

Chronic Fluoxetine Treatment Partly Attenuates the Long-Term Anxiety and Depressive Symptoms Induced by MDMA ('Ecstasy') in Rats

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Use of the drug 3,4-methylenedioxymethamphetamine (MDMA, 'Ecstasy') can have long-term adverse effects on emotion in both humans and laboratory animals. The present study examined whether chronic treatment with the antidepressant drug fluoxetine could reverse such effects. Male Wistar rats were briefly exposed to MDMA (4 × 5 mg/kg over 4 h) or vehicle on 2 consecutive days. Approximately 9–12 weeks later, half of the rats received a dose of approximately 6 mg/kg/day fluoxetine in their drinking water for a 5-week period. Fluoxetine administration reduced fluid intake and body weight in MDMA and vehicle pretreated rats. After several weeks of fluoxetine treatment, rats were assessed on the social interaction test, the emergence test of anxiety and the forced swim model of depression. MDMA pretreated rats showed reduced social interaction, increased anxiety on the emergence test, and increased immobility and decreased active responses in the forced swim test. Fluoxetine treatment reversed MDMA-induced anxiety in the emergence test and depressive-like effects in the forced swim test, yet exhibited no effects on the social interaction test. MDMA pretreated rats had decreased 5-HT and 5-HIAA levels in limbic and cortical regions, and decreased density of serotonin transporter sites in the cortex. Fluoxetine treatment did not greatly affect 5-HT levels in MDMA pretreated rats, but significantly decreased 5-HIAA levels in all brain sites examined. Postmortem blood serum levels of fluoxetine and norfluoxetine did not differ in MDMA and vehicle pretreated rats. These results indicate that fluoxetine may provide a treatment option for some of the deleterious long-term effects resulting from MDMA exposure.

Neuropsychopharmacology (2004) **29**, 694–704, advance online publication, 12 November 2003; doi:10.1038/sj.npp.1300347

Keywords: MDMA; ecstasy; anxiety; depression; serotonin; 5-HT; 5-HIAA; fluoxetine; antidepressant

INTRODUCTION

3,4-Methylenedioxymethamphetamine (MDMA: 'Ecstasy') is an illicit drug of growing popularity in many countries. The long-term physical and psychological effects of this drug are a matter of some concern, with evidence that MDMA has adverse effects on serotonin (5-HT) containing neurons in humans and laboratory animals (Boot *et al*, 2000; Parrott, 2001; Ricaurte *et al*, 2000). There is a need to better understand the deleterious psychological effects that may follow from this neurotoxic action.

Recent studies have linked MDMA use in humans to long-term psychological problems including depressed mood

(MacInnes *et al*, 2001; Morgan, 2000; Parrott *et al*, 2002) and increased anxiety (Gamma *et al*, 2000; McGuire, 2000; Parrott *et al*, 2002, 2000; Schifano *et al*, 1998; Verkes *et al*, 2001; Wareing *et al*, 2000). However, human studies rarely provide convincing proof of a causal role for MDMA in such effects. The methodology of many studies can be criticized with typical problems including the self-selection of subjects, insufficient consideration of pre-MDMA psychopathologies, and the fact that most MDMA users consume many drugs other than MDMA (Boot *et al*, 2000; Cole *et al*, 2002).

Studies with laboratory animals can help to resolve confounds inherent in human research. In recent work, we have discovered a 'MDMA syndrome' in rats given brief exposure to the drug. When tested weeks or months following brief exposure to MDMA, rats show decreased social interaction, increased anxiety on the elevated plus maze and emergence tests, poorer memory in the object recognition test and depressive-like symptoms in the forced swim test (Gurtman *et al*, 2002; McGregor *et al*,

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Received 16 May 2003; revised 16 September 2003; accepted 07 October 2003

Online publication: 9 October 2003 at <http://www.acnp.org/citations/Npp10090303217/default.pdf>

2003a,b; Morley *et al*, 2001). These behavioral changes are associated with changes in the regional density of the serotonin transporter (SERT), 5-HT_{2A/2C}, and 5-HT_{1B} receptors (McGregor *et al*, 2003a) and, to a certain extent, changes in tissue levels of 5-HT and its metabolite 5-HIAA (Gurtman *et al*, 2002; McGregor *et al*, 2003a,b).

Surveys indicate that many MDMA users appear to be seeking professional help for MDMA-related emotional problems (Topp *et al*, 1999). The mainstay for treatment of anxiety and depression are specific serotonin re-uptake inhibitor (SSRI) drugs such as fluoxetine (Prozac) (Vaswani *et al*, 2003; Wong *et al*, 1995). However, if MDMA permanently damages 5-HT neurons, it is uncertain whether SSRI drugs will have efficacy in treating MDMA users, even after periods of abstinence from MDMA. In the present study, we attempted to model this situation: rats were pretreated with MDMA and, 1 month later, commenced chronic treatment with fluoxetine. After a minimum of 3 weeks of fluoxetine treatment, they were assessed in animal models of social interaction, anxiety, and depression. Brains were assessed for neurotransmitter content and SERT density. Blood levels of fluoxetine and its principal active metabolite norfluoxetine were assayed using HPLC.

Clearly, in studies involving chronic administration of SSRIs, the route and frequency of administration may critically influence experimental outcomes. Daily intraperitoneal injection of fluoxetine may not allow steady-state levels to be achieved, because of the extremely long half-life of fluoxetine and its primary metabolite norfluoxetine (Benmansour *et al*, 1999; Vaswani *et al*, 2003). Delivery via an osmotic mini-pump may provide capacity for only 2 weeks of continuous dosing with fluoxetine. In the present study, where we wished to maintain rats on fluoxetine for more than 30 days, we presented the fluoxetine in the drinking water of the rats (Silva and Brandao, 2000). A low concentration was used that was anticipated to be well tolerated by the rats, yet be at a sufficient level to have potential therapeutic effects.

METHODS

Subjects

The subjects were 51 inbred male albino Wistar rats bred in our own facility, weighing on average 332 ± 8.6 g at the start of testing. Rats were housed in groups of no more than eight per cage during all phases of the experiment. Food (Young's Stock Feeds Rat and Mouse Breeder cubes, Allied Feeds, Sydney) was freely available to all subjects during all phases of the experiment. Water was freely available to all subjects except during fluoxetine treatment, when 26 subjects had their water replaced by fluoxetine solution (see below). The colony room temperature was controlled at 22°C with a 12h reverse light cycle (lights on at 20:30h). All behavioral testing was conducted during the dark cycle. All experimentation was approved by the University of Sydney Animal Ethics Committee, in accordance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*.

Experimental Procedures

Acute drug treatment. (\pm)3,4-Methylenedioxymethamphetamine hydrochloride was supplied by the Australian Government Analytical Laboratories (Pymble, NSW), and was diluted in 0.9% saline. Acute administration of MDMA involved procedures reported previously (Gurtman *et al*, 2002; McGregor *et al*, 2003a,b; Morley *et al*, 2001). Rats received a 5 mg/kg i.p. injection of MDMA ($n=26$) or saline ($n=25$) every hour for 4 h on 2 consecutive days, giving a cumulative dose of 40 mg/kg. This dose regime of MDMA produces long-term behavioral and neurochemical effects (Gurtman *et al*, 2002; McGregor *et al*, 2003a,b; Morley *et al*, 2001).

During MDMA or vehicle administration, individual rats were placed in standard operant chambers ($30 \times 50 \times 25.5$ cm) with three aluminum walls, one Perspex wall and a metal grid floor. The walls of the chambers were fitted with two passive infrared detectors that were triggered by movements of the head and body of the rats, as well as gross locomotion. Activity counts were recorded by a Macintosh computer running 'WorkbenchMac' data acquisition software. The chambers were enclosed in wooden sound attenuation boxes. Room temperature was maintained at an ambient temperature of 28°C by a reverse-cycle air conditioner. High temperatures may exacerbate the neurotoxic effects of MDMA in rats (Malberg and Seiden, 1998) and better simulate the conditions under which MDMA is often taken by humans.

The body temperature of all rats was recorded each hour with a Braun Thermoscan Instant Thermometer (IRT 1020) at the time of each injection. This procedure provides a rapid reading of body temperature and has been used previously with minimal stress compared to other procedures (Gurtman *et al*, 2002; McGregor *et al*, 2003b; Morley *et al*, 2001). Following the 4 h drug administration period, all rats were housed individually in the colony overnight and replaced back in their home cages the following morning. This procedure prevents the possible lethal effects of 'aggregation toxicity' sometimes seen with group housing following high-dose stimulant treatment (Green *et al*, 1995).

The MDMA administration phase was staggered over 3 weeks, controlling for age and weight at the time of MDMA treatment. The interval between MDMA treatment and subsequent fluoxetine treatment and behavioral testing of rats varied by up to 3 weeks across subjects.

Chronic fluoxetine treatment. At 9–12 weeks following MDMA treatment, the MDMA and vehicle groups were subdivided so that half received chronic fluoxetine (FLX) treatment, while the other half continued to receive standard drinking water. This resulted in four groups: VEH ($n=12$), VEH/FLX ($n=13$), MDMA ($n=13$), and MDMA/FLX ($n=13$). Rats were re-housed into cages of 6–7, in a way that ensured minimal weight differences between treatment groups. There were four fluoxetine-treated home cages and four receiving plain drinking water. Each cage contained approximately equal numbers of MDMA and vehicle pretreated rats.

Fluoxetine hydrochloride ((\pm)-N-methyl- γ -(4-[trifluoromethyl]-phenoxy)-benzenepropanamine) was obtained from Sigma (St Louis, USA). A target dose of 7 mg/kg/day

fluoxetine was chosen on the basis of effective doses for modifying behavior with chronic administration in previous studies (Berton *et al*, 1999; Contreras *et al*, 2001; Durand *et al*, 1999; File *et al*, 1999; Griebel *et al*, 1999; Jones *et al*, 2002; Silva and Brandao, 2000; To *et al*, 1999). Estimating that a 500 g rat would drink approximately 20 ml of fluoxetine solution per day, the drug was dissolved in tap water at a concentration of 0.175 mg/ml, to approach the target dose of 7 mg/kg/day. The chronic fluoxetine regime was maintained throughout the testing period, a total of 37 days, until the rats were killed at the end of the experiment. Body weights and fluid intake were recorded regularly throughout the period of fluoxetine administration.

Social interaction test. At 3 weeks following the start of fluoxetine treatment and a total of 12–15 weeks following MDMA administration, pairs of rats were assessed in the social interaction test, as described previously (File and Hyde, 1978; McGregor *et al*, 2003a). Pairs of rats were tested together, with each pair of approximately equal body weight and from the same treatment condition, but from a different home cage. Owing to uneven group numbers, one rat from each of the MDMA and MDMA/FLX conditions was tested twice in the social interaction test, with a different partner each time.

Pairs of rats were placed in a square black Perspex box ($52 \times 52 \times 40 \text{ cm}^3$) dimly lit with red light (40 W). A video camera located above the apparatus allowed live scoring of the interactions in an adjacent room by an experimenter who was blind to group allocations. Each social interaction session lasted for 10 min during which the total duration of social interaction and number of interaction bouts were scored by the observer using ODLog software (www.macropodsoftware.com). The test arena was wiped down with 10% ethanol in between each test session. Behaviors that were recorded as social interaction included sniffing, adjacent lying, following, crawling over/under, and mutual grooming.

Emergence test. At 1 day following the social interaction test, the rats were tested in the emergence test as described previously (McGregor *et al*, 2003a; Minor *et al*, 1994). The apparatus consisted of a black wooden rectangular arena ($96 \times 100 \times 40 \text{ cm}^3$) with a black wooden hide box ($24 \times 40 \times 15 \text{ cm}^3$) placed in the top right corner of the arena. The open part of the arena was illuminated with a fluorescent light. A video camera was mounted above the arena and connected to a video recorder, allowing live scoring by an observer in an adjacent room. Analysis was accomplished using ODLog data-logging software with an observer blind to group assignment. 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, with the following behaviors scored: (a) Emergence latency: the time taken for the rat to fully emerge from the hide box, (b) Open field time: the time spent exploring the open field, and (c) Risk assessment: the time spent with part but not all of the head/body protruding from the hide box. After each test session, the apparatus was thoroughly wiped down with a damp cloth containing 10% ethanol.

Forced swim test. At 8 days following the emergence test, the rats were exposed to the forced swim test as described previously (Blokland *et al*, 2002; McGregor *et al*, 2003b; Porsolt *et al*, 1978). Rats were placed in cylindrical clear Perspex tubes (40 cm high \times 17 cm diameter) filled to a height of 27.5 cm with water at a temperature of 23°C. This water height was chosen to prevent the animal from touching the bottom of the container, while at the same time preventing escape from the apparatus (Detke and Lucki, 1996). The tubes were located in a room illuminated with a 40 W dim red light, and were cleaned and refilled with fresh water in between each trial. A miniature video camera was located near the apparatus with pictures relayed to a 'blind' observer in an adjacent room, who scored using ODlog software. Behaviors scored included swimming, climbing, and immobility. Rats were tested for 5 min on each of 2 consecutive days.

Neurochemical analysis. At 1 week after the forced swim test, all rats were decapitated using a guillotine, their brains rapidly removed, and five brain regions of interest manually dissected out over dry ice, using methods previously reported (McGregor *et al*, 2003b). Samples from the prefrontal cortex, striatum, hippocampus, amygdala, and hypothalamus were stored in a freezer at -80°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 000g for 10 min at 4°C and a 20 μl aliquot of the resulting supernatant fluid was then analysed by high-performance liquid chromatography (HPLC).

The HPLC system consisted of a Shimadzu ADVP module (Kyoto, Japan) equipped with SIL-10 autoinjector with sample cooler and LC-10 on-line vacuum degassing solvent delivery unit. Chromatographic control, data collection, and processing were carried out using Shimadzu Class VP data 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-hydroxyindole acetic acid (5-HIAA), 5-HT, and 5-HMeT were separated by a Merck LiChrospher 100 RP-18 reversed-phase column. Quantification was achieved via a GBC LC-1210 electrochemical detector (Melbourne, Australia) equipped with a glassy carbon working electrode set at +0.75 V. The calibration curve of each standard was obtained by the concentration *vs* the area ratio of the standard and internal standard.

SERT binding. Samples of prefrontal cortex from the contralateral side of the brain to that used for HPLC analysis were used to assay SERT density. This analysis was performed for four randomly selected rats from each of the four groups. The samples were individually homogenized in 40-vol ice-cold Tris/HCl buffer (120 mM NaCl, 5 mM KCl, pH 7.4) and centrifuged (20 000g, 20 min, 4°C). Supernatants were discarded and the pellet re-suspended in 40-vol Tris/HCl and centrifuged again at 15 000g (10 min, 4°C). Prior to the third centrifugation (20 000g, 10 min,

4°C), re-suspended pellets were incubated for 15 min at 35°C to remove endogenous 5-HT. Final pellets were re-suspended in 15-vol Tris/HCl buffer and added to the reaction mix, which consisted of one of six concentrations of ³H-citalopram (84.2 Ci/mmol, Perkin-Elmer, Australia) ranging from 0.3 to 11 nM. Nonspecific binding was detected in the presence of 1 μM Fluoxetine HCl. The reaction was carried out for 1 h at room temperature, and terminated by the addition of 4 ml ice-cold Tris/HCl, followed by rapid filtration through a Whatman GF/B filter paper (presoaked in 0.01% polyethyleneimine, 1 h, 4°C). Filters were washed twice, transferred to scintillation vials, liquid scintillant added, and samples counted the next day.

Analysis of serum fluoxetine. At the time of decapitation, blood was collected in prechilled tubes, allowed to clot, and serum separated from cells by centrifuging (3300g, 4°C, 15 min). Serum samples were stored at -20°C and thawed just prior to analysis. For analysis, 0.5 ml of serum was spiked with 20 μl of internal standard in an Eppendorf tube, to give a final serum concentration of 2 μmol/l. The sample was then diluted with 0.5 ml of 0.1 M KH₂PO₄ buffer (pH 6.0) and mixed gently. The SPEC-DAU micro-disc SPE cartridges (Varian, Melbourne, Australia) were connected to a Vac Elut and conditioned with 0.5 ml methanol, followed by 0.5 ml of 0.1 M KH₂PO₄ buffer (pH 6.0). Serum samples were then applied to each cartridge. The sample was allowed to run through the disc at a low flow rate of no more than 1 ml/min. The cartridge was then rinsed with 0.5 ml 1 M acetic acid, followed by 0.5 ml methanol. The disc was dried under vacuum for about 2 min. The tips of the Vac Elut delivery needles were wiped and a rack with labeled collection microtubes was placed in the Vac Elut. The analytes were eluted with 0.5 ml of dichloromethane-isopropanol-ammonia (80:20:2 v/v), at a flow rate of no more than 1 ml/min. The elutant was then dried under vacuum in a SpeedVac vacuum evaporator (Savant Instruments, Farmingdale, NT, USA) and the dried residue was re-dissolved in 50 μl of mobile phase. The mixture was then vortexed and centrifuged to remove particulates, the supernatant transferred to micro insert vials and 20 μl of reconstituted solution was automatically injected into the HPLC system. Serum calibration curves of 100–4000 nmol/l of each analyte were also prepared and extracted similarly. The concentrations of fluoxetine and norfluoxetine in the unknown samples were calculated from the least-squares linear regression equation of the calibration curve.

Chromatographic separation of fluoxetine, norfluoxetine, and the internal standard clomipramine was accomplished using the previously described HPLC system on a Waters Symmetry C₈ 5 μm (2.1 × 150 mm) micro-bore reverse-phase column (Waters, Australia) coupled with a 3 mm Opti-Guard C₈ pre-column (Optimize Technologies, Alpha Resources, Thornleigh, Australia). The mobile phase consisted of a mixture of 67 mmol/l potassium phosphate buffer (pH 3.0) and acetonitrile (67:33 v/v). The flow rate was maintained isocratically at 0.3 ml/min. The eluate from the HPLC column was directed via a GBC LC1200 UV-VIS detector (Melbourne, Australia) monitored at 226 nm. The total run time was 15 min.

Statistical analysis. For the MDMA treatment phase, repeated-measures ANOVA was used to compare locomotor activity and body temperature in MDMA, and vehicle-treated rats, across the 4 h of testing on each day of treatment. Differences between groups for each hour of testing were subsequently analysed using *post hoc* contrasts.

For subsequent behavioral and neurochemical variables, data from the four experimental groups (VEH, VEH/FLX, MDMA, MDMA/FLX) were compared using one-way analysis of variance (ANOVA), followed by Fisher's PLSD *post hoc* comparisons. Fluid consumption and body weight during fluoxetine administration were analysed via repeated-measures ANOVA with group and time as the independent variables. Log transformation of data was occasionally performed when significant skew was evident in the raw data.

Data were analysed using Statview 5.0 software for Macintosh, with significance levels set at 0.05 for all tests.

RESULTS

Locomotor Activity

Locomotor activity during acute MDMA administration is shown in Figure 1. On day 1, repeated-measures ANOVA showed a significant overall effect of drug treatment ($F_{1,49} = 15.54$, $P < 0.001$) and a significant treatment by time interaction ($F_{3,147} = 99.28$, $P < 0.0001$). Subsequent analysis showed that locomotor activity was significantly lower during the first hour in MDMA-treated rats than controls, but was significantly higher in hour 2, hour 3, and hour 4 of testing (Figure 1).

A similar pattern was observed on day 2, with a significant overall effect of drug treatment ($F_{1,49} = 27.79$, $P < 0.0001$) and a significant treatment by time interaction ($F_{3,147} = 45.01$, $P < 0.0001$). MDMA-treated rats exhibited less locomotor activity than controls in the first hour, but greater activity in hour 2, hour 3, and hour 4 (Figure 1).

Body Temperature

The effects of MDMA on body temperature are shown in Figure 1. On day 1, repeated-measures ANOVA showed a significant overall effect of drug treatment ($F_{1,49} = 177.89$, $P < 0.0001$) and a significant treatment by time interaction ($F_{4,196} = 79.98$, $P < 0.0001$). Subsequent analysis showed that MDMA-treated rats showed no difference to controls in predrug baseline temperature, but exhibited higher temperatures than vehicle-treated rats on day 1 at hour 1, hour 2, hour 3, and hour 4 (Figure 1).

Similar results were obtained on day 2 with a significant overall effect of drug treatment ($F_{1,49} = 139.42$, $P < 0.0001$) and a significant treatment by time interaction ($F_{4,196} = 53.73$, $P < 0.0001$). There were no group differences in temperature at baseline or hour 1, but significant differences at hour 2, hour 3, and hour 4 (Figure 1).

Fluoxetine Intake and Body Weight

The intakes of the water and fluoxetine solutions are shown in Figure 2. Data were not collected across days on which behavioral testing occurred, as on these days rats did not

have continuous access to fluids. Rats given chronic fluoxetine treatment consumed an average of 17.98 ml of fluoxetine solution per rat per day. This is equivalent to an average dose of 6.2 mg/kg of fluoxetine per day. Repeated measures ANOVA revealed that this was significantly lower intake than the average 30.23 ml per rat per day consumed by rats receiving water over the same period ($F_{1,24} = 78.11$, $P < 0.0001$). Fluid intake increased significantly over time in both treatment groups ($F_{24,144} = 10.88$, $P < 0.0001$), with this pattern significantly different between the two treatment groups ($F_{24,144} = 2.55$, $P < 0.001$).

Body weight results are shown in Figure 2. When weight across the four groups from 2 days prior to fluoxetine administration until the 33rd day of fluoxetine administration was compared, a significant overall group effect was evident ($F_{3,47} = 34.91$, $P < 0.0001$). *Post hoc* tests showed that both of the groups given fluoxetine (VEH/FLX and MDMA/FLX) gained significantly less weight than the groups given

water (VEH and MDMA). There was no significant difference in weight gain between group VEH/FLX and group MDMA/FLX or between group VEH and group MDMA over the 33 days.

Social Interaction Test

Social interaction results are shown in Table 1. A significant overall group effect on social interaction time was obtained ($F_{3,22} = 6.48$, $P < 0.01$). *Post hoc* tests showed that both of the MDMA pretreated groups spent less time in social interaction than either of the vehicle pretreated groups. There was no significant difference between groups MDMA and MDMA/FLX or groups VEH and VEH/FLX, thus indicating the absence of a fluoxetine treatment effect on

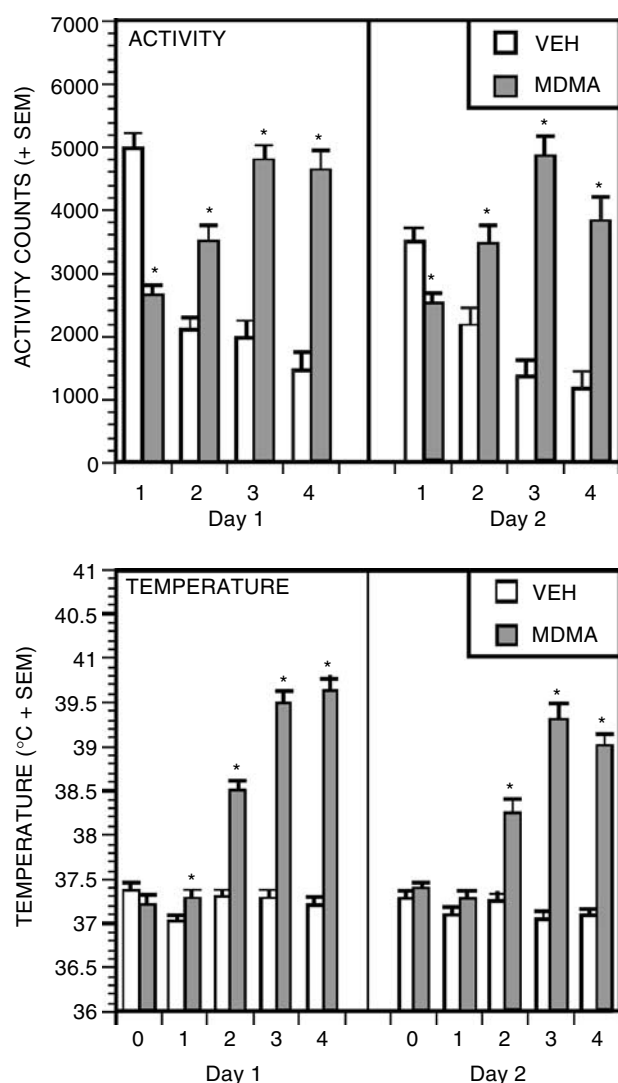


Figure 1 Mean locomotor activity counts (upper) and body temperature (lower) for vehicle (VEH) and MDMA-treated rats during day 1 and day 2 of drug treatment. Activity counts are shown for each of the 4 h of testing. Body temperature was measured immediately prior to drug administration (0) and at 1, 2, 3, and 4 h following the drug. * $P < 0.05$, comparing MDMA and vehicle-treated groups.

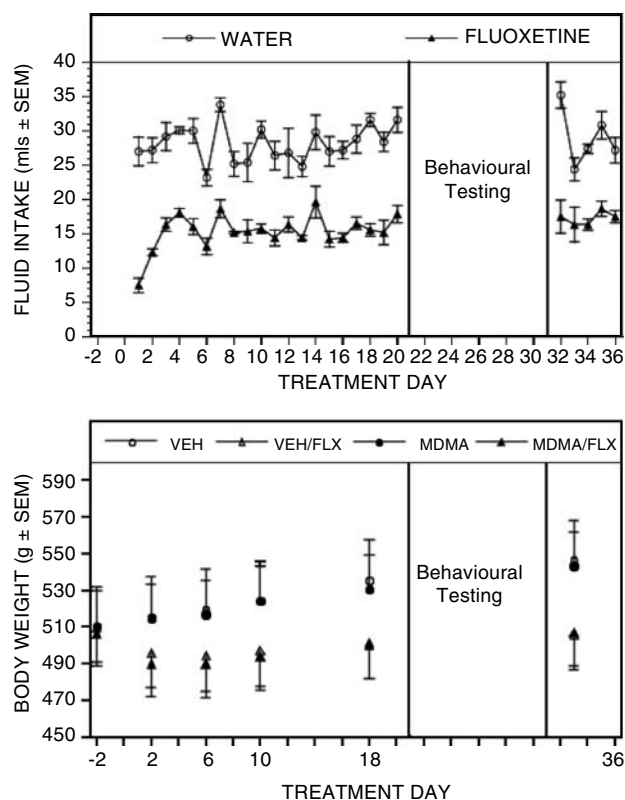


Figure 2 Mean fluid intake for water- and fluoxetine-treated rats (upper), and mean body weight for all treatment groups (lower) throughout the fluoxetine-treatment phase. Data for fluid intake during the behavioral testing period are not included, as rats did not have full 24 h access to fluids during this period. VEH = vehicle, FLX = fluoxetine.

Table 1 Results From Social Interaction Test ($n = 6-7$ per group)

	VEH	VEH/FLX	MDMA	MDMA/FLX
Interaction time (s)	126.03 (7.27)	136.15 (3.10)	100.51 (8.63) ^{a,b}	99.79 (7.48) ^{a,b}
Interactions (no.)	69.67 (3.40)	69.33 (3.56)	62.14 (4.88)	63.14 (3.99)

Data represent mean (SEM).

^aSignificantly different from the VEH group ($P < 0.05$).

^bSignificantly different from the VEH/FLX group ($P < 0.05$).

this model. There was no significant group effect for the number of social interactions ($F < 1$).

Emergence Test

The results of the emergence test are shown in Table 2. Data for three rats were lost due to a computer error. There were significant overall group effects for emergence latency ($F_{3,44} = 3.29$, $P < 0.05$) and for time spent in the open field (log transformed) ($F_{3,44} = 4.56$, $P < 0.01$). *Post hoc* tests showed that the MDMA group had higher emergence latencies and lower open field times than each of the other three groups (MDMA/FLX, VEH, VEH/FLX). No significant differences in risk assessment were observed between groups ($F > 1$).

Forced Swim Test

Results from each of the two days of the forced swim test are shown in Figure 3. There were significant overall group effects on day 1 for immobility ($F_{3,47} = 6.37$, $P < 0.001$), swimming (log transformed) ($F_{3,47} = 4.48$, $P < 0.01$), and climbing ($F_{3,47} = 2.99$, $P < 0.05$). *Post hoc* analysis showed that the MDMA group displayed greater immobility and less swimming than either the VEH or VEH/FLX groups, and showed less climbing than the VEH group. The MDMA/FLX group also showed less swimming than the VEH/FLX group.

On day 2 of testing, there were significant overall group effects for immobility ($F_{3,47} = 4.02$, $P < 0.05$) and swimming (log transformed) ($F_{3,47} = 5.19$, $P < 0.01$), but not climbing ($F < 2$). *Post hoc* analysis showed that MDMA group displayed greater immobility than the VEH or VEH/FLX groups, and less climbing than the VEH group. The MDMA group showed less swimming than the VEH, VEH/FLX, and MDMA/FLX groups.

HPLC Analysis of Brain Neurotransmitters

The levels of 5-HT, 5-HIAA, the 5-HIAA/5-HT ratio, and DA for the five brain regions investigated are presented in Table 3.

Prefrontal cortex. Results from the prefrontal cortex indicated a significant overall group effect for 5-HT ($F_{3,47} = 4.61$, $P < 0.01$), 5-HIAA ($F_{3,47} = 16.96$, $P < 0.001$), and the 5-HIAA/5-HT ratio ($F_{3,47} = 24.65$, $P < 0.001$), but not DA ($F < 1$). *Post hoc* analysis indicated that MDMA treatment significantly decreased 5-HT and 5-HIAA levels,

while fluoxetine treatment significantly decreased 5-HIAA levels (Table 3). Fluoxetine treatment also significantly decreased the 5-HIAA/5-HT ratio.

Striatum. Analysis of the striatum revealed the significant overall group effects for 5-HIAA ($F_{3,45} = 9.20$, $P < 0.001$) and the 5-HIAA/5-HT ratio ($F_{3,45} = 24.21$, $P < 0.001$), but not for 5-HT ($F_{3,45} = 1.51$, $P > 0.1$) or DA ($F_{3,45} = 1.74$, $P > 0.1$). *Post hoc* analysis indicated that both MDMA and fluoxetine treatments significantly decreased 5-HIAA levels (Table 3).

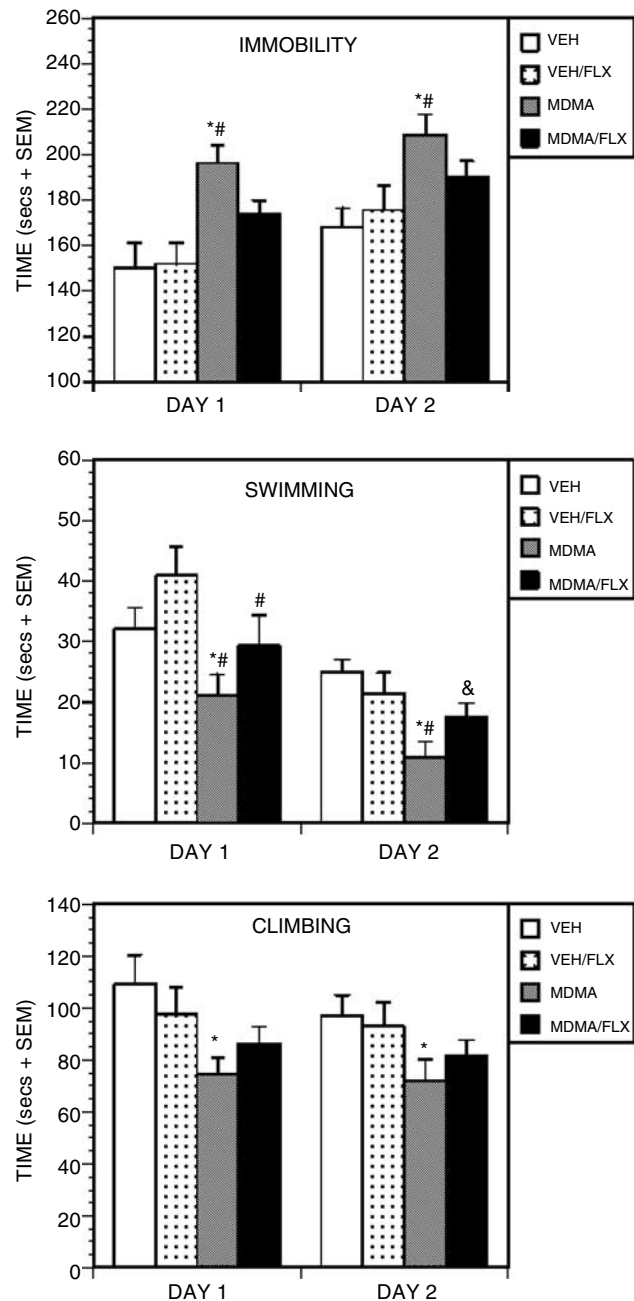


Figure 3 Mean time spent immobile (upper), swimming (middle), and climbing (lower) over the 2 days of the forced swim test. VEH = vehicle, FLX = fluoxetine. ^{*} $P < 0.05$, relative to VEH group, [#] $P < 0.05$, relative to VEH/FLX group, [&] $P < 0.05$, relative to MDMA group.

Table 2 Results From Emergence Test ($n = 12-13$ Per Condition)

	VEH	VEH/FLX	MDMA	MDMA/FLX
Latency (s)	90.85 (13.69)	85.60 (22.26)	146.05 (25.72) ^{a,b}	101.75 (15.29) ^c
Open time (s)	71.36 (11.17)	66.32 (10.58)	42.70 (11.26) ^{a,b}	62.33 (8.57) ^c
Risk assessment (s)	55.20 (5.06)	63.53 (7.11)	55.27 (5.22)	60.14 (7.01)

Data represent mean (SEM).

^aSignificantly different from the VEH group ($P < 0.05$).

^bSignificantly different from the VEH/FLX group ($P < 0.05$).

^cSignificantly different from the MDMA group ($P < 0.05$).

Table 3 Results of HPLC Analysis ($n = 12-13$ Per Condition)

Region	Measure	VEH	VEH/FLX	MDMA	MDMA/FLX
Prefrontal	5-HT	357.1 (19.6)	360.0 (15.6)	301.5 (9.2) ^{a,b}	297.0 (15.5) ^{a,b}
Cortex	5-HIAA	141.4 (6.9)	101.7 (5.0) ^a	122.4 (5.8) ^{a,b}	86.1 (5.7) ^{a,c}
	Ratio	0.40 (0.02)	0.29 (0.01) ^a	0.41 (0.01) ^b	0.29 (0.01) ^{a,c}
	DA	187.1 (28.2)	173.5 (20.5)	159.7 (18.1)	208.9 (37.3)
Striatum	5-HT	420.7 (15.5)	443.2 (30.0)	371.9 (19.5) ^b	405.6 (20.1)
	5-HIAA	445.0 (26.8)	308.0 (18.2) ^a	318.1 (19.4) ^a	273.6 (18.2) ^{a,c}
	Ratio	1.11 (0.07)	0.70 (0.03) ^a	1.04 (0.05) ^b	0.68 (0.03) ^{a,c}
	DA	9224 (470)	8050 (669)	9676 (564)	8262 (465)
Hippocampus	5-HT	367.8 (14.7)	310.5 (8.0) ^a	240.6 (18.4) ^{a,b}	238.1 (19.4) ^{a,b}
	5-HIAA	271.7 (12.0)	176.9 (7.8) ^a	169.9 (14.4) ^a	129.7 (9.4) ^{abc}
	Ratio	0.75 (0.04)	0.57 (0.01) ^a	0.71 (0.03) ^b	0.56 (0.02) ^{a,c}
	DA	31.8 (3.6)	28.3 (4.5)	30.4 (4.7)	27.6 (5.8)
Amygdala	5-HT	715.1 (52.8)	758.8 (23.9)	588.0 (25.8) ^{a,b}	655.9 (47.4)
	5-HIAA	323.6 (20.6)	239.2 (12.1) ^a	277.8 (20.9)	233.3 (23.4) ^a
	Ratio	0.48 (0.04)	0.32 (0.02) ^a	0.47 (0.02) ^b	0.39 (0.02) ^{a,c}
	DA	268.2 (37.2)	286.1 (47.7)	275.3 (24.4)	248.6 (34.0)
Hypothalamus	5-HT	807.7 (70.7)	711.9 (29.6)	742.1 (29.7)	719.7 (52.9)
	5-HIAA	359.1 (23.7)	235.5 (12.2) ^a	313.1 (17.3) ^b	230.4 (15.3) ^{a,c}
	Ratio	0.46 (0.032)	0.33 (0.02) ^a	0.42 (0.02) ^b	0.33 (0.02) ^{a,c}
	DA	478.3 (28.3)	416.6 (49.1)	398.5 (41.2)	392.5 (32.5)

Data represent mean (SEM). Units of measurement are ng/mg wet tissue. Ratio = ratio of 5-HIAA/5-HT.

^aSignificantly different from the VEH group ($P < 0.05$).

^bSignificantly different from the VEH/FLX group ($P < 0.05$).

^cSignificantly different from the MDMA group ($P < 0.05$).

Fluoxetine treatment also significantly decreased the 5-HIAA/5-HT ratio.

Hippocampus. Results from the hippocampus indicated significant overall group effects for 5-HT ($F_{3,47} = 17.64$, $P < 0.001$), 5-HIAA ($F_{3,47} = 30.96$, $P < 0.0001$), and the 5-HIAA/5-HT ratio ($F_{3,47} = 12.37$, $P < 0.001$), but not DA ($F < 1$). *Post hoc* analysis indicated that fluoxetine treatment significantly decreased 5-HIAA levels (Table 3), and the 5-HIAA/5-HT ratio.

Amygdala. Results from the amygdala indicated a significant overall group effect for 5-HT ($F_{3,47} = 3.66$, $P < 0.05$), 5-HIAA ($F_{3,47} = 4.35$, $P < 0.01$), and the 5-HIAA/5-HT ratio ($F_{3,47} = 3.09$, $P < 0.05$), but not DA ($F < 1$). *Post hoc* analysis indicated that MDMA treatment significantly decreased 5-HT and 5-HIAA levels, while fluoxetine treatment significantly decreased 5-HIAA levels (Table 3). Fluoxetine treatment also significantly decreased the 5-HIAA/5-HT ratio.

Hypothalamus. Results from the hypothalamus indicated a significant overall group effect for 5-HIAA ($F_{3,37} = 12.21$, $P < 0.001$) and the 5-HIAA/5-HT ratio ($F_{3,37} = 11.874$, $P < 0.001$), but not for 5-HT ($F < 1$) or DA ($F < 1$). *Post hoc*

analysis indicated that fluoxetine treatment significantly decreased 5-HIAA levels (Table 3), and the 5-HIAA/5-HT ratio.

SERT Binding

SERT density could not be determined in the MDMA/FLX or VEH/FLX group due to competition between the fluoxetine in the brain tissue and the radioligand used for the assay (^3H -citalopram). Comparison between the MDMA and VEH groups showed significantly lower SERT density (Bmax) in the MDMA group ($F_{1,6} = 19.94$, $P < 0.01$). The mean values for Bmax were 481.3 ± 22.4 fmol/mg protein in the vehicle group compared to 324.4 ± 27.1 fmol/mg protein in the MDMA group. There were no significant differences in receptor affinity (Kd) between groups ($F < 1$). The mean values were 1.83 ± 0.21 nmol in the vehicle groups and 1.76 ± 0.09 nmol in the MDMA group.

Serum Fluoxetine and Norfluoxetine

Rats in the MDMA and VEH groups exhibited no traces of fluoxetine or norfluoxetine in their serum. There were no significant differences between the VEH/FLX and MDMA/

FLX groups in serum fluoxetine (281 ± 44 and 235 ± 36 nmol/l, respectively) or norfluoxetine (1209 ± 123 and 1206 ± 99 nmol/l, respectively, $F_s < 1$).

DISCUSSION

The present results suggest that fluoxetine may reverse some but not all of the long-term adverse changes resulting from brief MDMA exposure. The effects of fluoxetine are relatively modest in magnitude and do not include an amelioration of MDMA-induced social interaction deficits. This partial therapeutic outcome is of interest, as it raises the possibility that MDMA dysfunction may involve disparate neuronal systems, only some of which may be positively corrected by fluoxetine treatment.

Acute Drug Effects

As frequently reported in previous studies, MDMA treatment produced significant hyperthermia and hyperactivity at an ambient temperature of 28°C. This is consistent with our previous results using identical treatment regimes (Gurtman *et al*, 2002; McGregor *et al*, 2003b; Morley *et al*, 2001).

Rats given fluoxetine in their drinking water over several weeks consumed significantly less fluid than rats given water only. This agrees with the conclusions of at least one previous study, albeit one in which specific intake data were not presented (Silva and Brandao, 2000). The inhibition of fluid intake may reflect the well-documented anorexic effects of fluoxetine (Caccia *et al*, 1992a; Goudie *et al*, 1976; Stein *et al*, 1978): rats are prandial drinkers so that lowered food intake would also lead to lower water intake. Alternatively, reduced fluid intake may have resulted from a conditioned taste aversion produced by fluoxetine (Prendergast *et al*, 1996). Fluoxetine-treated rats also gained significantly less weight than controls, an effect that is consistent with other animal (Berton *et al*, 1999; Caccia *et al*, 1997; Durand *et al*, 1999) and human studies (Wong *et al*, 1995).

Fluoxetine-treated rats consumed an average of 6.2 mg/kg of fluoxetine per day. Fluid consumption could not be formally compared between the VEH/FLX and MDMA/FLX groups, as home cages contained equivalent number of rats from each group. However, no significant differences in weight gain were observed between these two groups, suggesting similar levels of intake. Moreover, there were no significant differences in serum fluoxetine or norfluoxetine levels between these two groups at the end of testing.

Serum levels of fluoxetine and norfluoxetine were comparable to those seen in previous studies, where the drug has been injected or gavaged into rats (Caccia *et al*, 1990; Durand *et al*, 1999). For example, Durand *et al* (1999) injected 10 mg/kg fluoxetine once a day for 3 weeks, and after a 30 h washout reported plasma levels of 314 ng/ml norfluoxetine, similar to the 1209 nmol/l (equivalent to 409 ng/ml) reported here. Caccia *et al* (1990) noted fluoxetine levels of approximately 300 nmol/l and norfluoxetine levels of 500 nmol/l at 6 h following oral administration of a single 10 mg/kg dose of fluoxetine to rats.

Although the method of fluoxetine administration used here is rarely seen in the literature (Silva and Brandao, 2000), it provides a convenient method compared to repeated injection or osmotic mini-pump.

Fluoxetine and MDMA Behavioral Effects

MDMA pretreatment resulted in significant behavioral effects on the social interaction, emergence, and forced swim tests, confirming our previous reports of an MDMA 'syndrome' in rats (Gurtman *et al*, 2002; McGregor *et al*, 2003a,b; Morley *et al*, 2001). As the behavioral deficits in the current study were observed approximately 8–13 weeks post-MDMA administration, these effects must reflect the enduring neural changes.

A reduction in social interaction in MDMA pretreated rats has now been reported across six different studies from two different laboratories with various MDMA treatment schedules (Bull *et al*, 2003; Fone *et al*, 2002; Gurtman *et al*, 2002; McGregor *et al*, 2003a,b; Morley *et al*, 2001). Interestingly, chronic fluoxetine administration had no remedial effect on this social interaction deficit with almost identical levels of social interaction for the MDMA/FLX and MDMA groups (Table 1). This suggests that, whatever the mechanism underlying the chronic adverse effects of MDMA on social behavior, it is not reversed by chronic SSRI treatment. It is possible that this social effect may be linked to alterations in the density of specific 5-HT receptors (Bull *et al*, 2003; McGregor *et al*, 2003a), and therefore not specifically amenable to treatments, such as fluoxetine, which principally increase the levels of synaptic 5-HT.

Fluoxetine given to control rats (group VEH/FLX) was without apparent effect in the social interaction test, in agreement with some previous reports (File *et al*, 1999; To *et al*, 1999). It remains possible, of course, that a higher dose fluoxetine regime might have been effective in the social interaction test or that the dosing regime used here could have been anxiolytic, had different testing conditions been used. It is notable that chronic paroxetine treatment is anxiolytic in the social interaction test when the test is conducted under brightly lit novel conditions (Lightowler *et al*, 1994).

Increased anxiety was observed in the emergence test in MDMA pretreated rats, in agreement with our previous reports. In MDMA pretreated rats, fluoxetine acted to both decrease the emergence latency and to increase time spent in the open field, an apparent anxiolytic effect. This was despite the lack of an anxiolytic effect of fluoxetine in the vehicle pretreated rats, confirming reports that have used other rat strains (Durand *et al*, 1999; Pare *et al*, 2001). It therefore appears that fluoxetine has some capacity to reverse the long-term anxiogenic effects of MDMA in exploration-based models of anxiety.

In the forced swim test, MDMA pretreated rats displayed significantly fewer active escape attempts (climbing and swimming) and significantly greater immobility. This agrees with our previous study (McGregor *et al*, 2003b) and suggests that MDMA pretreatment leads to a deficit in active coping responses under stressful conditions. Considering that the forced swim test is the most widely used tool for preclinical antidepressant activity (Cryan *et al*, 2002), these

results strengthen the claim that a neurotoxic dose of MDMA leads to increased depressive-like symptoms in animals. This converges with increasing human research that links MDMA consumption with depression (MacInnes *et al*, 2001; Morgan, 2000; Parrott *et al*, 2002). The magnitude of the MDMA effect is marginal but reliable, consistent with a report showing that MDMA users manifest mild rather than severe symptoms of depression (MacInnes *et al*, 2001).

In the forced swim test, fluoxetine treatment significantly increased swimming in MDMA pretreated rats. Fluoxetine also reduced immobility in MDMA pretreated rats, with group MDMA/FLX showing immobility levels that did not significantly differ from vehicle pretreated rats (groups VEH and VEH/FLX). Taken together, these findings provide evidence of some ameliorating effects of chronic fluoxetine treatment on the apparent depressive-like effects of MDMA in the forced swim test.

Although there was a trend for fluoxetine to increase swimming in vehicle pretreated rats (group VEH/FLX), this did not reach significance. This agrees with observations from acute treatment studies that high fluoxetine doses may be required for an antidepressant effect in the forced swim model (Cryan *et al*, 2002). Strain factors may also be a consideration: at least one recent study has failed to find an effect of chronic SSRI treatment on forced swim immobility in Wistar rats, despite clear effects being apparent in the Wistar-Kyoto strain (Tejani-Butt *et al*, 2003). Antidepressant effects in intact animals with fluoxetine have been frequently reported in the Sprague–Dawley strain (Detke *et al*, 1997; Detke and Lucki, 1996; Page *et al*, 1999), but infrequently, if ever, with Wistar strain rats. However, there is also at least one reported failure of chronic SSRIs to affect forced swim behavior in Sprague–Dawley rats at doses that clearly affected 5-HT turnover (Connor *et al*, 2000), suggesting that procedural factors in forced swim testing may also be important.

Interestingly, it has been reported that a more than 90% depletion of cerebral 5-HT with the 5-HT synthesis inhibitor PCPA completely prevented the antidepressant action of fluoxetine in the forced swim test (Page *et al*, 1999). Clearly, the much more modest depletion of 5-HT produced in the present study with MDMA still allowed fluoxetine to have a marked behavioral effect in not only the forced swim test but also in other tests. It is interesting to speculate as to whether the efficacy of fluoxetine would be absent in rats subjected to a more severe MDMA dose regime, resulting in a massive loss of cerebral 5-HT.

Neurochemical Results

In the present study, MDMA pretreated rats showed significantly reduced levels of 5-HT and 5-HIAA in most brain regions assayed. This agrees with the widely reported depleting effects of MDMA on 5-HT (Battaglia *et al*, 1987; Commins *et al*, 1987; O'Shea *et al*, 1998) and our own previous reports. MDMA-treated rats also showed a decreased density of SERT-binding sites in prefrontal cortex, in agreement with many previous studies (Battaglia *et al*, 1987; Lew *et al*, 1996; McGregor *et al*, 2003a; O'Shea

et al, 1998). SERT density via ^3H -citalopram binding could not be detected in groups MDMA/FLX and VEH/FLX, due to the long half-life of fluoxetine and norfluoxetine. A considerable drug washout period would have been required for SERT density to be accurately identified in these groups using standard receptor-binding assays (Durand *et al*, 1999).

Neurochemical analysis revealed that fluoxetine significantly reduced 5-HIAA levels in all brain regions examined. This effect of fluoxetine mirrors previous reports (Caccia *et al*, 1992a, 1992b; Durand *et al*, 1999; Hrdina, 1987) and likely reflects decreased reuptake of 5-HT in fluoxetine-treated rats, preventing metabolism of 5-HT to 5-HIAA by intraneuronal monoamine oxidase A. Chronic fluoxetine treatment also caused a modest yet significant reduction in 5-HT in the hippocampus, as has been previously noted (Caccia *et al*, 1992b). This may reflect fluoxetine-induced reductions in 5-HT synthesis (Muck-Seler *et al*, 1996). The large effect of fluoxetine on 5-HIAA coupled to modest effects on 5-HT brought about a significant reduction in 5-HT utilization measures (5-HIAA/5-HT ratio) in all the five brain regions examined. This confirms that the dose of fluoxetine administered in this experiment was successful in altering 5-HT uptake and metabolism, and possibly 5-HT synthesis.

An interesting question is whether the behavioral differences evident between groups MDMA and MDMA/FLX can be explained at the neurochemical level. Clearly, the major difference between these two groups was the much lower rate of 5-HT utilization in the MDMA/FLX group. Most likely then, the increased synaptic availability of 5-HT in the MDMA/FLX group has helped reverse the functional effects of 5-HT depletion produced by MDMA in the emergence and forced swimming tests. The exact neuroanatomical sites underlying such effects require further investigation.

CONCLUSIONS

The present study indicates that fluoxetine has some remedial effects on the anxiety and depressive symptoms resulting from MDMA use. Results are not so encouraging, however, with respect to the social interaction test. This suggests that MDMA may have adverse effects on a broader range of functional systems than fluoxetine acts upon. The three behavioral tests used in this study model different emotional states, and it is possible that fluoxetine is only of use in specific stress-related behaviors, even in a compromised system. As the recreational consumption of MDMA continues to grow around the world, there is increasing concern as to the long-term neural and behavioral toxicity of the drug. While there is as yet no ultimate causal link between MDMA use and functional deficits in humans, convergent data from both human and animal research make this prospect likely. This is the first study to our knowledge that has investigated the possibility of a systematic treatment of these long-term deficits, and it is hoped that the data that have emerged will provide some insight into avenues for successful treatment of human MDMA users who encounter long-term emotional problems.

ACKNOWLEDGEMENTS

This work was supported by an NH&MRC grant to Iain S McGregor and Glenn E Hunt. We are grateful to Varian for the gift of solid-phase extraction columns, to Ljiljana Sokolic for technical assistance, and to Darek Figa and Debbie Brookes for their assistance with animal care.

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