kirby et al., 1995; Baumann et al., 2007). In addition, 5-HTP-B administration increases brain 5-HT to a greater extent than in rats pretreated with MDMA (Fig. 5). Indeed, autoradiograms of the 5-HTP-B/5,7-DHT group showed no evidence of 5-HT depletion, suggesting that, following 5,7-DHT administration, more 5-HT nerve terminals may survive than previously suspected.

Our data emphasize the difficulty in determining the effects of MDMA or 5,7-DHT when measuring endpoints

Table 2. Predicted effects of different models on 5-HT nerve terminal endpoints

Model	Extracellular 5-HT	Reactive gliosis	5-HT content	SERT binding	5-HTP-B "refill"
Neuroadaptation	No change	No	Decreased	Decreased	Yes
Neurotoxicity	No change	Yes	Decreased	Decreased	No
Mixed	No change	Yes	Decreased	Decreased	Yes

Effects of different models on 5-HT nerve terminal endpoints are summarized.

such as 5-HT content, SERT protein expression, binding or function. A great deal of significance has been attached to changes in SERT binding levels as an indicator of serotonergic "neurotoxicity" (Green et al., 2003). However, SERT binding is subject to various regulatory processes, and changes in SERT binding levels cannot, as a single line of evidence, connote a "neurotoxic" effect. For example, chronic treatment of rats with 5-HT selective reuptake inhibitors reduces SERT binding to a similar extent as MDMA (~90%) (Benmansour et al., 1999), yet such treatments are not considered to be neurotoxic. An ideal end-point to assess the integrity of 5-HT nerve terminals is one that is selective for 5-HT nerve terminals, but one that also is not subject to regulatory changes. None of the end-points used to date meet these requirements. Until such time as a "gold standard" end-point is identified and validated, it will not be possible to unambiguously determine which model (neuroadaptive, neurotoxic or mixed) best describes the effect of high-dose MDMA on rat brain serotonergic systems. Regardless of the mechanism by which high-dose MDMA depletes brain 5-HT, our findings show that administration of 5-HTP-B can apparently restore brain 5-HT concentrations, suggesting that this approach might be clinically useful in abstinent MDMA users who experience cognitive and psychiatric morbidity due to persistent 5-HT deficits (Thomasius et al., 2006).

CONCLUSION

In summary, three major findings emerge from this study. First, is difficult to ascertain the effect of MDMA on 5-HT nerve terminals by measuring tissue 5-HT, SERT binding or SERT function, since the magnitude of effect differs among these end-points and can be manipulated by changing vitro assay conditions. Second, the loss of brain 5-HT produced by MDMA can be substantially reversed by administration of the 5-HT precursor 5-HTP, suggesting that MDMA causes neuroadaptation rather than loss of 5-HT nerve terminals. Finally, following 5,7-DHT administration, more 5-HT nerve terminals may survive than previously suspected.

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