

# Pharmacokinetic Profile of Single and Repeated Oral Doses of MDMA in Squirrel Monkeys: Relationship to Lasting Effects on Brain Serotonin Neurons

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A large body of data indicates that ( $\pm$ )3,4-methylenedioxymethamphetamine (MDMA, 'ecstasy') can damage brain serotonin neurons in animals. However, the relevance of these preclinical data to humans is uncertain, because doses and routes of administration used in animals have generally differed from those used by humans. Here, we examined the pharmacokinetic profile of MDMA in squirrel monkeys after different routes of administration, and explored the relationship between acute plasma MDMA concentrations after repeated oral dosing and subsequent brain serotonin deficits. Oral MDMA administration engendered a plasma profile of MDMA in squirrel monkeys resembling that seen in humans, although the half-life of MDMA in monkeys is shorter (3 vs 6–9 h). MDMA was biotransformed into MDA, and the plasma ratio of MDA to MDMA was 3–5/100, similar to that in humans. MDMA accumulation in squirrel monkeys was nonlinear, and plasma levels were highly correlated with regional brain serotonin deficits observed 2 weeks later. The present results indicate that plasma concentrations of MDMA shown here to produce lasting serotonergic deficits in squirrel monkeys overlap those reported by other laboratories in some recreational 'ecstasy' consumers, and are two to three times higher than those found in humans administered a single 100–150 mg dose of MDMA in a controlled setting. Additional studies are needed on the relative sensitivity of brain serotonin neurons to MDMA toxicity in humans and non-human primates, the pharmacokinetic parameter(s) of MDMA most closely linked to the neurotoxic process, and metabolites other than MDA that may play a role.

*Neuropsychopharmacology* (2006) **31**, 339–350. doi:10.1038/sj.npp.1300808; published online 6 July 2005

**Keywords:** MDMA; serotonin; pharmacokinetics; plasma; amphetamines

## INTRODUCTION

( $\pm$ )3,4-Methylenedioxymethamphetamine (MDMA, 'ecstasy') has become a popular recreational drug in many developed countries (Degenhardt *et al*, 2004; EMCDDA, 2003; Johnston *et al*, 2004), with annual and lifetime use rates in the US now comparable to those of cocaine (Johnston *et al*, 2004). A large body of data, summarized below, indicates that MDMA has the potential to damage to brain serotonin (5-hydroxytryptamine, 5-HT) neurons in various animal species, including non-human primates (Steele *et al*, 1994; Kalant, 2001; Green *et al*, 2003).

However, the clinical relevance of much of these preclinical data remains uncertain because doses and routes of administration used in animals have generally differed from those used by humans (Hegadoren *et al*, 1999; Cole and Sumnall, 2003).

MDMA's potential to damage brain 5-HT neurons first became apparent when Schmidt (1987) reported that animals treated with single doses of MDMA developed lasting reductions in various markers of brain 5-HT neurons. Numerous subsequent studies have shown that MDMA-treated animals developed long-term (2 weeks) depletions of brain 5-HT, its major metabolite, 5-hydroxyindoleacetic acid (5-HIAA), its rate-limiting enzyme, tryptophan hydroxylase (TPH), its membrane transporter (5-HTT), and its vesicular monoamine transporter, VMAT<sub>2</sub> (see Steele *et al*, 1994; Gibb *et al*, 1994; Lew *et al*, 1997; Green *et al*, 2003, for reviews). Furthermore, morphologic studies reveal swollen, distorted, seemingly fragmented 5-HT immunoreactive (IR) axons within days of MDMA treatment, followed by marked, lasting reductions in 5-HT-

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Received 3 November 2004; revised 22 April 2005; accepted 27 April 2005

Online publication: 25 May 2005 at <http://www.acnp.org/citations/Npp052505040516/default.pdf>

IR axon density weeks later (O'Hearn *et al*, 1988; Ricaurte *et al*, 1988b; Wilson *et al*, 1993; Axt *et al*, 1994). Considered along with results of tract-tracing studies demonstrating that MDMA, like 5,7-dihydroxytryptamine (5,7-DHT), can produce lasting reductions in anterograde axonal transport (Callahan *et al*, 2001), neurochemical and anatomic data have led a number of investigators to conclude that MDMA has the potential to damage brain 5-HT axons and axon terminals (Schmidt, 1987; Battaglia *et al*, 1987; Ricaurte *et al*, 1988b; Slikker *et al*, 1988, 1989; Kleven *et al*, 1989; Wilson *et al*, 1989; Insel *et al*, 1989; Gibb *et al*, 1994; Axt *et al*, 1994; Lew *et al*, 1997; Green *et al*, 2003), without damaging 5-HT nerve cell bodies in the brain stem raphe nuclei. For reasons yet to be elucidated, the above-mentioned toxic effects of MDMA on 5-HT nerve fibers, like those of 5-7-DHT (Rowland *et al*, 1993; Bendotti *et al*, 1994), are not consistently associated with a glial reaction or a positive silver staining response (Wilson *et al*, 1993; O'Callaghan and Miller, 1994; Pubill *et al*, 2003; Thomas *et al*, 2004).

Despite the fact that long-term effects of MDMA have been demonstrated in a number of different experimental animals including rats, guinea pigs, cats, squirrel monkeys, cynomolgous monkeys, rhesus monkeys, and baboons (see Steele *et al*, 1994; Lew *et al*, 1997; Green *et al*, 2003, for reviews), their relevance to humans is uncertain. In particular, it is unclear if doses and dosing regimens of MDMA tested in animals translate to those used by humans. In addition to dose comparisons, questions about dosing parameters can be addressed, at least in part, by characterizing plasma concentrations of MDMA that produce long-term serotonergic deficits in animals and determining how these compare with those that develop in humans. The single previous study that measured plasma concentrations of MDMA in non-human primates (Bowyer *et al*, 2003) was not designed to simulate a human use pattern, but showed that repeated intramuscular 10 mg/kg doses of *d*-MDMA that led to profound loss of brain serotonergic neuronal markers were associated with approximately 10-fold higher plasma MDMA concentrations than those typically seen in humans after single MDMA doses.

The purpose of the present studies was to further bridge the gap between controlled studies of MDMA's long-term effects in laboratory animals and recreational use of MDMA ('ecstasy') in the community. To this end, we used non-human primates and administered MDMA orally, using a schedule of drug administration intended to simulate human use patterns. Specifically, we characterized the pharmacokinetic profile of single and repeated MDMA doses, and examined the relationship between plasma drug concentrations that develop acutely and regional brain 5-HT deficits observed in the same squirrel monkeys 2 weeks later.

## MATERIALS AND METHODS

### Animals

Adult squirrel monkeys (*Saimiri sciureus*) of both genders, ranging in weight from 0.63–1.42 kg, were used. Animals were housed in pairs in standard steel cages, in an ambient temperature of  $26 \pm 3^\circ\text{C}$  and 20–40% humidity, with free

access to food and water. The colony room was maintained on a 14:10 h light:dark cycle (lights on: 0700 h). The facilities for housing and care of the animals are accredited by the American Association for the Assessment and Accreditation of Laboratory Animal Care. Animal care and experimental manipulations were approved by the Institutional Animal Care and Use Committee at the Johns Hopkins University School of Medicine, and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### Drugs and Chemicals

These were obtained from the following sources: ( $\pm$ )3,4-methylenedioxymethamphetamine hydrochloride (MDMA) and ( $\pm$ )3,4-methylenedioxyamphetamine HCl (MDA) (National Institute on Drug Abuse, Rockville, MD, USA), 5-hydroxytryptamine creatinine sulfate complex (5-HT), 5-hydroxyindoleacetic acid dicyclohexylammonium salt (5-HIAA), dopamine hydrochloride, 3,4-dihydroxyphenylacetic acid (DOPAC), sodium octyl sulfate, and ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, St Louis, MO, USA), sodium phosphate, citric acid, phosphoric acid, perchloric acid, and sodium chloride (NaCl) (JT Baker, Phillipsburg, NJ, USA). The authenticity of the MDMA sample used in the present studies was confirmed using gas chromatographic/mass spectroscopic methods. For administration, MDMA was dissolved in normal saline (NaCl; 0.9% w/v) at a volume of 1 ml/kg, and doses are expressed as the salt weight, unless otherwise stated.

### Single Dose/Route of Administration Study

**Oral vs subcutaneous dosing.** For this experiment, four squirrel monkeys were used. Three of the four animals had participated in prior substituted amphetamine toxicity studies (Table 1); all other experiments reported in this

**Table 1** Information on Drug Treatment of Squirrel Monkeys Used in Initial Study Comparing the Pharmacokinetic Profile of MDMA after Subcutaneous (s.c.) and Oral (p.o.) Administration

| Monkey | Time interval between single s.c. and p.o. doses (weeks) | Time elapsed since prior drug treatment (weeks) | Prior treatment                         |
|--------|--|---|---|
| 1      | 11.5   | 63  | MDMA<br>2 mg/kg, every 3 h $\times$ 3   |
| 2      | 7  | 9.5   | MDMA<br>2 mg/kg, every 3 h $\times$ 3   |
| 3      | 10   | 204   | MDMA<br>5 mg/kg, twice daily $\times$ 4 |
| 4      | 4  | No prior drug treatment                         | None                                    |

Both routes of administration were tested in the same four animals, with subcutaneous administration always preceding oral administration. The time interval between s.c. and p.o. administration of single dose of MDMA was, on average, approximately 8 weeks (times for each animal are shown above). Three of the four monkeys used in this experiment had prior drug exposure. For these animals, the time elapsed since their prior drug treatment is shown, along with the drug used. On average, these animals had not been treated with any other drug for approximately 92 weeks.

paper used drug-naïve animals. Animals with a history of substituted amphetamine exposure were used for this initial experiment because the primary outcome measures were plasma concentrations of MDMA and MDA, rather than measures of 5-HT neurotoxicity, and, to our knowledge, there is no evidence that remote (in the present case,  $92 \pm 100$  weeks; range: 9.5–203 weeks) prior exposure to MDMA or its analogs alters its pharmacokinetic profile. Further, since a goal of the study was to compare the pharmacokinetics of MDMA in squirrel monkeys to those in humans in controlled studies, which invariably require previous MDMA exposure, use of animals with prior MDMA exposure was deemed reasonable. For this experiment, each monkey received two doses of MDMA (on average, 8 weeks apart, Table 1), one dose being administered subcutaneously (s.c.) and the second orally by gavage (p.o.).

In all cases, subcutaneous administration preceded oral administration, with an average of 8 weeks between s.c. and p.o. dosing. For subcutaneous administration, drug solution was injected under the skin in the midscapular region. For oral administration, animals were placed in a plexiglass restraining chair, and a number 8 French feeding tube was inserted and used to administer the drug orogastrically. Mean ( $\pm$  SEM) doses used for this experiment were  $7.32 \pm 0.32$  mg/kg; s.c. and  $7.40 \pm 0.29$  mg/kg; p.o., calculated to be 'equivalent' to approximately a 2 mg/kg dose in a 70 kg human being, using interspecies dose scaling methods (for discussion of these methods, including their limitations, see Mordenti and Chappell, 1989; Ings, 1990; Boxenbaum and DiLea, 1995; Mahmood, 1999, 2002; Mahmood *et al*, 2003). Each monkey was administered a dose calculated using their individual weight at the time of administration. The fact that the mean dose differed slightly between s.c. and p.o. administration was due to slight differences in individual animal weights at the times of the two drug treatments. Blood from control and experimental animals was assayed in parallel.

### Repeated Oral Dose Studies

Drug-naïve monkeys were used in studies designed to test the neurotoxic potential of sequential oral MDMA doses, intended to simulate a recreational use pattern (Weir, 2000; Irvine *et al*, 2003; Parrott, 2005). In the first study, 15 monkeys were treated with MDMA and five untreated animals served as controls. For this experiment, separate groups of monkeys were administered a total of three equal doses of MDMA, at 3 h intervals, at one of four dosing levels (2.4, 4.3, 6.4, or 8.6 mg/kg). As indicated above, we selected a 3 h dosing interval to simulate the dosing interval used by humans at dance parties, where it is not uncommon for individuals to take multiple doses over a 12 h period (Weir, 2000; Irvine *et al*, 2003; Parrott, 2005). Given that the elimination half-life of MDMA in squirrel monkeys is approximately one-half of that in humans (see Results/Discussion), use of a comparable dosing interval would tend to underestimate that amount of MDMA likely to accumulate in humans as a consequence of repeated MDMA ingestion. No lethality was observed at any of the lower dosing regimens tested (2.4, 4.3, and 6.4 mg/kg regimens). After the highest dosing regimen tested (8.6 mg/kg), two of

six animals died shortly after drug treatment, and one received only two of the three planned doses and was, therefore, excluded from the analysis.

In the second sequential oral dose study, designed to explore the relationship between dose regimens of MDMA, plasma profiles of MDMA (and MDA), and indexes of brain serotonergic toxicity, we used additional drug-naïve animals. For these studies, three additional groups of monkeys ( $n = 3$  per group) were treated with three of the four dose regimens of MDMA used above (2.4, 4.3, and 6.4 mg/kg) and were compared to controls ( $n = 5$ ). Plasma samples were obtained at various times after oral MDMA administration and analyzed for their content of MDMA and MDA; 2 weeks later, brains of the same animals were assessed to determine the status of serotonergic neuronal markers. Tissues from control and experimental animals were assayed in parallel.

In a third study involving repeated doses, designed to test the effect of two (instead of three) sequential doses, three drug-naïve squirrel monkeys received two equal doses of 4.3 mg/kg. p.o., with a 3 h interval between the two doses. As in the second study described above, blood was collected at regular intervals after each dose for determination of plasma MDMA and MDA levels. After 2 weeks, the animals were killed for determination of regional brain 5-HT neuronal markers.

In a fourth study that tested for a possible confounding effect of isoflurane (see anesthesia, below) anesthesia on plasma MDMA/MDA concentrations, squirrel monkeys ( $n = 4$ ) received MDMA by oral gavage at a dose of 3.6 mg/kg, followed by plasma sampling with or without anesthesia. In anesthetized monkeys, blood was collected at regular intervals after MDMA administration for the determination of plasma MDMA and MDA concentrations. In unanesthetized monkeys (same four monkeys tested at least 4 weeks later), blood was collected only at the 2 h time point after MDMA administration. This particular time point was selected because results of an initial study in anesthetized monkeys showed that plasma levels of MDMA and MDA remained relatively stable from 1.5 to 3 h after a 3.6 mg/kg oral dose.

### Blood Sampling

For collection of blood samples used in the determination of plasma drug concentrations, animals were removed from their home-cages, transported to a surgery suite in a metal carrying box, and anesthetized with isoflurane, as below. While under isoflurane anesthesia, the animals were lain on a heating pad (maintained at  $37^\circ\text{C}$  by circulating warm water via a heat therapy pump (Gaymar Industries Inc., Orchard Park, NY, USA)), and placed on a steel surgical table. Blood samples were collected at 5, 15, and 30 min; 1, 2, 3, 4, 5, and 6 h during single dose experiments, and at 30 min; 1, and 2 h 50 min after each dose during sequential dose experiments. At each time point, approximately 0.5–1.0 ml of blood were withdrawn from the femoral vein using a disposable, sterile 3 ml syringe and 23 G 1" needle. Blood samples were dispensed into 5 ml Vacutainer<sup>®</sup> haematology tubes, containing 0.057 ml 15% EDTA solution (Becton-Dickinson, Franklin Lakes, NJ, USA), and stored on ice for up to 30 min, until centrifuged. Samples were centrifuged at 1100g for 10 min at  $4^\circ\text{C}$  (Sorvall RC2-B, Kendro Laboratory

Products, Newtown, CT, USA), plasma was withdrawn using a 5 3/4 Pasteur pipette and decanted into a 1.5 ml microcentrifuge tube. Sodium metabisulfite (250 mM) was added at a volume of 30  $\mu$ l/ml of plasma, as in Segura *et al* (2001) to minimize oxidation of the compounds of interest. Samples were vortexed for 10 s, then stored at  $-20^{\circ}\text{C}$  until further processed.

### Plasma Preparation

Prior to determination of plasma levels of MDMA and MDA, plasma samples were subjected to solid phase extraction (SPE), to enable plasma filtration and purification of the compounds of interest, following the method of Herráez-Hernández *et al* (2001).

### Determination of Concentrations of MDMA and MDA

Plasma concentrations of MDMA and its metabolite, MDA, were determined using HPLC with fluorescence detection, following the method of Sadeghipour and Veuthey (1997), with minor modifications. Briefly, the mobile phase consisted of sodium phosphate (20 mM), sodium octyl sulfate (0.25 mM), and acetonitrile (15% v/v), and was adjusted to pH 3.8 with phosphoric acid, filtered, and degassed. The flow rate was 1 ml/min and the HPLC system consisted of a pump (LC-6A, Shimadzu Scientific Instruments Inc., Columbia, MD, USA) linked to an automatic sample injector (Shimadzu SIL-10Ai; loop 50  $\mu$ l) and a stainless steel base deactivated  $\text{C}_{18}$  column (NUCLEOSIL 100-5  $\text{C}_{18}$  Nautilus,  $250 \times 4.6$  mm, Macherey-Nagel Inc., Easton, PA, USA), stored at  $40^{\circ}\text{C}$  (Shimadzu CTO-6A column oven). Detection was performed using an RF-10AXL fluorescence detector (Shimadzu), operated at an excitation wavelength of 285 nm and emission wavelength of 320 nm, and the emitted wavelengths were monitored via an integrator (Shimadzu Chromatopac C-R7A), which provided calculation of sample peak heights, thus enabling comparison with peak heights of external standards. As with neurochemical assays, samples from control and experimental animals were assayed in parallel.

### HPLC Determination of Regional Brain 5-HT and DA and Their Metabolites

At 2 weeks after drug administration, regional brain levels of 5-HT and 5-HIAA, and striatal levels of DA and DOPAC, were determined via HPLC with electrochemical detection, as described previously (Ricaurte *et al*, 1992).

### 5-HTT Binding

The density of 5-HTT-binding sites in regional brain homogenates was determined with tritiated paroxetine, using previously described methods (Scanzello *et al*, 1993).

### Quantitative Autoradiography

Half-hemisphere coronal sections (20  $\mu$ m) at the level of the hippocampus were thaw-mounted onto gelatin-coated microscope slides and then labeled with 50 pM [ $^{125}\text{I}$ ]RTI-55 (Perkin-Elmer Life Sciences, Billerica, MA) by incubating

for 60 min at room temperature. [ $^{125}\text{I}$ ]RTI-55 was used because it binds with high affinity to both DA and 5-HT transporters, and because it labels DA and 5-HT transporters in a region-specific manner, with the vast majority of RTI-55 binding in striatal regions (caudate, putamen) representing binding to DA transporters, and the bulk of RTI-55 binding in extra-striatal regions representing binding to 5-HT transporters (Laruelle *et al*, 1993; Staley *et al*, 2001). Thus, [ $^{125}\text{I}$ ]RTI-55 was an attractive ligand because it would concomitantly give us information on dopamine and 5-HT transporter binding in the same animal/section. Autoradiographs were digitized with a Dage CCD 72 camera and MCID Elite 6.0 image analysis system (Amersham Biosciences Niagara Inc, St Catharines, Ontario, Canada), as previously described (Fischer *et al*, 1995).

### Pharmacokinetic Parameters

Peak plasma concentrations ( $C_{\text{max}}$ ), times of peak plasma concentration ( $T_{\text{max}}$ ), area under the concentration-time curve (AUC), and the elimination half-lives ( $t_{1/2}$ ) of MDMA were obtained using the software program WinNonlin<sup>TM</sup> (Pharsight Co., Mountain View, CA). Data were fitted to a noncompartmental model with first-order output and elimination.

### Anesthesia

For all studies requiring collection of blood samples (except for a control study in which the possible confounding effect of anesthesia on plasma MDMA and MDA levels was examined), isoflurane anesthesia was used. Isoflurane (2%) was delivered at a rate of 2 l/min using a vaporizer coupled to a nonrebreathing apparatus. Each animal was manually restrained on a covered surgical table, a small feline anesthesia mask was placed over the nose and mouth of the monkey and maintained in this position while isoflurane was administered. At the end of the procedure, the mask was removed and the animal was returned to its home cage.

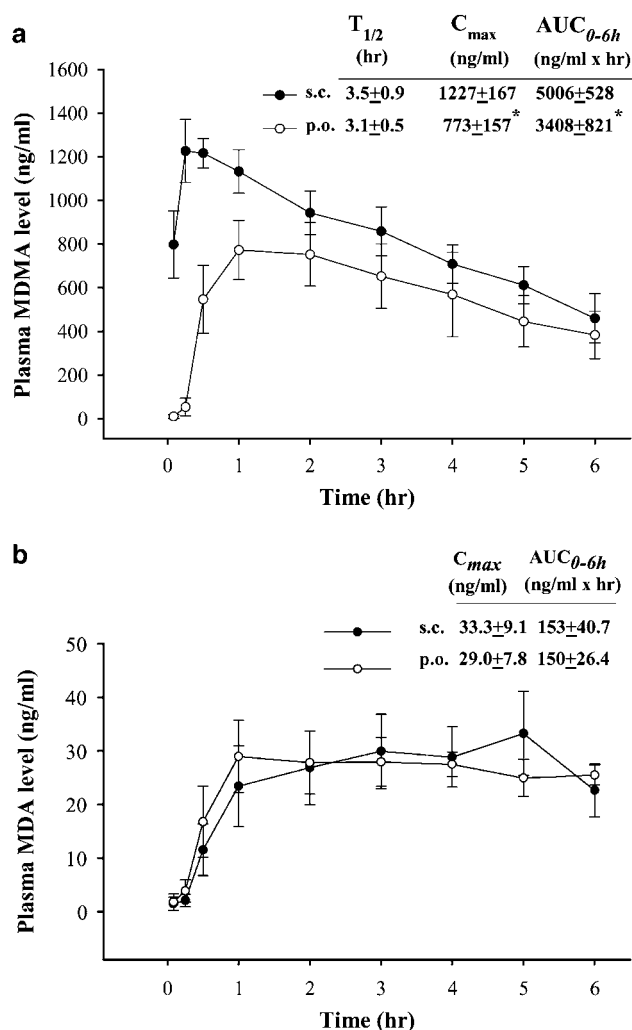
### Statistics

A mixed model repeated measures ANOVA was used to analyze the results of the route of administration study. Results of the repeated dosing studies were analyzed using a one-way analysis of variance, followed by LSD *post hoc* range tests, where appropriate. When group variances were unequal, Dunnett's T3 *post hoc* range was used. The significance of differences between means of two groups was determined using a two-tailed Student's *t*-test. Correlations were explored using Pearson's product moment correlation. Recognizing that the Pearson correlation assumes a bivariate normal distribution, and that our sample size was relatively small, we also calculated a nonparametric version of the Pearson product correlation coefficient, the Spearman's rho correlation coefficient. Results were considered significant when  $p < 0.05$ , using a two-tailed test. Data analysis was performed using the Statistical Program for the Social Sciences (SPSS for Windows, Release 10.5).

## RESULTS

Compared to oral administration, subcutaneous administration of MDMA to squirrel monkeys led to a significantly higher peak level ( $C_{\max}$ ) and larger integrated area under the time-concentration curve ( $AUC_{0-6h}$ ) of MDMA (Figure 1). In contrast, the plasma concentration-time profile of MDMA's metabolite, MDA, was similar after the two routes of administration (Figure 1, inset). The elimination half-life ( $t_{1/2}$ ) of MDMA was comparable after oral and subcutaneous administration (s.c.:  $3.5 \pm 0.9$  h; p.o.:  $3.1 \pm 0.5$  h); the  $t_{1/2}$  of MDA could not be calculated because plasma drug sampling was terminated before concentrations of MDA in the plasma had begun to decline (6 h after MDMA administration).

In light of the observed impact of route of administration on MDMA's plasma concentration-time profile in squirrel monkeys, and given the fact that humans invariably take MDMA orally rather than systemically (Hegadoren *et al*,



**Figure 1** Plasma concentrations of MDMA and MDA in squirrel monkeys following administration of a single dose of MDMA by either an oral or subcutaneous route. Squirrel monkeys were administered MDMA at a dose of 7.4 mg/kg. Blood was collected at various intervals after drug administration for determination of plasma concentrations of MDMA (a) and MDA (b). Results are shown as mean  $\pm$  SEM ( $n = 4$ ). \*Designates significant difference from subcutaneous route,  $P < 0.05$ .

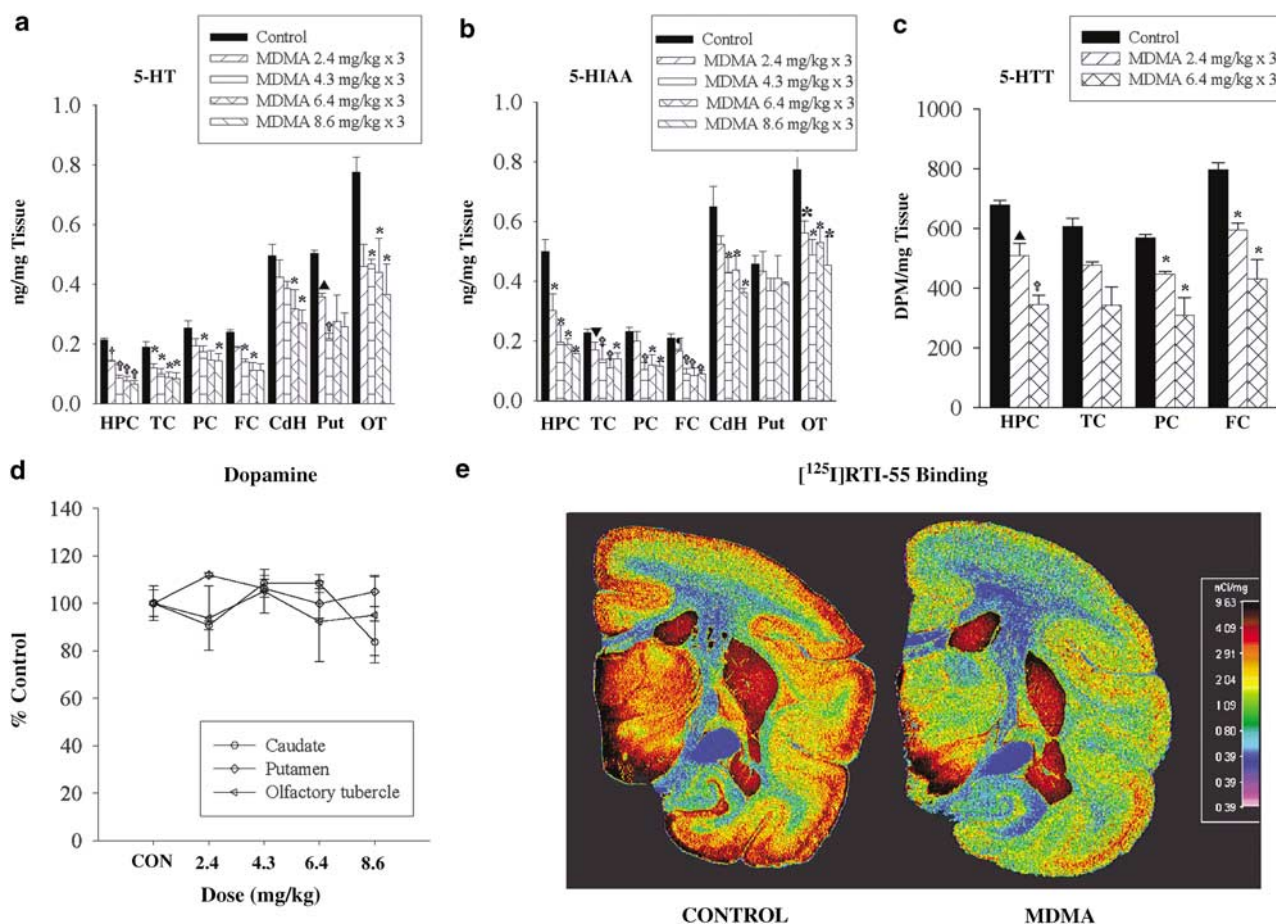
1999; Cole and Sumnall, 2003), we performed a dose-ranging study using the oral route of administration. Principal outcome measures were markers of brain monoaminergic neurons 2 weeks after the last dose of MDMA. These studies were carried out in drug-naïve squirrel monkeys. Animals ( $n = 3-6$  per group) received a total of three equal oral doses of MDMA, at 3 h intervals, at one of four dosing levels (2.4, 4.3, 6.4, or 8.6 mg/kg). This particular repeated dosing regimen of MDMA was employed in order to simulate a common human recreational MDMA use pattern (Weir, 2000; Irvine *et al*, 2003; Parrott, 2005). At 2 weeks after MDMA treatment, monkeys showed dose-related reductions in regional brain 5-HT, 5-HIAA, and the 5-HTT (Figure 2a, b and c, respectively). There were no significant effects of MDMA on brain dopamine (DA), at any of the doses tested (Figure 2d). The lowest dose regimen of MDMA (2.4 mg/kg regimen) produced significant serotonergic deficits in the hippocampus, temporal cortex, and olfactory tubercle; higher dose regimens of MDMA (4.3 mg/kg and above) produced greater effects in additional brain regions (Figure 2). At the higher doses, MDMA-induced serotonergic deficits appeared to plateau at approximately 50–60%.

Having characterized the dose-response function of repeated oral doses of MDMA in non-human primates, we next examined the relationship between plasma MDMA concentrations and MDMA-induced serotonergic deficits, with an eye toward determining how plasma concentrations of MDMA that produce lasting serotonergic deficits in monkeys compare to those that develop in recreational 'ecstasy' users. For these studies, different groups of monkeys ( $n = 3$  per group) were treated with three of the four dose regimens of MDMA used above (2.4, 4.3, and 6.4 mg/kg regimens). Measurements of MDMA and MDA plasma concentrations at regular intervals after drug administration revealed dose- and time-related increases (Figure 3a, Table 1), with evidence of a plateau effect at the higher doses. In particular, after the two higher dose regimens (4.3 and 6.4 mg/kg), neither the  $C_{\max}$  values ( $2069 \pm 314$  and  $2359 \pm 172$  ng/ml, respectively) nor the  $AUC_{0-9h}$  ( $9422 \pm 1145$  and  $11444 \pm 512$  ng ml $^{-1}$  h, respectively) were significantly different from one another. Squirrel monkeys metabolized MDMA to MDA, with a ratio of MDA to MDMA of approximately 3–5/100 (Figure 3a, Table 1). As in our initial study (Figure 2), MDMA produced dose-related serotonergic deficits 2 weeks later (Figure 3b), again with evidence of a plateau effect at the higher doses.

Analysis of the relationship between plasma drug concentrations and the extent of regional brain 5-HT depletion observed 2 weeks later showed high and direct correlations for the  $C_{\max}$  and  $AUC_{0-9h}$  of MDMA (Figure 3c). There was also a high correlation for the  $C_{\max}$  of MDA, but not for its  $AUC_{0-9h}$  (not shown).

Notably, squirrel monkeys showed evidence of nonlinear MDMA accumulation, with MDMA levels exceeding those anticipated if the effects of increasing or repeated doses were simply additive (Figure 3a, Table 1).

To determine if three sequential doses of MDMA are necessary to produce serotonergic deficits 2 weeks later in monkeys, we tested the effects of two sequential doses. We used a dose of 4.3 mg/kg; p.o., administered twice, with 3 h



**Figure 2** Effect of different repeated oral doses of MDMA on regional brain 5-HT (a), 5-HIAA (b), serotonin transporter (5-HTT) (c), DA (d), and [ $^{125}$ I]RTI-55-labeled binding sites (e) in squirrel monkeys 2 weeks after drug treatment. Each dose of MDMA was tested in a different group of drug-naïve squirrel monkeys, and was administered three times, at 3 h intervals. Results shown are the mean  $\pm$  SEM ( $n=3-5$  per group). \*Designates significant difference from control; Designates significant difference from control and 2.4 mg/kg group;  $\blacktriangle$  Designates significant difference from control and 4.3 mg/kg group;  $\nabla$  Designates significant difference from control, 4.3 and 6.4 mg/kg groups;  $\dagger$  Designates significant difference from 4.3, 6.4, and 8.6 mg/kg groups;  $\ddagger$  Designates significant difference from control, 4.3, 6.4, and 8.6 mg/kg groups. (e) [ $^{125}$ I]RTI-55-labeled binding site distribution is shown in a coronal plane of a brain from a control monkey and a monkey treated with MDMA (4.3 mg/kg regimen, as above), 2 weeks previously. Scale on right shows density of binding sites designated by color expressed in nCi/mg tissue. Note reductions in [ $^{125}$ I]RTI-55 binding in 5-HT innervated regions such as hippocampus, temporal cortex, and dorsal neocortex; also note lack of change in the caudate and putamen, where [ $^{125}$ I]RTI-55 binding is predominately to DA transporters. HPC: hippocampus; TC: temporal cortex; PC: parietal cortex; FC: frontal cortex; CdH: caudate (head); PUT: putamen; OT: olfactory tubercle.

interval between doses. As shown in Figure 4, two doses of MDMA also produced significant 5-HT deficits 2 weeks later.

To ascertain if anesthesia, used to facilitate blood sampling in the studies detailed above, might have influenced the metabolism of MDMA and, potentially, altered its plasma concentrations, we compared plasma concentrations of MDMA and MDA in monkeys that underwent blood sampling with and without isoflurane anesthesia. Results of these studies showed no significant anesthesia effect. In particular, plasma levels of MDMA and MDA were nearly identical with and without isoflurane anesthesia (Figure 5).

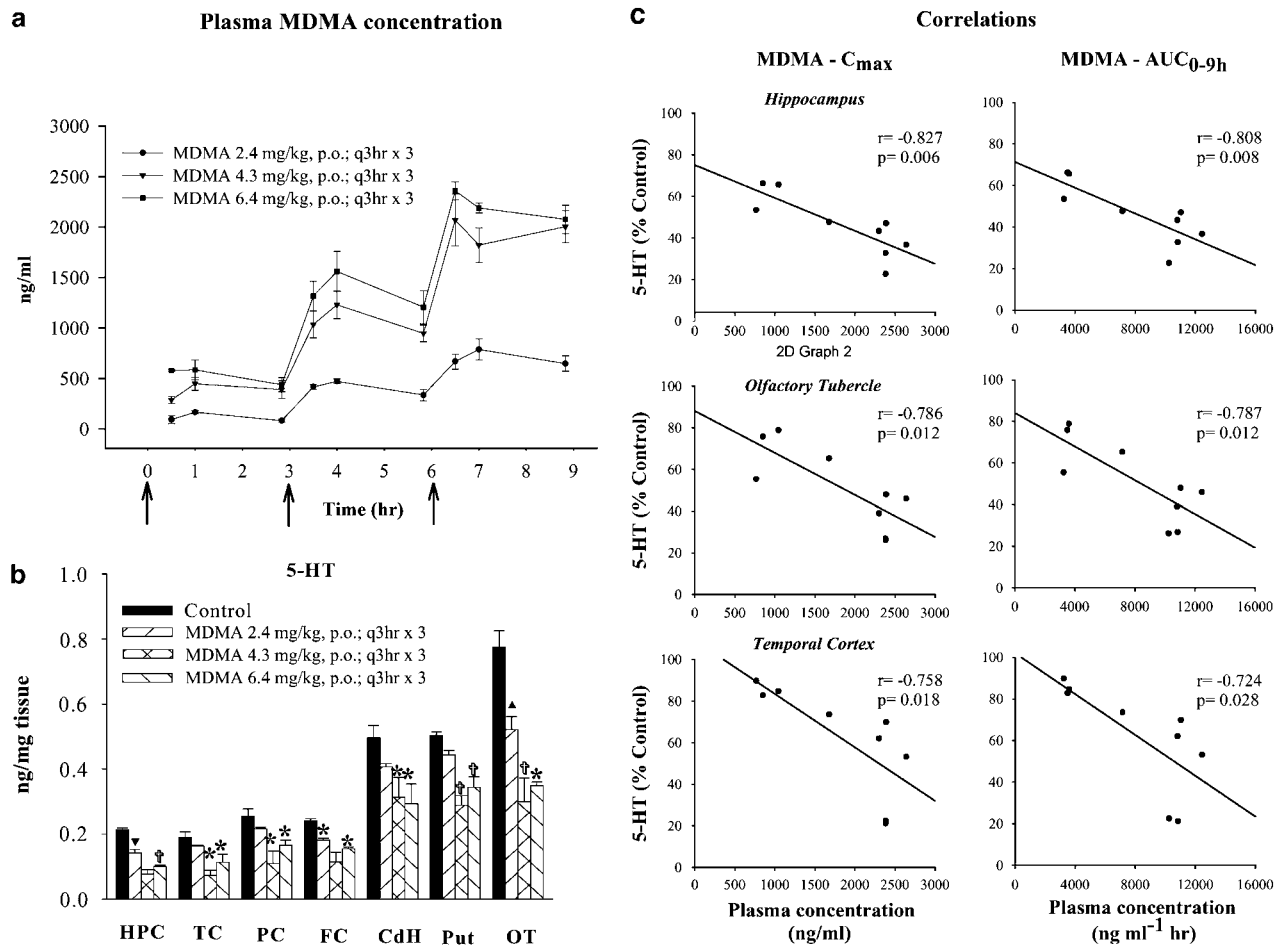
## DISCUSSION

The overall aim of the present study was to further bridge the gap between controlled animal studies of MDMA in the research laboratory and the recreational 'ecstasy' use in the community. To this end, we examined the pharmacokinetic

profile of MDMA after oral administration, and tested the effects of an MDMA dosing regimen intended to simulate a recreational use pattern. We also explored the relationship between acute plasma MDMA concentrations and decrements in 5-HT neuronal markers 2 weeks later. Principal findings are discussed, in turn, below.

## Route of Administration

Comparison of plasma concentration-time profiles of MDMA after oral vs subcutaneous administration revealed distinct differences. Specifically, compared to subcutaneous administration, oral administration produced a slower and significantly lower  $C_{max}$ , as well as a smaller  $AUC_{0-6h}$ , of MDMA. Interestingly, the time-concentration profile of MDA, a metabolite of MDMA in animals and humans, was similar after both routes of MDMA administration. What influence, if any, these pharmacokinetic differences exert on MDMA's lasting serotonergic effects is presently unclear. However, they raise the possibility that MDA, known to



**Figure 3** Plasma MDMA concentrations (a), regional brain serotonin (5-HT) levels (b), and correlations between peak plasma concentrations ( $C_{max}$ ) and area under the time–concentration curve ( $AUC_{0-9h}$ ) and regional brain 5-HT deficits (c) in squirrel monkeys administered MDMA 2 weeks previously. MDMA was administered orally in doses ranging from 2.4 to 6.4 mg/kg. Each dose was administered three times, at 3 h intervals. Results shown are the mean  $\pm$  SEM ( $n = 3$ –5 per group). For determination of plasma MDMA concentrations, blood was collected at various intervals after each dose. After 2 weeks, the animals were killed for determination of regional brain 5-HT neuronal markers. \*Designates significant difference from control; ▲Designates significant difference from control and 2.4 mg/kg group; ▼Designates significant difference from control and 4.3 mg/kg group; †Designates significant difference from control, 4.3 and 6.4 mg/kg groups; ‡Designates significant difference from 4.3, 6.4, and 8.6 mg/kg groups. HPC: hippocampus; TC: temporal cortex; PC: parietal cortex; FC: frontal cortex; CdH: caudate; PUT: putamen; OT: olfactory tubercle. R = Pearson correlation coefficient. Similarly, high correlations were observed with Spearman's rho correlation coefficient (not shown).

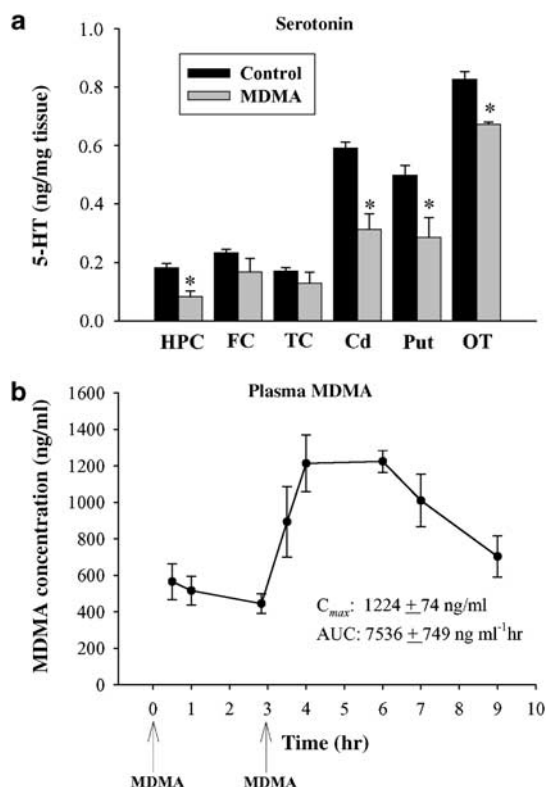
have toxic potential toward brain 5-HT neurons (Ricaurte *et al*, 1985), may be more closely linked to long-term effects of MDMA, because most previous studies indicate that route of administration does not substantially impact MDMA's lasting serotonergic effects (Slikker *et al*, 1988, 1989; Finnegan *et al*, 1988; Kleven *et al*, 1989; Insel *et al*, 1989; Ali *et al*, 1993), although one study showed modest increases in 5-HT deficits after subcutaneous administration (Ricaurte *et al*, 1988a). Alternatively, sustained concentrations of MDMA (or MDA) hours after administration, rather than initial high peak MDMA levels, such as those seen after subcutaneous administration (Figure 1), may be more closely related to the neurotoxic process. Regardless of which pharmacokinetic parameter is most closely linked to MDMA's long-term effects, the pharmacokinetic profile differences presently observed suggest that, if a goal is to elucidate possible long-term effects of MDMA in humans, the oral route of administration may be preferable

in preclinical studies, because it avoids the higher initial MDMA  $C_{max}$ , as well as the greater AUC, observed after subcutaneous administration, and because it engenders a pharmacokinetic profile that more closely resembles that seen in humans (Helmlin *et al*, 1996; de la Torre *et al*, 2000, 2004; Pacifici *et al*, 2002). Although in our initial route of administration/pharmacokinetic study we cannot definitively exclude an order effect (because the subcutaneous route was tested before the oral route), we do not believe it accounts for the observed differences because, on average, 8 weeks had elapsed between subcutaneous and oral MDMA testing (Table 1).

### Repeated Oral Doses and Nonlinear MDMA Accumulation

Repeated oral dosing of MDMA, intended to simulate a common recreational use pattern (Weir, 2000; Irvine *et al*,

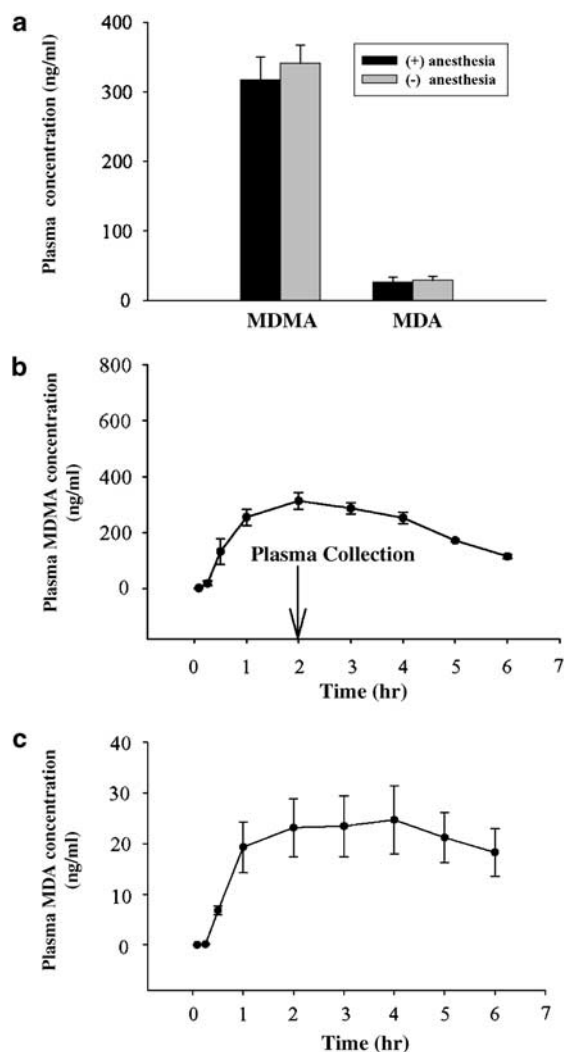




**Figure 4** Regional brain serotonin (5-HT) (a) and plasma MDMA concentrations (b) in squirrel monkeys ( $n=3$ ) administered two doses of MDMA 2 weeks previously. MDMA was administered orally at a dose of 4.3 mg/kg. There was a 3 h interval between the two doses. Arrows designate time of MDMA administration. Results shown are the mean  $\pm$  SEM. For determination of plasma MDMA concentrations, blood was collected at various intervals after each dose. \*Designates significant difference from control ( $P<0.05$ ). HPC: hippocampus; TC: temporal cortex; PC: parietal cortex; FC: frontal cortex; CdH: caudate; PUT: putamen; OT: olfactory tubercle.

2003; Parrott, 2005), produced dose-related serotonergic deficits in monkeys 2 weeks later. Further, measurement of MDMA plasma concentrations revealed dose- and time-related increases, with increases in plasma MDMA levels after single doses ranging from 2.4 to 7.4 mg/kg and repeated doses of 2.4 and 6.4 mg/kg exceeding those that would be expected based solely upon the aggregate increase in dose (Table 2, Figure 3a). These results are in agreement with previous findings in rodents indicating that MDMA has a propensity to accumulate in a nonlinear fashion, both in plasma and brain (Chu *et al*, 1996). These results are also in line with findings in humans suggesting nonlinear MDMA accumulation after single doses of increasing magnitude (50–150 mg) (de la Torre *et al*, 2000), as well as after repeated doses of the same magnitude (two doses of 100 mg given 4 h apart) (Pacifi *et al*, 2001; Farre *et al*, 2004). In contrast to MDMA, MDA (a metabolite of MDMA), did not accumulate in a nonlinear fashion, which is in keeping with recent evidence of mechanism-based inactivation of CYP<sub>450</sub> 2D6 enzyme activity by MDMA (Heydari *et al*, 2004).

Of note, after repeated oral dosing, plasma MDMA concentrations (Figure 3a) and serotonergic deficits



**Figure 5** Comparison of plasma concentrations of MDMA and MDA after blood sampling with and without isoflurane anesthesia (a). Squirrel monkeys were administered MDMA at a dose of 3.6 mg/kg, p.o. In anesthetized monkeys ( $n=4$ ), blood was collected at regular intervals after MDMA administration for determination of plasma concentrations of MDMA (b) and MDA (c). In unanesthetized monkeys ( $n=4$ ), blood was collected 2 h after MDMA administration for determination of plasma concentrations of MDMA and MDA. This particular time point for plasma collection and dose of MDMA were selected because results of an initial study in anesthetized monkeys showed that plasma levels of MDMA and MDA remained relatively stable from 1.5 to 3 h after a 3.6 mg/kg oral dose.

(Figure 3b, and also 2a) appear to plateau with higher doses, at least those used in these experiments. We do not know why this occurs, but suspect that some aspect of the drug absorption process may become saturated. While this phenomenon is not yet understood, it is noteworthy because it underscores the fact that plasma MDMA concentration is a key determinant of subsequent effects. Specifically, although both lasting serotonergic deficits and plasma MDMA concentrations are 'dose-dependent' or 'dose-related' in a general way, it appears that, ultimately, it is the plasma drug concentration, rather than drug dose *per se*, that determines the extent of subsequent lasting serotonergic deficits.



**Table 2** Predicted Vs Observed AUC Values for MDMA and MDA after (A) Single Doses of MDMA of Increasing Magnitude and (B) Three Sequential Doses of MDMA of the Same Magnitude Given at 3 h Intervals

| Dose (mg/kg)             | MDMA (AUC)<br>(ng ml <sup>-1</sup> h) |             | MDA (AUC)<br>(ng ml <sup>-1</sup> h) |          |
|--------------------------|---------------------------------------|-------------|--------------------------------------|----------|
|                          | Predicted                             | Observed    | Predicted                            | Observed |
| (A) Single dose          |                                       |             |                                      |          |
| 2.4                      |                                       | 314 ± 23    |                                      | 55 ± 4   |
| 3.6                      | 471                                   | 811 ± 73    | 82                                   | 75 ± 17  |
| 4.3                      | 563                                   | 938 ± 204   | 98                                   | 61 ± 17  |
| 6.4                      | 838                                   | 1373 ± 201  | 146                                  | 123 ± 6  |
| 7.4                      | 968                                   | 2285 ± 444  | 169                                  | 114 ± 21 |
| (B) Repeated doses       |                                       |             |                                      |          |
| Levels after second dose |                                       |             |                                      |          |
| 2.4                      | 628                                   | 1442 ± 48   | 110                                  | 152 ± 63 |
| 4.3                      | 1876                                  | 3941 ± 575  | 122                                  | 170 ± 33 |
| 6.4                      | 2747                                  | 5211 ± 522  | 246                                  | 302 ± 19 |
| Levels after third dose  |                                       |             |                                      |          |
| 2.4                      | 942                                   | 3451 ± 103  | 164                                  | 279 ± 15 |
| 4.3                      | 2814                                  | 9422 ± 1145 | 183                                  | 345 ± 46 |
| 6.4                      | 4120                                  | 11444 ± 512 | 369                                  | 500 ± 22 |

'Predicted' AUC values assume simple cumulative or additive effects of dose increments and do not take into account expected metabolic degradation. Predicted AUC values are based on the initially observed AUC value. Thus, after observing an AUC<sub>0-3h</sub> of 314 ng/ml h after a single 2.4 mg/kg dose of MDMA, the predicted AUC after the 7.4 mg/kg dose would be 7.4/2.4 × 314 ng/ml h, or 968 ng/ml h. As is evident from the data below, the observed AUC values after single doses of increasing magnitude were consistently higher than those predicted. For repeated or sequential doses, observed AUC values were also consistently higher than expected concentrations. For example, after the first dose of 2.4 mg/kg, a plasma concentration of 314 ng/ml h was observed; therefore, the predicted AUC after the second dose of 2.4 mg/kg (again, not taking into account expected metabolic degradation) would be 314 ng/ml h × 2, or 628 ng/ml h. As can be seen above, the observed AUC<sub>0-6h</sub> was 1442 ng/ml h, again suggesting nonlinear MDMA accumulation. Note that the same is not the case for MDA (except, possibly, after third dose). Please note that single dose data for the 3.6 and 7.4 mg/kg doses was derived from the anesthesia and route of administration studies, respectively.

### Correlation between Plasma MDMA Levels and Lasting Brain 5-HT Deficits

The present data indicate that plasma concentrations of MDMA are highly correlated with regional brain 5-HT depletions observed 2 weeks later (Figure 3c). To our knowledge, this is the first such demonstration, one that raises the possibility that plasma MDMA concentration may be an good predictor of possible long-term effects of MDMA in humans. At first glance, our results in non-human primates seem at odds with those of Chu *et al* (1996) in rats, where no significant correlation between MDMA level and 5-HT concentration was observed. However, as emphasized by those authors, they measured 5-HT levels acutely (ie

within hours after MDMA administration, at a time when 5-HT content is not necessarily an indicator of 5-HT axon terminal integrity), whereas we measured 5-HT neuronal markers 2 weeks after MDMA.

### Comparison to Plasma MDMA Concentrations in 'Ecstasy' Consumers

Published reports (Peters *et al*, 2003; Lora-Tamayo *et al*, 2004; also see Samyn *et al*, 2002) indicate that some recreational 'ecstasy' consumers who come to the attention of authorities have plasma concentrations of MDMA that overlap those seen here, in monkeys that subsequently develop lasting deficits in brain 5-HT neuronal markers. Findings in asymptomatic 'ecstasy' consumers of (Irvine *et al*, unpublished observations) are in general agreement with those in symptomatic individuals who presented to emergency rooms, or for some reason (eg driving impairment) came to the attention of authorities in the wake of 'ecstasy'/MDMA use. Knowledge of plasma MDMA concentrations in individuals who use 'ecstasy' in a recreational setting may be particularly relevant, because variables unique to the setting of drug ingestion (dance party) could influence plasma MDMA concentrations, as well as its metabolism and, possibly, its acute and long-term effects.

### Comparison to Laboratory Human Studies

Comparison of plasma profiles of MDMA and MDA in squirrel monkeys to those previously reported in humans given MDMA in a controlled laboratory setting reveals similarities and differences. As indicated above, nonlinear MDMA accumulation appears to take place in both squirrel monkeys and humans (de la Torre *et al*, 2000; Pacifici *et al*, 2001; Farre *et al*, 2004). The plasma ratio of MDA to MDMA, 3–5/100, is also similar in the two species (de la Torre *et al*, 2000; Pacifici *et al*, 2002; Farre *et al*, 2004). One difference appears to be that 'equivalent' single doses produce higher plasma concentrations of MDMA in the squirrel monkey than in the human. For example, the 7.4 mg/kg dose in the monkey, estimated to be comparable to a 2 mg/kg dose in a human by the allometric method (for discussion of the method, including its limitations, see Mordenti and Chappell, 1989; Ings, 1990; Boxenbaum and DiLea, 1995; Mahmood, 2002; Mahmood *et al*, 2003) produced a C<sub>max</sub> of 773 ng/ml (Figure 1), whereas the 'equivalent' dose in humans (150 mg given to two subjects weighing approximately 75 kg) produced a C<sub>max</sub> ranging from 442 to 487 ng/ml (de la Torre *et al*, 2000). The higher plasma MDMA concentration in squirrel monkeys than humans could reflect limits of sample size, limitations of interspecies dose scaling methods using an allometric equation, or effects of stress of our experimental procedures (Johnson *et al*, 2004).

### Effect of Two Sequential Doses

In monkeys, sequential or closely spaced repeated dosing (and the consequent sustained levels of MDMA and/or MDA, Figure 3a) is likely to have played a role in the observed lasting serotonergic deficits. However, three sequential doses of MDMA do not appear to be necessary

to produce long-term (2 weeks) effects, since two sequential doses (4.3 mg/kg; p.o.; 3-h interval between doses) also produced lasting serotonergic deficits (Figure 5). As evidenced by subjects who participated in a recent clinical study conducted in a naturalistic setting (Irvine *et al*, 2003), recreational 'ecstasy' users often use sequential doses hours apart, suggesting that our findings in non-human primates may be relevant to such individuals. However, additional studies (using single and repeated doses) in non-human primates are necessary to further assess the risks associated with MDMA exposure, and future clinical and preclinical research should address potentially important differences between single and multiple doses of MDMA, both with regard to pharmacokinetics and long-term effects.

### 'Threshold' Level

The present studies do not permit conclusive statements about a 'threshold' plasma concentration of MDMA associated with brain serotonergic neurotoxicity, because even the lowest dose regimen tested produced lasting serotonergic effects, and because our sample size of non-human primates was necessarily small. However, mean ( $\pm$ SD) peak plasma MDMA concentrations after the lowest repeated dose regimen that produced long-term effects were  $787 \pm 129$  ng/ml (range: 654 to 1046 ng/ml, Figure 3). As mentioned above, these plasma MDMA concentrations overlap those found in some recreational 'ecstasy' consumers. Further, they are two to three times higher than maximal concentrations ( $C_{max}$ ) that develop in humans (190–487 ng/ml) administered a single 100–150 mg dose of MDMA in a controlled laboratory setting (Helmlin *et al*, 1996; de la Torre *et al*, 2000, 2004; Pacifici *et al*, 2001, 2002; Kraemer and Maurer, 2002). The dose of MDMA required to produce desired subjective effects in humans ranges from 80 to 160 mg (Anderson *et al*, 1978). Although we do not yet know which pharmacokinetic parameter is most closely linked to MDMA's long-term effects, it appears that plasma MDMA concentrations in recreational users are on the order of those that produce lasting serotonergic deficits in non-human primates (present study) and rodents (Colado *et al*, 1995).

### Study Limitations

Several potential limitations of our studies should be recognized. First, the full significance of the relationship between plasma concentrations of MDMA (and/or MDA) and lasting serotonergic deficits is uncertain, because the mechanism of MDMA-induced toxicity toward brain 5-HT neurons requires a better understanding. Second, MDMA is biotransformed to metabolites that were not measured in this study (3, 4-dihydroxymethamphetamine (HHMA) and 4-hydroxy- 3-methoxymethamphetamine (HMMA)), and these metabolites could be implicated in MDMA's long-term effects (if they cross the blood-brain barrier in their conjugated form or if they are formed centrally) (see Jones *et al*, 2005). Third, some aspect of MDMA metabolism in squirrel monkeys and humans may differ, although non-linear MDMA accumulation and comparable proportions of MDA and MDMA are observed in both species. Fourth, the threshold oral dose of MDMA that elicits physiological

effects in squirrel monkeys is not known; thus, a point of reference for toxic doses of MDMA in squirrel monkeys is currently unavailable, although in other non-human primate species behaviorally active doses range from 0.01 to 5.6 mg/kg (depending on route and frequency of administration and outcome measure in question (Kamien *et al*, 1986; Thompson *et al*, 1987; Slikker *et al*, 1989; Frederick and Paule, 1997; Fantegrossi *et al*, 2004)).

### Conclusions

Route of administration influences the pharmacokinetic profile of MDMA, but not that of its metabolite, MDA. Repeated oral administration of MDMA to squirrel monkeys results in dose-related serotonergic deficits 2 weeks later. MDMA (but not MDA) accumulates in a nonlinear fashion in squirrel monkeys, and peak levels are highly correlated with lasting serotonergic deficits documented in the same animals 2 weeks later. Plasma MDMA concentrations associated with lasting effects on brain 5-HT neurons in squirrel monkeys overlap those found in some recreational 'ecstasy' consumers, and are only two to three times higher than those that develop in humans given a single 100–150 mg dose of MDMA in a controlled setting. Additional research is needed to determine the relative sensitivity of human and monkey brain 5-HT neurons to MDMA, the pharmacokinetic parameter(s) of MDMA or its metabolites most closely linked to the neurotoxic process, and metabolites other than MDA that may play a role.

### ACKNOWLEDGEMENTS

We thank Christopher Mehl for his guidance in using the pharmacokinetic program (Pharsight Corp., Mountain View, CA). Portions of this work were presented in abstract form (G Ricaurte, International Congress of Therapeutic Drug Monitoring and Clinical Toxicology, Basel, Switzerland, September 2003). This work was supported by USPHS Grants DA5707 and DA00206 (GAR) and the National Health and Medical Research Council of Australia (RJI).

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