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Increased Anxiety 3 Months after Brief Exposure to MDMA ('Ecstasy') in Rats: Association with Altered 5-HT Transporter and Receptor Density

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Male Wistar rats were treated with 3,4-methylenedioxymethamphetamine (MDMA, 'Ecstasy') using either a high dose (4 × 5 mg/kg over 4 h) or low dose (1 × 5 mg/kg over 4 h) regimen on each of 2 consecutive days. After 10 weeks, rats were tested in the social interaction and emergence tests of anxiety. Rats previously given either of the MDMA dose regimens were significantly more anxious on both tests. After behavioral testing, and 3 months after the MDMA treatment, the rats were killed and their brains examined. Rats given the high-, but not the low-, dose MDMA treatment regimen exhibited significant loss of 5-hydroxytryptamine (5-HT) and 5-HIAA in the amygdala, hippocampus, striatum, and cortex. Quantitative autoradiography showed loss of SERT binding in cortical, hippocampal, thalamic, and hypothalamic sites with the high-dose MDMA regime, while low-dose MDMA only produced significant loss in the medial hypothalamus. Neither high- nor low-dose MDMA affected $5HT_{1A}$ receptor density. High-dose MDMA increased $5HT_{1B}$ receptor density in the nucleus accumbens and lateral septum but decreased binding in the globus pallidus, insular cortex and medial thalamus. Low-dose MDMA decreased $5HT_{1B}$ receptor density in the hippocampus, globus pallidus, and medial thalamus. High-dose MDMA caused dramatic decreases in cortical, striatal, thalamic, and hypothalamic $5HT_{2A/2C}$ receptor density, while low-dose MDMA tended to produce similar effects but only significantly in the piriform cortex. These data suggest that even brief, relatively low-dose MDMA exposure can produce significant, long-term changes in 5-HT receptor and transporter function and associated emotional behavior. Interestingly, long-term 5-HT depletion may not be necessary to produce lasting effects on anxiety-like behavior after low-dose MDMA.
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INTRODUCTION

MDMA (3,4-methylenedioxymethamphetamine, 'Ecstasy') is a drug with unique empathogenic properties and one of the most popular illicit recreational drugs in the world. For more than 15 years it has been known that MDMA and several closely related amphetamine derivatives produce depletion of the neurotransmitter 5-hydroxytryptamine (5-HT) in the brains of laboratory animals (Battaglia *et al*, 1987; Commins *et al*, 1987; Ricaurte *et al*, 1985). This loss of 5-HT reflects a primary toxic effect of these drugs on 5-HT axons leading to loss of 5-HT terminals, but not cell bodies (Callahan *et al*, 2001; Scallet *et al*, 1988). There is controversy, however, over whether similar effects are seen in humans who use MDMA. Brain imaging studies suggesting loss of 5-HT innervation in human MDMA users (McCann *et al*, 1998; Semple *et al*, 1999) have been subjected to some recent criticism (Kish, 2002).

Nonetheless, there is growing clinical evidence that human MDMA users, particularly heavy users, are more prone to a variety of psychological problems, including anxiety, depression, and memory impairment (Morgan, 2000; Parrott, 2001; Schifano *et al*, 1998; Topp *et al*, 1999). Given the important role of 5-HT in cognition and mood, these clinical manifestations are consistent with presumed 5-HT depletion (Boot *et al*, 2000). These clinical effects have been mirrored in recent preclinical studies from our laboratory where Wistar rats briefly exposed to MDMA were shown to have long-term increases in anxiety-like behavior in the social interaction, elevated plus maze and emergence tests 1–3 months post-MDMA

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(Gurtman *et al*, 2002; Morley *et al*, 2001). MDMA-treated rats also displayed inferior memory in an object recognition test (Morley *et al*, 2001). Post-mortem neurochemical analysis showed that these rats had approximately 40–50% loss of 5-HT in cortex, hippocampus, amygdala, and striatum (Gurtman *et al*, 2002).

Recent work has also shown that MDMA causes long-term decreases in social interaction in Lister rats, but in the absence of significant depletion in brain 5-HT (Fone *et al*, 2002). Long-term increases in anxiety-like behavior have also been seen in rats given relatively modest doses of MDMA (5 mg/kg on 2 consecutive days) that might not be expected to produce 5-HT depletion (Morley *et al*, 2001). Taken together, these results raise the possibility that MDMA may produce long-term emotional and behavioral effects that are not necessarily linked to a long-term loss of 5-HT. Rather it may be other neural adaptations to MDMA that underlie the long-term effects of the drug (Green and McGregor, 2002).

A variety of 5-HT receptor subtypes are known to play a role in anxiety-like behavior, for example 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2C}, and 5-HT_3 (Griebel, 1995). The 5-HT transporter (SERT) is also known to play an important role in anxiety, and is the key target for the antidepressant drugs commonly used to treat anxiety disorders in humans (Lesch *et al*, 1996; Rausch *et al*, 2001). This suggests that changes in anxiety-like behavior in rats given MDMA may be related to changes at the 5-HT receptor and/or transporter level. High-dose MDMA produces loss of SERT binding sites in rats (Battaglia *et al*, 1991, 1987; Lew *et al*, 1996) and primates (Jagust *et al*, 1996; Scheffel *et al*, 1998) while brain imaging studies suggest a similar loss of SERT in human MDMA users (McCann *et al*, 1998; Semple *et al*, 1999).

A small number of studies have also tracked long-term changes in specific 5-HT receptors following MDMA administration. There have been reports that 5-HT_{1A} receptor density in the forebrain may be increased with MDMA treatment (Aguirre *et al*, 1998, 1995). Transient upregulation of 5-HT_{1B} receptors has also been reported after high-dose MDMA (Sexton *et al*, 1999). Similarly, a short-term decline in 5-HT_{2A} and/or 5-HT_{2C} receptors has been shown following MDMA treatment (Reneman *et al*, 2002b; Scheffel *et al*, 1992).

In the present study, we administered rats either a high- or low-dose MDMA regime over 2 successive days. Ten weeks later, we examined anxiety-like behavior in the social interaction and emergence tests. Rats were then killed and their brains assayed for 5-HT using HPLC, and for density of SERT, the dopamine transporter (DAT), 5-HT_{1A} receptors, 5-HT_{1B} receptors and 5-$HT_{2A/2C}$ receptors using quantitative autoradiography. It was predicted that rats given the low-dose MDMA treatment may not show 5-HT depletion but might show marked alterations in 5-HT transporters or receptors, changes that could conceivably underlie any increased anxiety-like behavior seen.

METHODS

Subjects

The subjects were 36 inbred male albino Wistar rats (Concord Hospital breeding facility) aged 3 months at the beginning of the experiment and weighing 374 $\pm$ 17 g. The rats were housed in groups of 6–8 per cage for the duration of the experiment with food and water freely available. The temperature in the colony room was controlled at 22°C and a 12 h reverse light cycle was in operation. All behavioral testing was conducted during the dark cycle. All experimentation was approved by the University of Sydney animal ethics committee and was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experimental Procedures

Drug. (+/−)3,4-Methylenedioxymethamphetamine was supplied by the Australian Government Analytical Laboratories (Pymble, NSW). It was diluted in 0.9% saline and injected i.p. at a dose of 5 mg/kg at a volume of 1 ml/kg.

Drug administration. During the drug administration period, individual rats were placed in standard operant chambers ($30 \times 50 \times 25.5\,cm^3$) with aluminium side and back walls, a Perspex front wall, and a metal grid floor. The chambers were placed inside wooden sound attenuation boxes. The ambient temperature in the room in which the chambers were located was maintained at 28 $\pm$ 1.0°C. High ambient temperatures are thought to promote the neurotoxic effects of MDMA (Broening *et al*, 1995; Malberg *et al*, 1996; Malberg and Seiden, 1998).

The drug administration session lasted for 4 h. Rats were given an initial dose of MDMA or vehicle and placed in the chambers. Each hour, rats were briefly removed from the test chambers to administer their next injection of MDMA or vehicle.

Rats in the high-dose MDMA group ($n = 12$) were given a 5 mg/kg i.p. dose of MDMA every hour for 4 h on each of 2 consecutive days to give a cumulative total dose of 40 mg/kg (20 mg/kg/day). This dose regime is intended to simulate a weekend of heavy MDMA use in a human user, given that the typical self-administered human dose is 1–2 tablets, equivalent to 1–4 mg/kg MDMA (Boot *et al*, 2000). The half-life of MDMA is approximately 2.2 h in the rat (Fitzgerald *et al*, 1990) so that the 4×5 mg/kg regime may produce peak MDMA concentrations similar to that produced by a single 15 mg/kg injection of MDMA.

Rats in the low-dose MDMA group ($n = 12$) received a single 5 mg/kg i.p. injection of MDMA, intended to simulate the more typical human use of 1–2 tablets of MDMA (Boot *et al*, 2000). The single MDMA injection was followed by three injections of saline every hour over 4 h on each of 2 consecutive days to give a cumulative total dose of 10 mg/kg (5 mg/kg/day). Rats in the vehicle group received an injection of saline every hour for 4 h on each of the 2 days. Each treatment condition was equally represented within the home cages.

This regime of drug administration has been used several times in our laboratory and is known to produce a robust hyperthermia and hyperactivity in rats given the high-dose MDMA treatment but no significant hyperthermia or hyperactivity in rats in the low-dose MDMA treatment (Gurtman *et al*, 2002; Morley *et al*, 2001). The high-dose regime is known to produce long-term effects on anxiety as well as substantial, lasting 5-HT depletion (Gurtman *et al*,

2002; Morley *et al*, 2001). The low-dose regime produces alterations in anxiety-like behavior (Morley *et al*, 2001) but effects on 5-HT have yet to be determined.

Following the 4 h drug administration sessions, rats were returned to their home cages in the animal colony.

Social interaction test. At 10 weeks following drug administration, pairs of rats were assessed in the social interaction test. Testing occurred in a square clear Perspex box ($52 \times 52 \times 40\,cm^3$) dimly lit with red light (40 W). A miniature video camera was placed vertically above the box. The camera was connected to a video recorder and monitor in a neighbouring room where the interactions of the rats were recorded onto videotape. The experimenter remained outside the test room during testing and the test arena was wiped down with 10% ethanol in between each test session.

Testing was performed across 2 consecutive days, with each rat tested with a different partner on each of the 2 days. Partners were selected so as to be of approximately equal body weight and from the same treatment condition (high-dose MDMA, low-dose MDMA, or vehicle) but from a different home cage. Data for a total of 12 pairs from each condition were obtained.

Each social interaction session lasted for 10 min. The total duration of social interaction and the number of interactions during this 10 min period was scored from video by an observer using ODLog software (www.macropodsoftware.com). Behaviors that were recorded as social interaction included sniffing, adjacent lying, following, crawling over/under, and mutual grooming (Gurtman *et al*, 2002; Morley *et al*, 2001).

Emergence test. At 1 week after the social interaction test, rats were tested in the emergence test. The apparatus consists of a white Perspex-walled rectangular arena ($96 \times 100 \times 40\,cm^3$) with a black wooden hide box ($24 \times 40 \times 15\,cm^3$) placed in the top left corner of the arena. The open part of the arena was illuminated with red light (40 W) and a video camera was mounted above the arena and connected to a video recorder.

Rats were initially placed inside the wooden hide box (which had a hinged lid through which the rat could be placed inside the box). Testing continued for 5 min, during which time the experimenter remained outside the test room.

Subsequent video analysis by an experimenter blind to group assignment scored the latency of rats to emerge from the hide box and the duration of time spent in the open field. 'Risk assessment' behavior was also scored, which refers to the time rats spent with their head poking out of the hide box but with the majority of the body remaining inside the box. Analysis was accomplished using ODLog data logging software from Macropod software (www.macropodsoftware.com).

After each test session the apparatus was thoroughly wiped down with a damp cloth containing 10% ethanol.

Neurochemical analysis. At 12 weeks following MDMA or vehicle treatment, 18 of the 36 rats used in the study ($n = 6$ per group) were decapitated using a guillotine, and their brains rapidly removed for neurochemical analysis. Five regions of interest were manually dissected out and frozen over dry ice using a method derived from that of Harkin *et al* (2001). Samples from the olfactory bulb, prefrontal cortex, striatum, hippocampus, and amygdala were individually placed in centrifuge tubes and stored in a freezer at $-80^{\circ}C$ until assayed.

Tissue samples were weighed and then homogenized with a 500 µl ice-cold solution of 0.2 M perchloric acid containing 0.1% cysteine and 200 nmol/l of internal standard 5-hydroxy-*N*-methyltryptamine (5-HMeT). The homogenate was centrifuged at 15 000 *g* for 10 min at 4°C and a 20 µl aliquot of the resulting supernatant fluid was then analyzed for biogenic amines by high-performance liquid chromatography (HPLC) with electrochemical detection as described previously (Gurtman *et al*, 2002; Schworer *et al*, 1987).

Briefly, the GBC HPLC system (Melbourne, Australia) comprised an LC 1610 auto-injector, an LC 1150 multi-solvent delivery pump, an LC 1210 electrochemical detector (ECD), and WinChrom data management software. The mobile phase consisted of 0.1 mol/l phosphate buffer (pH 3.0), PIC B-8 octane sulfonic acid (Waters, Australia) 0.74 mmol/l, sodium EDTA (0.3 mmol/l), and methanol (12% v/v). The flow rate was maintained at 1 ml/min. Dopamine, 5-HIAA, 5-HT, and 5-HMeT were separated by a Merck LiChrospher 100 RP-18 reversed phase column. Quantification was achieved via ECD equipped with a glassy carbon working electrode set at a potential of +0.75 V versus an Ag/AgCl reference electrode. The calibration curve of each standard was obtained by the concentration versus the area ratio of the standard and internal standard.

Quantitative autoradiography. Also at 12 weeks following MDMA or vehicle treatment, the remaining 18 of the 36 rats were killed by decapitation, and their brains removed and rapidly frozen over dry ice, and then stored at $-80^{\circ}C$. Coronal sections (14 µm) were cut posterior to anterior on a Leica cryostat at the level of the dorsal raphe (AP −8.00 mm from bregma), caudal amygdala (−3.60), rostral amygdala (−2.30), and nucleus accumbens (+1.20). Sections were thaw mounted on gelatin/chrome alum-coated sides (two sections per slide), air dried, and then stored at $-80^{\circ}C$ until required. In all cases, at least four sections/rat/region were analyzed for each binding site, and at least two sections/rat/region were utilized to define nonspecific binding.

Serotonin and DAT. Binding of [^{125}I] RTI-55 (Lew *et al*, 1996) was used to assess binding density for both the DAT and serotonin transporter (SERT). DAT binding was carried out at the level of the nucleus accumbens only, while SERT binding was carried out at all levels. Sections were allowed to defrost, and then preincubated for 30 min in phosphate buffer (10 mM NaH_2PO_4, 0.1 M sucrose, pH 7.4) at room temperature. DAT binding was defined with 50 pM [^{125}I] RTI-55 in the presence of 50 nM fluoxetine, while SERT binding was defined with 50 pM [^{125}I] RTI-55 in the presence of 1 µM mazindol. Nonspecific binding was determined with 10 µM GBR 12909 or 10 µM fluoxetine, respectively. Following incubation for 1 h at room temperature, slides were washed (1 × 1 min, then 2 × 20 min) in ice-cold buffer, followed by one dip in ice-cold distilled water

and dried under a gentle stream of cool air. Slides were stored overnight in a desiccator, and then apposed for 6 h to Kodak X-OMAT AR film in the presence of standard microscales. Autoradiographs were developed using Kodak D-19 developer and fixed with Ilford Hypam Rapid Fixer.

5-HT$_{1A}$ and 5-HT$_{1B}$ receptors. Binding to 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors by [^{125}I]cyanopindolol was performed with an adaptation of published procedures (Offord *et al*, 1988; Sexton *et al*, 1999). In brief, sections were preincubated for 15 min in ice-cold Tris/HCl buffer (50 mM Tris, 2.5 mM $MgCl_2$, 150 mM NaCl, pH 7.4) containing 10 μM pargyline and 20 μM isoprenaline (to prevent binding to β-adrenoceptors). Slides were then transferred to the incubation buffer containing 30 pM [^{125}I]cyanopindolol alone (total) or with the addition of 300 nM CP 93129 (5-HT$_{1B}$ agonist, to define 5-HT$_{1A}$ binding), 10 μM 8-OH-DPAT (5-HT$_{1A}$ agonist, to define 5-HT$_{1B}$ binding), or 20 μM 5-HT (nonspecific binding). Following a 2 h incubation at room temperature, slides were washed (2 × 1 min) in ice-cold buffer, then dipped in ice-cold distilled water, and dried as above. Slides were apposed to film for 6 days in the presence of standard microscales and processed as above.

5-HT$_{2A/2C}$ receptors. Binding to 5-HT$_{2A/2C}$ receptors was visualized using [^{125}I] DOI as previously described (Compan *et al*, 1998a, b). Sections were preincubated for 30 min at RT in a Tris/HCl buffer (50 mM Tris, 4 mM $CaCl_2$, 0.1% ascorbate, pH 7.4) containing 0.1% bovine serum albumin. Slides were then incubated at room temperature for 1 h in 0.1 nM [^{125}I] DOI in the presence or absence of 100 μM unlabeled DOI to determine nonspecific binding. Slides were then washed (2 × 10 min) in ice-cold buffer, dipped in ice-cold distilled water, and dried as above. Slides were apposed to film for 6 days in the presence of standard microscales and processed as above.

Image analysis. Autoradiographic images were captured and analyzed using the Scion Image Analysis Program (v. 1.62, Scion Corporation, USA, PC version of NIH Image). The density of binding was calculated by converting the optical density of the image to dpm/mm^2 with the aid of a standard curve generated with calibrated microscales, as previously described (Chen and Lawrence, 2000). Individual brain nuclei were identified with reference to a stereotaxic atlas (Paxinos and Watson, 1997).

Statistics. Statistical analyses of data from the two anxiety tests, the HPLC assays, and the binding assays were achieved by comparing MDMA and vehicle groups using one-way ANOVA followed by Fisher's PLSD *post hoc* tests. The significance level for all statistical tests was set at $P<0.05$.

RESULTS

Social Interaction test

The results of the social interaction tests conducted 10 weeks post-MDMA are shown in Table 1. There was an overall effect of group on social interaction time ($F_{2,33}=31.80$, $P<0.001$) and on the total number of interactions ($F_{2,33}=5.03$, $P<0.05$). *Post hoc* tests showed that rats in the low- and high-dose MDMA treatment groups spent significantly less time in social interaction than controls. Rats in the high-dose MDMA group also showed a significantly lower total number of interactions than controls.

Table 1 Results from the Social Interaction Test Conducted 10 Weeks Post-MDMA

Group	Time in social interaction (s)	Number of interactions (*n*)
Vehicle	120.63 (8.05)	56.25 (3.27)
Low MDMA	72.98 (4.03)[a]	47.00 (3.42)
High MDMA	56.31 (4.85)[a,b]	42.67 (2.28)[a]

Data represent mean (SEM) for $n=12$ social interaction tests per group.
[a]$P<0.05$ relative to vehicle group.
[b]$P<0.05$ relative to low-MDMA group.

Table 2 Results from the Emergence Test Conducted 11 Weeks Post-MDMA

Group	Emergence latency (s)	Time in open field (s)	Risk assessment (s)
Vehicle	45.46 (4.96)	73.43 (8.82)	9.00 (0.61)
Low MDMA	91.03 (11.90)[a]	63.16 (10.92)	8.83 (0.86)
High MDMA	118.61 (18.88)[a]	52.65 (10.24)	9.08 (0.61)

Data represent mean (SEM) for $n=12$ per group.
Maximum emergence latency = 300 s due to 5 min test.
[a]$P<0.05$ relative to vehicle group.

Emergence Test

The results of the emergence test conduced 11 weeks post-MDMA are shown in Table 2. There was an overall effect of group on emergence latency ($F_{2,33}=7.80$, $P<0.01$). *Post hoc* tests showed that rats in both the low- and high-dose MDMA treatment groups took longer to emerge in the open field than controls. No significant differences were observed in open-field time or risk assessment.

Neurotransmitter Levels

The results of HPLC analysis of neurotransmitter content in key brain regions are shown in Table 3. There were no significant differences between groups on 5-HT or 5-HIAA levels within the olfactory bulb. However, significant overall group effects on 5-HT and 5-HIAA were obtained in the prefrontal cortex ($F_{2,15}=12.34$, $P<0.001$ and $F_{2,15}=10.07$, $P<0.01$), striatum ($F_{2,15}=5.69$, $P<0.05$ and $F_{2,15}=13.57$, $P<0.001$), hippocampus ($F_{2,15}=3.99$, $P<0.05$ and $F_{2,15}=6.25$, $P<0.05$), and amygdala ($F_{2,15}=6.22$, $P<0.05$ and $F_{2,15}=6.39$, $P<0.05$), Rats in the high-dose MDMA group showed significantly lower levels of 5-HT and 5-HIAA than rats in either the vehicle or low-dose MDMA groups in all four of these regions. The low-dose MDMA group and vehicle group did not differ significantly in any measures.

Table 3 Results of HPLC Analysis in Five Brain Areas Conducted 3 Months Post-MDMA

Region	Treatment	5-HT	5-HIAA	DA
Olfactory bulb	Vehicle	136.7 (18.4)	95.2 (9.3)	111.4 (39.2)
	Low MDMA	153.3 (24.8)	79.0 (11.0)	97.2 (14.8)
	High MDMA	147.7 (20.7)	73.2 (9.2)	92.3 (22.4)
Prefrontal cortex	Vehicle	521.4 (17.7)	172.8 (10.5)	285.3 (55.4)
	Low MDMA	489.6 (37.3)	173.9 (12.8)	369.0 (70.7)
	High MDMA	353.1 (15.4)[a,b]	118.6 (4.9)[a,b]	288.0 (142.9)
Striatum	Vehicle	522.2 (41.6)	350.0 (16.0)	7546.5 (372.5)
	Low MDMA	584.6 (52.0)	381.5 (42.1)	7959.0 (701.7)
	High MDMA	383.0 (30.0)[a,b]	251.1 (13.0)[a,b]	6426.8 (558.1)
Hippocampus	Vehicle	592.3 (15.3)	407.5 (35.4)	78.9 (23.3)
	Low MDMA	568.2 (25.5)	389.5 (19.7)	52.8 (14.9)
	High MDMA	485.3 (15.0)[a,b]	259.2 (26.2)[a,b]	62.1 (19.8)
Amygdala	Vehicle	880.4 (50.2)	174.8 (11.1)	235.3 (77.6)
	Low MDMA	897.8 (53.0)	160.4 (13.0)	180.4 (19.5)
	High MDMA	689.6 (54.8)[a,b]	120.1 (12.4)[a,b]	208.9 (45.9)

Data are mean (SEM) for $n = 6$ per group.
Data are in wt ng/g tissue.
[a]$P < 0.05$ relative to vehicle group.
[b]$P < 0.05$ relative to Low MDMA group.

Table 4 Dopamine and Serotonin Transporter Densities 3 Months Post-MDMA

	Vehicle	Low MDMA	High MDMA
DAT			
Caudate-putamen	145.06 (6.66)	139.85 (5.07)	139.39 (7.55)
Nucleus accumbens	151.98 (7.04)	139.73 (5.26)	138.10 (6.39)
Cingulate cortex	141.02 (8.09)	133.84 (3.12)	127.24 (6.19)
SERT			
Caudate-putamen	41.93 (4.59)	35.13 (3.53)	36.00 (3.37)
Nucleus accumbens	61.67 (5.90)	50.86 (7.33)	48.67 (3.69)
Lateral septum	71.64 (4.94)	74.05 (3.90)	60.00 (3.11)[b]
Cingulate cortex	56.59 (3.08)	55.45 (3.76)	44.46 (2.16)[a,b]
Basolateral amygdala	96.52 (6.00)	112.14 (5.85)	95.23 (5.30)[b]
Central amygdaloid nucleus	58.94 (3.59)	58.71 (2.31)	48.39 (5.36)
Medial amygdaloid nucleus	63.53 (2.59)	65.33 (2.26)	58.05 (3.22)
Posterolateral cortical amygdaloid nucleus	81.06 (6.00)	85.48 (4.98)	61.27 (8.68)[b]
Piriform cortex	40.31 (4.72)	57.12 (7.22)[a]	33.90 (2.66)[b]
Perirhinal/entorhinal cortical area	42.94 (4.46)	58.30 (6.57)[a]	31.69 (1.79)[b]
Retrosplenial cortex	47.27 (2.97)	47.22 (1.74)	41.99 (2.44)
Hippocampus	49.49 (3.25)	48.64 (3.33)	35.72 (2.49)[a,b]
Medial hypothalamic area	99.93 (4.29)	75.59 (6.44)[a]	64.74 (6.88)[a]
Medial thalamic nuclei	71.83 (3.90)	62.84 (3.99)	37.74 (3.20)[a,b]
Lateral thalamic nuclei	90.49 (5.67)	82.44 (5.56)	43.51 (5.07)[a,b]
Zona incerta	62.31 (9.02)	76.82 (4.38)	71.01 (2.20)
Entorhinal cortex	55.73 (4.59)	62.87 (2.01)	26.88 (4.32)[a,b]
Medial raphe	112.88 (4.08)	118.03 (6.41)	117.79 (2.72)
Dorsal raphe	127.56 (14.62)	120.81 (9.60)	119.62 (11.17)

Data are mean (SEM) for $n = 6$ per group.
Units of measurement are dpm/mm^2.
[a]$P < 0.05$ relative to vehicle group.
[b]$P < 0.05$ relative to Low MDMA group.

There were no significant differences between groups in dopamine content in any of the brain regions examined.

DAT Density

Quantification of [^{125}I] RTI-55 binding to DAT sites revealed no significant effects of either high- or low-dose MDMA treatment (Table 4).

SERT Density

Representative autoradiograms of SERT binding are shown in Figure 1. In contrast to the DAT binding, quantification of binding to SERT by [^{125}I] RTI-55 revealed several differences between groups (Table 4). The low-dose MDMA group differed from controls in three brain regions, having significantly lower SERT density in the medial hypothalamus but increased

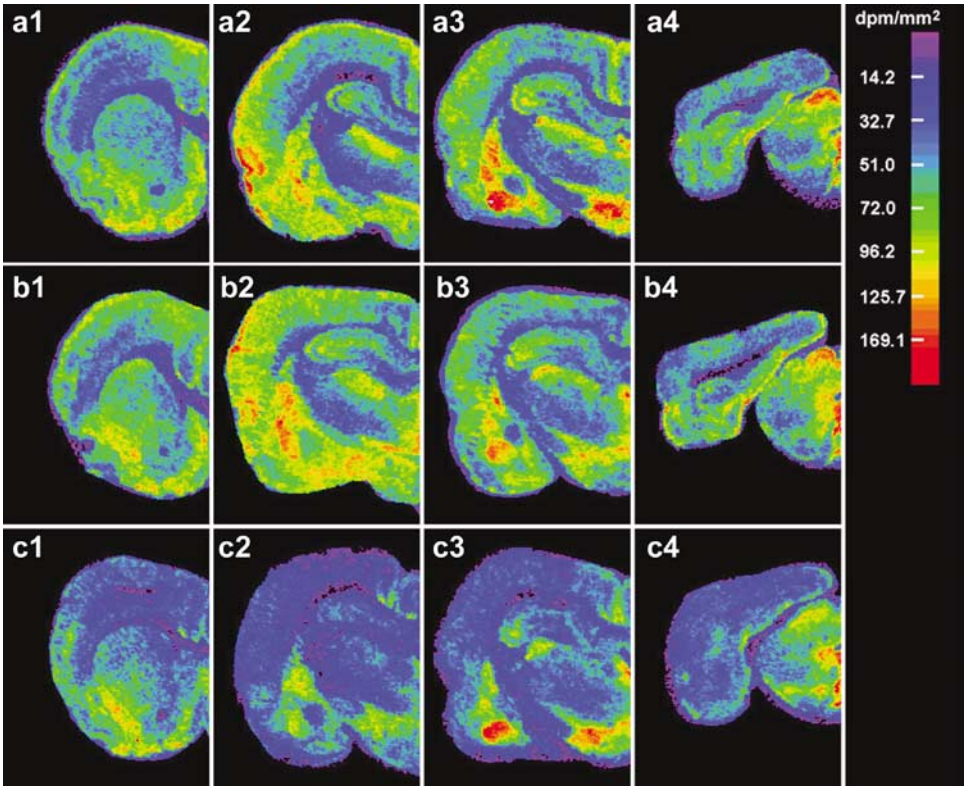

Figure 1 Representative autoradiograms demonstrating [^{125}I] RTI-55 binding to SERT of rats in the vehicle (a1–a4, top row), low-dose MDMA (b1–b4, middle row) and high-dose MDMA (c1–c4, bottom row) groups at the level of bregma +1.2 (far left column), bregma −2.30 (inner left column), bregma −3.60 (inner right column), and bregma −8.00 (far right column). See Table 4 for quantitative analysis of binding data.

SERT density in the piriform cortex and perirhinal/entorhinal cortex.

The high-dose MDMA group had lower SERT density than controls in nearly every brain region examined. This effect reached statistical significance in several brain regions including the cingulate cortex, hippocampus, entorhinal cortex, medial hypothalamic area, and the medial and lateral thalamic nuclei (Table 4). The high-dose MDMA group also had lower SERT density than the low-dose MDMA group in most brain regions. This difference was statistically significant in the lateral septum, cingulate cortex, basolateral amygdala, cortical amygdaloid nucleus, piriform cortex, perirhinal cortex, medial and lateral thalamic nuclei, and entorhinal cortex (Table 4).

5-HT$_{1A}$ Receptor Density

The results for 5-HT$_{1A}$ receptor binding are shown in Table 5. There were no significant differences in binding to 5-HT$_{1A}$ receptors between groups in any brain region examined.

Table 5 5-HT$_{1A}$ Receptor Density 3 Months Post-MDMA

	Vehicle	Low MDMA	High MDMA
Lateral septum	1.28 (0.11)	1.44 (0.07)	1.62 (0.16)
Cingulate cortex	0.55 (0.19)	0.66 (0.13)	0.35 (0.19)
Insular cortex	0.64 (0.22)	0.69 (0.10)	0.65 (0.19)
Piriform cortex	0.55 (0.06)	0.69 (0.11)	0.89 (0.32)
Retrosplenial cortex	0.57 (0.23)	0.33 (0.11)	0.32 (0.16)
Hippocampus (all)	1.37 (0.14)	1.15 (0.14)	1.26 (0.08)
Hippocampus (CA1)	1.53 (0.15)	1.35 (0.13)	1.46 (0.09)
Medial hypothalamic area	0.59 (0.07)	0.79 (0.15)	0.84 (0.18)
Lateral thalamic nuclei	0.33 (0.12)	0.44 (0.15)	0.61 (0.23)
Dorsal raphe	1.60 (0.32)	1.41 (0.24)	1.80 (0.24)

Data are mean (SEM) for $n = 6$ per group.
Units of measurement are dpm/mm^2.

5-HT$_{1B}$ Receptor Density

Representative autoradiograms of 5-HT$_{1B}$ binding are shown in Figure 2, and the densitometric data are presented in Table 6. The low-dose MDMA group showed a significantly lower density of 5-HT$_{1B}$ binding than controls in three brain regions: the globus pallidus, the hippocampus, and the medial thalamic nuclei (Table 6).

The high-dose MDMA group showed lower density of 5-HT$_{1B}$ receptors in the globus pallidus, insular cortex and medial thalamus but higher density in the nucleus accumbens. The high-dose MDMA group also showed significantly higher density than the low-dose MDMA group in the lateral septum.

5-HT$_{2A/2C}$ Receptor Density

Representative autoradiograms of 5-HT$_{2A/2C}$ binding are shown in Figure 3, and the densitometric data are presented in Table 7. The low-dose MDMA group showed a lower density of 5-HT$_{2A/2C}$ receptor binding than controls in most

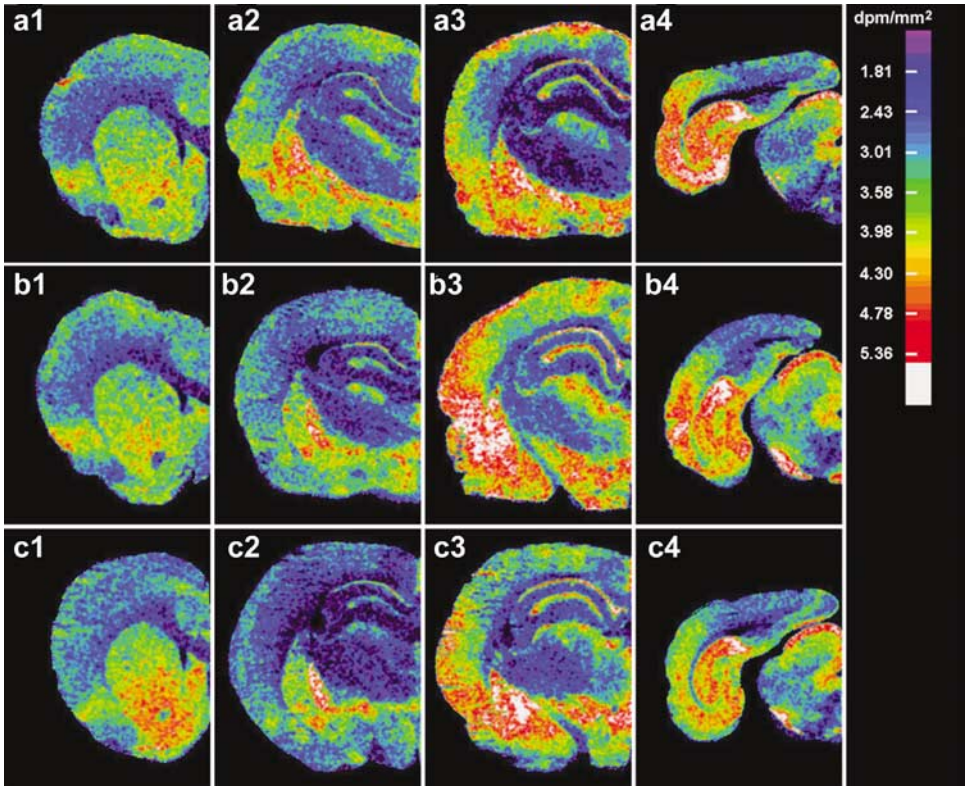

Figure 2 Representative autoradiograms demonstrating [^{125}I]cyanopindolol binding to 5-HT$_{1B}$ receptors of rats in the vehicle (a1–a4, top row), low-dose MDMA (b1–b4, middle row) and high-dose MDMA (c1–c4, bottom row) groups at the level of bregma +1.2 (far left column), bregma −2.30 (inner left column), bregma −3.60 (inner right column), and bregma −8.00 (far right column). See Table 6 for quantitative analysis of binding data.

Table 6 5-HT $_{1B}$ Receptor Density 3 Months Post-MDMA

	Vehicle	Low MDMA	High MDMA
Caudate-putamen	1.55 (0.26)	1.23 (0.18)	1.47 (0.17)
Nucleus accumbens	1.70 (0.20)	1.88 (0.18)	2.37 (0.20)[a]
Lateral septum	2.36 (0.37)	2.13 (0.15)	2.83 (0.23)[b]
Insular cortex	2.31 (0.07)	2.14 (0.11)	1.93 (0.12)[a]
Cingulate cortex	1.29 (0.40)	1.19 (0.34)	1.13 (0.19)
Globus pallidus	4.27 (0.12)	3.76 (0.09)[a]	3.70 (0.08)[a]
Amygdaloid nuclei	2.50 (0.12)	2.21 (0.09)	2.39 (0.14)
Medial amygdaloid nucleus	2.46 (0.11)	2.30 (0.21)	2.64 (0.27)
Piriform cortex	2.60 (0.10)	2.42 (0.07)	2.43 (0.10)
Perirhinal/entorhinal cortical area	1.83 (0.27)	1.94 (0.16)	2.19 (0.20)
Retrosplenial cortex	1.16 (0.16)	1.17 (0.16)	1.38 (0.17)
Hippocampus	1.36 (0.03)	1.09 (0.10)[a]	1.19 (0.08)
CA1 stratum oriens	2.85 (0.28)	2.44 (0.19)	2.61 (0.19)
Dentate gyrus granule layer	2.61 (0.20)	2.15 (0.19)	2.20 (0.21)
Medial hypothalamic area	2.74 (0.14)	2.42 (0.17)	2.60 (0.17)
Medial thalamic nuclei	2.52 (0.14)	2.08 (0.09)[a]	2.16 (0.12)[a]
Lateral thalamic nuclei	2.02 (0.12)	1.90 (0.15)	1.98 (0.11)
Entorhinal cortex	3.49 (0.81)	3.07 (0.54)	3.41 (0.30)
Dorsal raphe	3.10 (0.74)	2.75 (0.43)	3.11 (0.37)

Data are mean (SEM) for $n = 6$ per group.
Units of measurement are dpm/mm^2.
[a]$P < 0.05$ relative to vehicle group.
[b]$P < 0.05$ relative to Low MDMA group.

brain regions. However, the effect only reached statistical significance in the piriform cortex.

The high-dose MDMA group showed dramatically lower 5-HT$_{2A/2C}$ binding in nearly every brain region examined. The effect reached statistical significance in several brain regions, including the caudate-putamen, insular cortex, cingulate cortex, lateral septum, frontal cortex, and entorhinal cortex. In addition, the low- and high-dose MDMA groups differed significantly in binding density in many regions, including the caudate-putamen, lateral septum, medial hypothalamus, medial and lateral thalamic nuclei, and entorhinal cortex.

DISCUSSION

Behavioral Changes and 5-HT Depletion

The behavioral results obtained confirm previous reports from our laboratory that brief exposure to MDMA leads to a long-term increase in anxiety-like behavior in Wistar rats tested in the social interaction and emergence tests weeks or months after drug administration (Gurtman *et al*, 2002; Morley *et al*, 2001). Importantly, anxiogenic effects of MDMA were evident even in rats exposed to a relatively low dose of MDMA (2×5 mg/kg) 3 months previously, specifically confirming the results obtained by Morley *et al* (2001) using an identical treatment regime. Rats given the high-dose regime of MDMA showed an even greater anxiogenic response, in agreement with our previous findings (Morley *et al*, 2001), but in rats given the lower dose regime the anxiogenic response was nonetheless robust. The long-term increases in anxiety seen in rats following MDMA is in agreement with numerous recent reports of elevated anxiety in human MDMA users

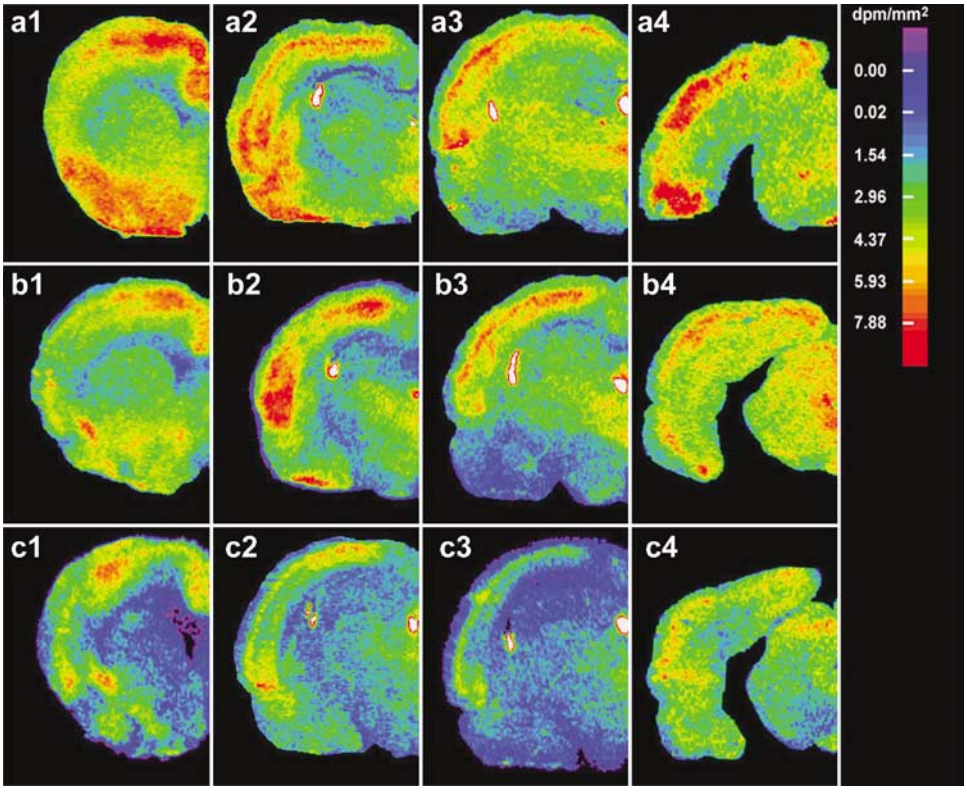

Figure 3 Representative autoradiograms demonstrating [^{125}I] DOI binding to 5-HT$_{2A/2C}$ receptors of rats in the vehicle (a1–a4, top row), low-dose MDMA (b1–b4, middle row), and high-dose MDMA (c1–c4, bottom row) groups at the level of bregma +1.2 (far left column), bregma −2.30 (inner left column), bregma −3.60 (inner right column), and bregma −8.00 (far right column). See Table 7 for quantitative analysis of binding data.

Table 7 5-HT $_{2A/2C}$ Receptor Density 3 Months Post-MDMA

	Vehicle	Low MDMA	High MDMA
Caudate-putamen	3.87 (0.60)	3.36 (0.39)	1.70 (0.62)[a,b]
Nucleus accumbens	6.13 (0.52)	5.09 (0.45)	4.01 (0.94)
Lateral septum	4.22 (0.56)	4.26 (0.17)	2.31 (0.63)[a,b]
Claustrum	10.18 (1.05)	9.87 (0.86)	7.39 (1.14)
Insular cortex	7.91 (0.71)	7.28 (0.41)	6.14 (0.37)[a]
Cingulate cortex	6.91 (0.73)	5.12 (0.61)	3.90 (0.86)[a]
Medial amygdaloid nucleus	4.79 (0.52)	4.41 (0.14)	3.98 (0.54)
Piriform cortex	5.16 (0.29)	3.84 (0.29)[a]	2.72 (0.12)[a,b]
Perirhinal/entorhinal cortical area	5.00 (0.21)	4.22 (0.33)	1.82 (0.49)[a,b]
Medial hypothalamic area	3.07 (0.33)	2.67 (0.53)	0.68 (0.08)[a,b]
Medial thalamic nuclei	3.34 (0.82)	3.56 (0.90)	0.47 (0.13)[a,b]
Lateral thalamic nuclei	3.42 (0.46)	3.37 (0.62)	0.50 (0.09)[a,b]
Entorhinal cortex	1.79 (0.21)	2.14 (0.26)	1.04 (0.37)[b]

Data are mean (SEM) for $n = 6$ per group.
Units of measurement are dpm/mm^2.
[a]$P < 0.05$ relative to vehicle group.
[b]$P < 0.05$ relative to Low MDMA group.

(Gamma *et al*, 2000; Parrott *et al*, 2000; Verkes *et al*, 2001; Wareing *et al*, 2000).

Higher-dose MDMA treatment was associated with the expected depletion of 5-HT and 5-HIAA 3 months later, although only a relatively modest depletion of 20–30% across the various regions was evident. Assessment at earlier time intervals after drug administration may have uncovered a greater 5-HT depletion, since gradual recovery of tissue 5-HT levels in the weeks and months following MDMA treatment is well established (Fischer *et al*, 1995; Sabol *et al*, 1996; Scanzello *et al*, 1993). Rats given the low-dose MDMA treatment had brain 5-HT and 5-HIAA levels that were indistinguishable from controls, suggesting that 5-HT depletion is not necessary for the long-term anxiogenic behavioral profile produced by MDMA. This result agrees with recent findings where long-term reductions in social interaction were seen in Lister rats after MDMA, without significant 5-HT depletion (Fone *et al*, 2002). This is not to deny that 5-HT depletion may have occurred in the low-dose MDMA group shortly after drug treatment although, notably, at least one previous study has failed to find any 5-HT depletion 1 week after rats were given 4 mg/kg MDMA once daily for 4 days (O'Shea *et al*, 1998). On balance then, this suggests that minimal or no 5-HT depletion occurred in the low-dose MDMA rats at any stage of the present experiment; however, changes in anxiety levels are clearly apparent.

SERT Binding

The present study found a clear loss of SERT binding sites in rats given the high-dose MDMA regime, in agreement with numerous previous studies showing 5-HT transporter loss in cortical, striatal, or hippocampal homogenates of rats given MDMA (Battaglia *et al*, 1987; Boot *et al*, 2002; Colado *et al*, 2001, 1995; Harkin *et al*, 2001; O'Shea *et al*, 1998; Scanzello *et al*, 1993). A smaller number of studies have depicted the regional loss of SERT binding following MDMA in brain slices using quantitative autoradiographic

methods similar to those used here (Battaglia *et al*, 1991; Boot *et al*, 2002; Fischer *et al*, 1995; Lew *et al*, 1996). Only two of these studies have rigorously quantified regional SERT in MDMA-treated rats, albeit in animals subjected to a much higher dose regime (20 mg/kg, twice a day for 4 consecutive days) and across different time intervals to those used in the present study (Battaglia *et al*, 1991; Lew *et al*, 1996). Nonetheless, our results are in broad agreement with these two previous studies showing that high-dose MDMA causes little loss of SERT binding in the basolateral amygdala, dorsal raphe and medial raphe, considerable loss of binding in cortical, hippocampal, septal and striatal sites, and profound loss of binding in specific thalamic nuclei and more posterior cortical regions (Battaglia *et al*, 1991; Lew *et al*, 1996).

Interestingly, in one of the previous studies (Lew *et al*, 1996). a progressive recovery of SERT binding was noted from 2 to 52 weeks following MDMA exposure, suggesting that the rats in the present study may have shown greater SERT loss at earlier time intervals following MDMA. Lew *et al* (1996) reported that more posterior cortical regions and lateral thalamic areas showed the least recovery of SERT at 52 weeks, a result that is consistent with the profound loss of SERT evident in the present study in the entorhinal cortex and thalamic nuclei 3 months post-MDMA.

The low-dose MDMA treatment in the present study had a much smaller effect on SERT: a significant reduction in binding at the level of the medial hypothalamus and a significant increase in SERT binding in the piriform and perirhinal cortex. The exact significance of the upregulation in these two sites is unclear. Given that both low- and high-dose MDMA groups exhibited increased anxiety-like behavior yet only the low-dose MDMA group showed this upregulation of SERT, it is unlikely that they can be linked to the increases in anxiety-like behavior. One previous study has shown that the entorhinal/perirhinal region is uniquely sensitive to chronic antidepressant effects on SERT (Hebert *et al*, 2001), and future studies may hopefully identify what makes the response of SERT in this region to pharmacological stimuli somewhat atypical.

In contrast, the consonant effects of low- and high-dose MDMA on SERT at the level of the medial hypothalamus suggest this region as a possible target for future investigations of MDMA-induced anxiety. A role for hypothalamic nuclei in determining anxiety in the social interaction, exploration, and predator-based models of anxiety is reasonably well established (Dielenberg *et al*, 2001; File *et al*, 1999; Shekhar and Katner, 1995).

DAT Binding

The failure to find any alteration in DAT binding density after MDMA treatment in the present study is in accord with previous reports in rats (Battaglia *et al*, 1991, 1987; Lew *et al*, 1996) and humans (Reneman *et al*, 2002a; Semple *et al*, 1999). There was also no significant depletion of tissue dopamine.

A very recent report has indicated a profound loss of DAT sites and other dopaminergic markers in squirrel monkeys and baboons given closely spaced low-dose injections of MDMA (Ricaurte *et al*, 2002). Thus it is conceivable, although yet to be established, that a dose regime different to that used in the present study might cause long-term alterations in DAT. However, this is unlikely to be the mechanism to explain the increase in anxiety-like behavior seen in the present study after MDMA.

5-HT_{1A} Receptor Binding

There was little evidence in the present study of alterations in 5-HT_{1A} receptors in rats given either the low- or high-dose MDMA treatment. High receptor density was seen in the septum, hippocampus, and dorsal raphe, as would be expected from previous autoradiographic studies of 5-HT_{1A} receptor distribution (Verge *et al*, 1986). This receptor has a demonstrated role in anxiety-like behavior in rodents, with targeted deletion of this receptor having effects on behavior in the elevated plus maze and other rodent anxiety models (Ramboz *et al*, 1998). This receptor also has a role in MDMA effects, inasmuch as MDMA generalizes to the 5-HT_{1A} agonist 8-OH-DPAT in the drug discrimination paradigm (Glennon and Young, 2000).

The failure of MDMA to have long-term effects in 5-HT_{1A} receptor density is in partial agreement with reports of no alteration in 5-HT_{1A} binding in the various brain regions following extensive serotonergic lesions with the neurotoxin 5,7-DHT (Compan *et al*, 1998a; Lawrence *et al*, 1993). However, our results contrast with one report of increased cortical and hypothalamic and decreased raphe 5-HT_{1A} density at 7 days following repeated MDMA (Aguirre *et al*, 1995). The difference in the time course of testing may explain the discrepancy in results here as well as the large difference in doses. In agreement with our results, no differential functional response to the 5-HT_{1A} agonist 8-OH-DPAT was evident 30–33 days after neurotoxic MDMA treatment (Granoff and Ashby, 2001; Mechan *et al*, 2001), although some differences were seen in a different study at 7 days post-treatment (Aguirre *et al*, 1998). Similarly, repeated pre-exposure to high doses of the 5-HT_{1A} agonist 8-OH-DPAT did not alter the locomotor response to MDMA in rats (Callaway and Geyer, 1992).

A cautionary note should be sounded concerning the relatively insensitive and nonselective nature of the radioligand used here to label 5-HT_{1A} receptors. Accordingly, unequivocal confirmation of the sensitivity (or lack of) of 5-HT_{1A} receptors towards MDMA treatment awaits further experimentation.

5-HT_{1B} Receptor Binding

Significant long-term changes in 5-HT_{1B} receptor density were seen after both the low- and high-dose MDMA treatments. An increase in density was seen in the nucleus accumbens and lateral septum of rats given the high-dose MDMA treatment. This agrees with the results of Compan *et al* (1998b) who found increased 5-HT_{1B} receptor density in the nucleus accumbens of rats 3 weeks after major 5-HT depletion accomplished by intra-raphe microinjection of 5,7-DHT. There is also a report of very transient upregulation of 5-HT_{1B} receptors in the striatum following neurotoxic MDMA treatment (Sexton *et al*, 1999) or following 5-HT depletion with intraventricular 5,7-DHT (Offord *et al*, 1988).

In both the low- and high-dose MDMA groups, significant downregulation of 5-HT_{1B} receptors was seen in the globus pallidus and the midline thalamus, with significant decreases also evident in the hippocampus in the low-dose group and in the insular cortex of the high-dose MDMA group. The 5-HT_{1B} receptor is often thought of as a terminal autoreceptor, so the loss of receptors in these areas may be consistent with a loss of 5-HT terminals, also suggested by the loss of SERT in some of these regions. However, the 5-HT_{1B} receptor also acts as a terminal heteroreceptor in some regions, and such receptors would be thought to be unaffected by 5-HT depletion.

The 5-HT_{1B} receptor plays a key role in the acute hyperactivity produced by MDMA (Bankson and Cunningham, 2002; Scearce-Levie *et al*, 1999). Our findings of downregulation of 5-HT_{1B} receptors is in agreement with the findings of a diminished locomotor response to a 5-HT_{1B} receptor agonist in rats pre-exposed to high-dose MDMA treatments (Callaway and Geyer, 1992).

Whether changes in the 5-HT_{1B} receptor might underlie the anxiety-like behavior seen in MDMA-treated rats is uncertain. The consequences of 5-HT_{1B} receptor deletion in mice include an increase in aggressive behavior (Saudou *et al*, 1994) and, if anything, a decrease in anxiety-like behavior (Malleret *et al*, 1999; Zhuang *et al*, 1999). On the other hand, overexpression of 5-HT_{1B} receptors in forebrain regions can increase anxiety-like behavior in rats (Clark *et al*, 2002), and a higher genetically endowed density of forebrain 5-HT_{1B} receptors was associated with greater anxiety in mice (Clement *et al*, 1996). It therefore appears that the downregulation of 5-HT_{1B} receptors seen (for the most part) in the present study with MDMA is difficult to reconcile with the increased anxiety produced by the drug.

5-$HT_{2A/2C}$ Receptor Binding

The most profound neuronal changes in the present study were seen with the 5-$HT_{2A/2C}$ receptors, with high-dose MDMA treatment causing a dramatic loss of this receptor in the cortex, septum and caudate-putamen, and low-dose MDMA treatment tending to have similar effects in many regions, although only significantly in the piriform cortex.

These results agree with previous findings, which have noted downregulation of 5-HT_{2A} receptors in both human MDMA users and rats given MDMA (Reneman *et al*, 2002b; Scheffel *et al*, 1992). However, both of these previous studies have suggested that recovery may occur, with receptor density returned to normal levels in rats within 21 days of MDMA treatment in one study (Scheffel *et al*, 1992). In the other study, rats given an aggressive dose regime of MDMA (10 mg/kg twice a day for 4 days), which produced near 90% depletion of 5-HT, were associated with downregulation of 5-HT_{2A} receptors at 3 but not 30 days following administration. Indeed, at 30 days following administration, upregulation of 5-HT_{2A} receptors was observed in the frontal cortex. Similar upregulation of 5-HT_{2A} was observed in the occipital cortex of abstinent human MDMA users, and this was interpreted at a compensatory response to serotonergic denervation (Reneman *et al*, 2000, 2002b). However, an alternative explanation concerns the increased availability to bind due to lack of competition with depleted endogenous 5-HT. It is also notable that in one human study recent users of MDMA (on average 3 weeks since the last tablet) showed significant reductions in 5-HT_{2A} binding in all brain regions examined, more in agreement with the current results (Reneman *et al*, 2002b). The discrepancy between the current results and those of previous studies may well be a reflection of the more moderate dosing regimes used here, which produced modest or no 5-HT depletion.

The 5-HT_{2A} receptor plays a role in the acute response to MDMA, with 5-HT_{2A} antagonists reducing the locomotor response to MDMA (Bankson and Cunningham, 2002; Kehne *et al*, 1996) and MDMA-induced hyperthermia (Mechan *et al*, 2002; Schmidt *et al*, 1990). A role for the 5-HT_{2C} receptor in acute MDMA effects is also suggested by the fact that a 5-HT_{2C} receptor antagonist potentiates MDMA-induced hyperactivity (Bankson and Cunningham, 2002). A diminished endocrine and emotional response to the 5-HT_{2C} receptor agonist mCPP has been reported in human MDMA users (McCann *et al*, 1999), which is also consistent with downregulation of that receptor with MDMA use.

The magnitude of loss of 5-$HT_{2A/2C}$ receptors in the present study was in line with the effects seen on anxiety-like behaviors, with a large, highly significant effect in the high-dose MDMA group and a more modest yet nonetheless significant effect in the low-dose MDMA group. Is it therefore conceivable that changes in one, other, or both of these receptors explain the magnification of anxiety-like behavior seen? Again, the answer to this question is not clear. Drugs acting as antagonists at 5-HT_{2C} receptors are clearly anxiolytic (Kennett *et al*, 1997; Martin *et al*, 2002) while the anxiolytic effects of 5-HT_{2A} acting drugs are less certain (Griebel *et al*, 1997). An anxiolytic action of antagonists at 5-HT_{2C} receptors suggests that loss of this receptor with MDMA treatment might have anxiolytic, rather than anxiogenic, effects.

Interestingly however, mutant mice lacking 5-HT_{2C} receptors take longer to emerge into a novel open field (Tecott *et al*, 1998), similar to the effect observed in MDMA-treated rats tested in the present study. Thus, a role for long-term alterations in 5-HT_{2C} receptors in MDMA-induced anxiety cannot be ruled out, although clearly much further work is required to confirm this.

Conclusions

As a recent commentary has noted (Green and McGregor, 2002), the role of 5-HT systems in anxiety is exceedingly complex, and defies simplistic explanations in terms of a one-to-one correspondence between anxiety and endogenous 5-HT levels. This conclusion is certainly reinforced in the present study, where reliable long-term changes in anxiety-like behavior were seen in rats following low-dose MDMA in the absence of any effects on tissue concentrations of 5-HT.

This raises the important question of the mechanism underlying the long-term anxiogenic effect of low-dose MDMA. Low-dose MDMA-treated rats displayed a variety of 5-HT transporter and receptor-related changes, any, all or none of which may be associated with altered anxiety. These include a reduction of SERT binding in the hypothalamus, decreased 5-HT_{1B} receptor binding in the hippocampus,

globus pallidus and thalamus, and decreased cortical 5-$HT_{2A/2C}$ binding. Any or all of these changes could conceivably be linked to alterations in anxiety-like behavior.

However, it is important to recognize that receptor binding and tissue monoamine concentrations give insight into only a small subset of the potential mechanisms whereby MDMA may exert a long-term influence on brain and behavior. Other useful avenues of enquiry could examine changes in the efficacy of 5-HT receptor subtypes, changes in the transcription of 5-HT-related receptors, and alterations of basal and stimulated 5-HT within the synapse. The ability of MDMA to alter endocrine function chronically (Forsling *et al*, 2002) is another avenue through which anxiety may be affected as well as global effects on brain energy metabolism (Darvesh *et al*, 2002). Thus, it would be premature to conclude that the present study has isolated the mechanism through which MDMA chronically increases anxiety in rats. However, it has provided some interesting clues that can be followed up in future studies.

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