

A Reduced Rate of *In Vivo* Dopamine Transporter Binding is Associated with Lower Relative Reinforcing Efficacy of Stimulants

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A slow onset of action has been hypothesized to weaken the reinforcing effects of drugs. The present study evaluated this hypothesis with slow-onset cocaine analogs, WIN 35428, RTI 31, and RTI 51. When cocaine or a cocaine analog was made available to rhesus monkeys ($n = 4$ or 5) for self-administration under a progressive-ratio (PR) schedule with a 1-h time-out between injections, all the drugs functioned as positive reinforcers. The maximum number of injections was in the order of cocaine > WIN 35428 > RTI 31 > RTI 51. In *in vivo* binding in rat striatum, equipotent doses of cocaine, WIN 35428, RTI 31, and RTI 51 were estimated to displace 25% of [³H]WIN 35428 binding at the dopamine transporters (DAT), respectively, 5.8, 22.4, 30.8, and 44.1 min after the intravenous injection. Further, relative reinforcing efficacy was correlated with rate of DAT binding such that slower displacement of [³H]WIN 35428 was associated with a weaker reinforcing effect. In *in vitro* binding in monkey brain tissue, the cocaine analogs had higher affinity for monoamine transporter sites, but similar affinity ratios of 5-HTT/DAT, compared to cocaine. Lastly, RTI 31 was shown to function as a positive reinforcer in drug-naïve rhesus monkeys under a fixed-ratio 1 schedule. Collectively, the data support the hypothesis that a slow onset at the DAT is associated with reduced reinforcing efficacy of DAT ligands. The data under both the PR and FR schedules, however, suggest that a slow onset at the DAT influence reinforcing effect only to a limited extent.

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INTRODUCTION

It is well established that cocaine can function as a positive reinforcer in humans and non-humans. The reinforcing effect of cocaine appears related to its action at the dopamine transporters (DAT) in the CNS. Drugs that inhibit the DAT, for instance, functioned as positive reinforcers (Bergman *et al*, 1989; Tella *et al*, 1997). Moreover, the potency of cocaine-like drugs in self-administration was positively correlated with binding affinity at the DAT *in vitro* (Ritz *et al*, 1987; Bergman *et al*, 1989). Simple potency as a reinforcer, however, does not seem to predict abuse potential particularly well. Abuse has rather been more strongly associated with efficacy as a reinforcer (see Brady and Griffiths, 1976). Reinforcing efficacy, or reinforcing strength, here refers to the

maximum reinforcing effect. Cocaine, for example, has a relatively weak affinity at the DAT, and low potency as a reinforcer, but is one of the most efficacious reinforcers in laboratory animals and is highly abused by humans. This dissociation between potency and efficacy as a reinforcer can also be found in other DAT inhibitors (Roberts *et al*, 1999; Woolverton *et al*, 2001). Thus, pharmacological factors other than or in addition to DAT inhibition appear to determine reinforcing efficacy.

Cocaine penetrates the brain quickly after peripheral administration and produces a 'high' in less than a minute (Fowler *et al*, 1998; Volkow *et al*, 2000). This relatively rapid onset of action seems to contribute to cocaine's high efficacy as a reinforcer. Balster and Schuster (1973) demonstrated that decreasing the infusion rate of intravenous (i.v.) cocaine decreased responding maintained by cocaine in a manner similar to a decrease of a unit dose in rhesus monkeys under a fixed-interval schedule. Comparable results have been described under a fixed-ratio 30 schedule (Panlilio *et al*, 1998). When reinforcing efficacy was compared for i.v. cocaine with different injection duration under a progressive-ratio (PR) schedule, an increase of injection duration significantly diminished

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reinforcing efficacy as it significantly decreased rate of DAT occupancy (Woolverton and Wang, 2004). Data from humans are also consistent with the conclusion that rate of onset affects reinforcing effects. Using positron emission tomography, Volkow and colleagues (2000) suggested that the rate of cocaine delivery was a reliable predictor for the intensity of subjective effects.

The hypothesis that a slow onset is associated with reduced reinforcing efficacy has been tested to a limited extent across DAT ligands. In two-drug comparisons, diminished reinforcing efficacy of a piperidine analog (Woolverton *et al*, 2002) or a tropane analog (Lile *et al*, 2002) relative to cocaine was attributed to its slower rate of onset. On the other hand, in a comparison across several drugs, no obvious relationship between rate of onset and reinforcing efficacy was observed (Lile *et al*, 2003). Therefore, the relationship between onset of action and reinforcing efficacy across DAT ligands remains equivocal. The present study was designed to further investigate the relationship between reinforcing efficacy and onset at the DAT across DAT ligands within a single chemical class (phenyltropanes). Reinforcing efficacy was measured using the maximum number of injections that a drug maintained under a PR schedule. This dependent measure has been shown to be comparable to the more traditional break point measure, and more amenable to statistical analysis (see Rowlett *et al*, 1996).

MATERIALS AND METHODS

All animal use procedures were approved by the University of Mississippi Medical Center's Animal Care and Use Committee and were in accordance with National Institutes of Health guidelines.

Self-Administration by Experienced Monkeys

Animals and apparatus. The subjects were five male rhesus monkeys (*Macaca mulatta*), L638, Rik2, AV88, L463, and L500, weighing between 9.9 and 11.5 kg at the beginning of the study. All the monkeys had histories of self-administration of cocaine and/or other stimulants. All monkeys were provided with sufficient food to maintain stable body weight (Teklad 25% Monkey Diet, Herlan/Teklad, Madison, WI) and had unlimited access to water. Fresh fruit was provided daily and a vitamin supplement was given three times a week. Lighting in the experimental room was cycled to maintain 21 h of light and 3 h of dark, with light on at 0700 h and off at 0400 h during the study. The monkeys were fit with restraint harnesses and tethers, and individually housed in the experimental cubicles equipped with response levers, stimulus lights, and infusion pumps, as described previously (see Wilcox *et al*, 2000). A Macintosh computer with custom interface and software controlled all events in an experimental session.

Procedure. Monkeys were anesthetized using ketamine and isoflurane and implanted with a silicone catheter (0.26 cm o.d. \times 0.076 cm i.d.; Cole-Parmer Co., Chicago, IL) in the jugular (internal or external) or femoral vein. The proximal end of the catheter was inserted into the vein and terminated in the vena cava near the right atrium. The

distal end was threaded subcutaneously to the back of the monkey where it exited through a small opening in the skin. The catheter was threaded through the tether, exited through the rear of the cubicle and connected to the peristaltic pump. Experimental sessions began at 1000 h each day and were conducted 7 days per week. At 30 min before each session started, catheters were filled with drugs for the sessions without infusing the drugs into monkeys. At the start of a session, the white lights were illuminated above both levers and responding on the right lever resulted in the delivery of a drug injection via the catheter for 10 s. During the injection, the white lights were extinguished and the red lights were illuminated. Pressing the left lever was counted but had no other programmed consequence.

Drugs were available under a PR schedule of reinforcement (see Wilcox *et al*, 2000). The PR schedule consisted of five components, four trials in each component, for a total of 20 trials/day. For four monkeys, the response requirement began at 200 responses/injection and doubled in each successive component. For monkey L638, responding was not well maintained under the PR 200, so the initial response requirement was reduced to a 50 and doubled in each successive component. A subject had 30 min to complete a trial (limited hold 30 min: LH 30 min). A trial ended with a 10-s drug injection or the expiration of the LH. It was rare that the LH interrupted completion of the response requirement under this PR schedule since the pattern of responding was mostly all or none in each trial in all monkeys. There was a 60 min time-out (TO 60 min) after each trial. If the response requirement was not completed for two consecutive trials (ie the LH expired), or the animal self-administered all 20 injections, the session ended.

In baseline sessions, cocaine or saline was available for an injection. The baseline dose (0.3 mg/kg/injection) of cocaine was available until responding was stable for three consecutive sessions (mean \pm 2 injections/session with no trends). Saline was then available daily until responding declined to $<$ 5 injections/session. Cocaine baseline was then re-established and, when stable, doses of cocaine or other test drugs were available for at least the number of sessions required for saline-maintained responding to decline and until responding was stable for three consecutive sessions (mean \pm 2 injections with no trend). The data from the last three sessions of stable responding were used for data analysis. After testing a dose of a drug, baseline sessions with cocaine and saline were inserted to assure the stability of responding. Cocaine (0.01–1.0 mg/kg/injection) was tested in consecutive daily sessions. However, because of evidence that responding maintained by the highest dose (0.03 mg/kg) of RTI 31 in one session affected responding in the session on the following day in monkeys L463, Rik2, and AV88, test sessions were conducted every other day for RTI 31 (0.0003–0.03 mg/kg/injection), WIN 35428 (0.001–0.3 mg/kg/injection), and RTI 51 (0.001–0.1 mg/kg/injection), with no session on intervening days. The drugs were tested in the order cocaine, RTI 31, WIN 35428, and RTI 51, with doses of each drug tested in a random order. The effect of high doses of cocaine was re-determined at the end of the study. All the compounds except RTI 51 were tested in five monkeys. RTI 51 was examined in four monkeys because monkey L638 lost its final catheter during the study.

Data analysis. The mean number of injections/session was calculated from the last three test sessions as a function of dose. The range of injections served as a measure of variability in individual subjects. A dose was considered to function as a reinforcer in a monkey if the mean number of injections was above saline levels and the ranges did not overlap. Log ED₅₀ values were calculated in individual monkeys over the ascending portion of the dose–response function using nonlinear regression analysis (GraphPad Prism 3.0). Mean ED₅₀'s were calculated for each drug by averaging these log values and taking the antilog. Relative potency was compared with ED₅₀ values using one-way repeated measures analysis of variance (ANOVA). The mean total intake/session was also calculated from the last three test sessions as a function of dose by multiplying the number of injections/session by the dose and by averaging them from the last three sessions.

For a group dose–response function for each drug, the mean number of injections per session was averaged as a function of dose across the four out of five monkeys because the drugs in the fifth monkey (L638) were tested under a different condition from the other monkeys. However, for statistical analysis to compare maximum responding across the drugs, normalized group dose–response functions for the drugs were obtained so that the fifth monkey (L638) was included. The number of injections was first normalized to the percentage of the maximum cocaine injections in each monkey. The normalized maximum responding for each drug in individual monkeys were, then, averaged across the monkeys as to the dose of the maximum responding, regardless of the absolute values of the doses. This was carried out because each drug maintained qualitatively comparable dose–response functions across monkeys but with the maximum responding seen at a different doses across subjects (see, Woolverton and Wang, 2004). Repeated one-way ANOVA with Student–Newman–Keul as a *post hoc* test was conducted on these normalized data to assess statistically significant differences among drugs in the maximum self-administration. Statistical analysis of RTI 51 in comparison to the rest of the drug was conducted on the data of four monkeys without monkey L638.

Acquisition of Self-Administration By Naïve Monkeys

Animals and apparatus. The subjects were four male rhesus monkeys (*M. mulatta*; M1389, 96R0661, 97R0111, and 96R0679). All the monkeys except M1389 were drug naïve at the beginning of the study. Monkey M1389 had been trained to respond under variable-ratio schedule of food reinforcement for 14 sessions and had self-administered cocaine in one session. Other aspects of animals and apparatus were the same as described for experienced monkeys.

Procedure. The acquisition of drug self-administration was tested under a fixed-ratio 1 (FR1) schedule. That is, pressing the active lever once resulted in a drug delivery. For all naïve monkeys, the right lever was active and the left lever inactive. Owing to his brief experience pressing the right lever, this arrangement was reversed in monkey M1389. Saline or RTI 31 were made available in successive sessions until responding was stable (the range of injections/session

over three consecutive sessions is within $\pm 20\%$ of the mean or ± 4 injections of the mean when the number of injections was less than 10). RTI 31 was selected for this experiment because of its slow rate of *in vivo* DAT binding and because it was readily available. Sessions began at noon each day, 7 days per week, and lasted for 4 h.

At the beginning of the study, saline was made available. When a monkey did not press levers for 1 h in the first session, a raisin or other piece of fruit was taped on the active lever every half an hour. Otherwise, no bait was used. Beginning with session two, no bait was taped on an active lever for the first half-hour. When the monkey did not initiate responding, baiting was repeated every half-hour over the session. The baiting procedure was used for a maximum of five sessions. Subsequent sessions were without bait for at least three sessions and until responding was stable. After saline sessions, 0.0015 mg/kg/injection of RTI 31 was available at least for the same number of sessions, and using the same protocol, as and when saline was available. If the dose of RTI 31 reliably maintained responding above saline level, the dose of RTI 31 was reduced to 0.0003 mg/kg/injection. After testing these two doses of RTI 31, saline was again substituted for RTI 31 until responding declined. If responding above saline levels was not established using 0.0015 mg/kg/injection of RTI 31, a higher dose (0.01 mg/kg) of RTI 31 was made available using the same protocol. After testing 0.01 mg/kg of RTI 31, 0.0015 mg/kg/injection of RTI 31 was retested and then 0.0003 mg/kg were tested.

Data analysis. The mean injections/session and total intake/session were calculated for individual monkeys from the last three test sessions. The range of injections served as a measure of variability in individual subjects. A dose of a drug was considered to function as a reinforcer if the mean injections/session was above levels seen with saline and the ranges did not overlap.

In Vivo Binding

The method for *in vivo* binding studies has been described previously (Stathis et al, 1995; Woolverton et al, 2002).

Animals and apparatus. Male Sprague–Dawley rats, weighing between 250 and 300 g, were initially housed in groups of three in standard plastic rodent cages under a 12 : 12 light : dark cycle (lights on at 0600 h). Food and water were available *ad libitum*.

Procedure. Rats were surgically implanted with polyethylene catheters (PE 10; 0.28 mm i.d. \times 0.61 mm o.d.; Becton Dickinson & Co.) into femoral veins under pentobarbital (50 mg/kg, intraperitoneally) anesthesia. The distal end of the catheter was threaded subcutaneously to exit the rat between the scapulae, flushed with heparin (20 IU/ml), and sealed by heat. After surgery, rats were individually housed at least for 2 days, and then used experimentally.

Binding at the DAT was studied for each drug *in vivo* via competition studies using [³H]WIN 35428 as the DAT radioligand. The pretreatment time between injection of a test drug and injection of [³H]WIN 35428 was first

determined for RTI 31, WIN 35428, and RTI 51. Catheterized rats were placed in a plastic restrainer. After establishing the patency of the catheter by flushing saline, a selected dose of each drug was injected via the catheter in an injection volume of 1.0 ml/kg delivered over 10 s and flushed with saline. The dose was selected based on ED₅₀ doses of *in vivo* binding in mice in a previous study (Scheffel *et al*, 1991). Between 5 and 30 min after a test drug injection, [³H]WIN 35428 (10 μCi/0.4 ml/rat) was injected. The rat was returned to a cage until killed by decapitation 45 min after [³H]WIN 35428 injection, the time at which specific [³H]WIN 35428 binding has been found to be asymptotic (Scheffel *et al*, 1991). Brains were immediately removed and the striatum (about 60 mg; high DAT density) and cerebellum (about 190 mg; nonspecific binding) were dissected (Kaufman and Madras, 1992). Tissue samples were weighed, placed into glass vials, and solubilized in Solvable™ (10 μl/mg tissue; Perkin-Elmer, Boston, MA) overnight. After solubilization, glacial acetic acid (1 μl/mg tissue) was added to neutralize Solvable™ and 200 μl aliquots were placed in 24-well scintillation plates. Microscint-20™ cocktail (Packard Instruments, Downers Grove, IL) was then added to each well and the preparation was allowed to sit at least 4 h to reduce chemiluminescence. Radioactivity was then counted using Packard Top Count™ scintillation counter (Downers Grove, IL). The time point at which the dose of a drug maximally inhibited the radioligand binding at the DAT was used as a pretreatment time to establish the dose–response function of each drug. For dose–response analysis, procedures were the same as above, except that various doses of a test drug were injected and [³H]WIN 35428 was injected after the defined pretreatment time for the test drug. An ED₅₀ for the inhibition of [³H]WIN 35428 binding at the DAT was calculated as described below.

To study the kinetics of *in vivo* DAT binding for a drug, [³H]WIN 35428 (10 μCi/0.4 ml/rat) was first injected to occupy the DAT and the ED₅₀ dose of a test drug was injected 45 min after the radioligand injection. The ED₅₀ dose was used to allow a comparison across drugs at functionally equivalent doses. At various time points after the test drug injection, rats were killed and the specific binding of [³H]WIN 35428 was measured. A study with saline was conducted in tandem at the same time points.

Data analysis. The binding ratio of [³H]WIN 35428 in the striatum and cerebellum (S/C) was calculated. When the binding ratio reached 1, it was considered that the drug completely displaced the specific binding of [³H]WIN 35428 to the DAT in the striatum. In dose–response functions, the ratio data were transformed to the percentage of saline data and analyzed using nonlinear regression analysis assuming a one-site interaction (Prism 3.0, GraphPad, San Diego, CA). The dose that inhibited one-half of [³H]WIN 35428 binding was expressed as the ED₅₀.

In the kinetic study, the displacement of [³H]WIN 35428 binding by the ED₅₀ dose of a test drug was expressed as the percentage of [³H]WIN 35428 binding after saline treatment of the same time point as a function of time. Data were transformed to log values to fit the displacement curves to straight lines. As WIN 35428, RTI 31, and RTI 51 showed

delay in displacing [³H]WIN 35428 binding at the DAT, a linear regression line in the displacement of [³H]WIN 35428 was obtained for each drug from the latest time point when the [³H]WIN 35428 binding was not reduced below 100% of the control binding (ie mean + SEM ≥ 100) to the time point of the maximum displacement of [³H]WIN 35428 binding (Prism 3.0, GraphPad, San Diego, CA). To compare onsets of the drugs at the DAT, the time when 25% (*T*₂₅) and 50% (*T*₅₀) of [³H]WIN 35428 binding had been displaced were estimated and compared across drugs. The time point for 25% of the DAT binding was used because cocaine showed 25% of the displacement of the radioligand binding 2 min after the i.v. injection, a time that appears particularly relevant to the reinforcing effect (Volkow *et al*, 2000; Woolverton *et al*, 2002). The time point of 50% of the DAT binding was chosen because it was the maximum binding achieved by an ED₅₀ dose. The correlation between the maximum responding under the PR schedule and the *T*₂₅, the *T*₅₀, or the slopes for the linear regression lines was obtained using the Pearson product-moment correlation (Prism 3.0, GraphPad, San Diego, CA). The differences among the slopes for the linear regression lines were analyzed using analysis of covariance with Bonferroni as a *post hoc* test (SPSS, SPSS Inc., Chicago, IL).

In Vitro Binding

Animals and apparatus. Brain tissues for rhesus monkeys (*M. mulatta*; caudate/putamen for the DAT; cerebellum for the NET; frontal cortex for the 5-HTT) were used. Each monkey had a history of drug self-administration and was drug free for at least 2 months before being killed. During that period, they were housed in stainless-steel primate cages with food and water available *ad libitum*. Monkeys were not killed specifically for the purposes of this experiment but as part of a general program to bank monkey brain tissue.

Procedure. Euthanasia and dissection were performed under veterinary supervision as described previously (Wee *et al*, 2004). In brief, monkeys were euthanized by an overdose of i.v. pentobarbital. Brains were collected and dissected immediately after killing and kept in an 80°C freezer until assay. On the day of binding study, frozen tissue was thawed, homogenized in Tris buffer (50 mM, pH 7.4), and centrifuged at 20 000g for 20 min at 4°C. The resulting pellet was collected. The washing procedure was repeated two or three times, depending on the assay. After centrifugation, the pellet was suspended in an incubation buffer at the appropriate tissue concentration.

Assay conditions were as published previously (Woolverton *et al*, 2002). The displacement of the radioligand binding (DAT: [³H]WIN 35428; NET: [³H]nisoxetine; 5-HTT: [³H]paroxetine) was performed with various concentrations of cocaine, RTI 31, WIN 35428, and RTI 51. Concentrations ranged from one too low to displace the radioligand, to a concentration that maximally inhibited the radioligand binding, with at least 12 test concentrations. Nonspecific binding was measured in the presence of 10 μM cocaine, 1 μM mazindol, and 1 μM sertraline for the DAT, NET, and 5-HTT transporters, respectively. All assays were initiated with the addition of tissue. Reactions (2 h for the

DAT; 4 h for the NET; 1 h for the 5-HTT) were terminated by rapid vacuum filtration. The collected membranes on the filters were rinsed with an ice-cold buffer and deposited into Packard Top Count™ deep well plates. Microscint-20™ cocktail (Packard Instruments, Downers Grove, IL) was added to each well and allowed to stand overnight. Radioactivity was determined using a Packard Top Count™ scintillation counter.

Data analysis. Data were analyzed by competition using nonlinear regression analysis (Prism 3.0, GraphPad, San Diego, CA). Data were fit to models assuming a one-site interaction and two sites of interaction. The best of fit was determined by comparing the sum of squares of residuals using the following equation: $F = ((SS_1 - SS_2)/(df_1 - df_2))/(SS_2/df_2)$, where SS_1 and df_1 are the sum of squares and degrees of freedom from a one-site model and SS_2 and df_2 are that from a two-site model. A two-site model was considered a significantly better fit if the F value was greater than that reported in the F-statistic table at $p < 0.05$ for the numerator of $df_1 - df_2$ and the denominator of df_2 . K_i values were calculated from IC_{50} values using the Cheng-Prusoff correction factor (Cheng and Prusoff, 1973).

The correlation between reinforcing potencies and *in vitro* binding affinities at the DAT, NET, 5-HTT, 5-HTT/DAT, or NET/DAT was measured using the Pearson product-moment correlation. The correlation between binding potencies *in vivo* and *in vitro* at the DAT was also conducted using the Pearson product-moment correlation. The correlation between reinforcing efficacy and *in vitro* binding affinities at the DAT, NET, 5-HTT, 5-HTT/DAT, or NET/DAT was also measured using the Pearson product-moment correlation. Correlation was performed on log- or square root-transformed data when the data violated the assumptions (ie equal variance, normal distribution, etc) for parametric analysis.

Drugs

Cocaine hydrochloride was provided by the National Institute on Drug Abuse (Rockville, MD). RTI 31 tartrate (3β-(4-chlorophenyl)tropane-2β-carboxylic acid methyl esters), WIN 35428 tartrate (3β-(4-fluorophenyl)tropane-2β-carboxylic acid methyl esters), and RTI 51 tartrate (3β-(4-bromophenyl)tropane-2β-carboxylic acid methyl esters) were synthesized as reported previously (Carroll *et al*, 1991; Figure 1). Pentobarbital, mazindol, and sertraline were purchased from Sigma (St Louis, MO). For the self-administration study, drugs were dissolved in 0.9% saline. Doses were expressed as the salt forms of the drugs. Radioligands [³H]WIN 35428, [³H]paroxetine, and [³H]nisoxetine were purchased from Perkin-Elmer (Boston, MA). For *in vitro* binding and *in vivo* binding studies, drugs were freshly prepared for each experiment.

RESULTS

Self-Administration by Experienced Monkeys

Cocaine, RTI 31, WIN 35428, and RTI 51 functioned as positive reinforcers (Figure 2). Group mean dose-response functions were biphasic, while dose-response functions in

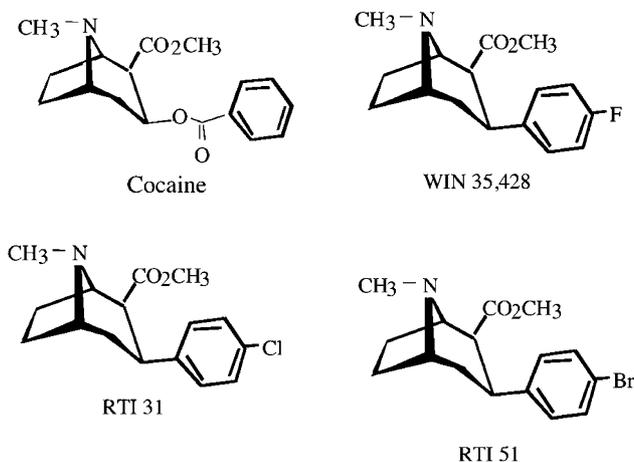


Figure 1 Chemical structures of cocaine and the phenyltropane analogs that were tested.

individual monkeys (not shown) were biphasic or sigmoidal. The dose that maintained the maximum number of injections differed by one-half log unit across the monkeys for each drug. Monkey L638 did not self-administer RTI 31 (≤ 3 injections) when the initial response requirement for the PR was 200 but, when it was reduced to 50 in that monkey, RTI 31 maintained responding marginally above saline level at 0.01 mg/kg/injection. The ED_{50} values of the compounds were statistically different and the order of potency was $RTI\ 31 > RTI\ 51 > WIN\ 35428 \gg cocaine$ (Table 1). In a group mean comparison (Figure 2), the rank order of the maximum self-administration was $cocaine > WIN\ 35428 > RTI\ 31 > RTI\ 51$. Cocaine maintained significantly higher number of injections than any other compound and WIN 35428 maintained significantly higher number of injections than RTI 51. Intake of all the drugs dose dependently increased to the maximum (group mean; cocaine, 11.6 mg/kg/session; WIN 35428, 2.1 mg/kg/session; RTI 51, 0.27 mg/kg/session; RTI 31, 0.17 mg/kg/session).

Acquisition of Self-Administration by Drug-Naïve Monkeys

In the acquisition study, two (M1389, 96R0661) of four monkeys pressed a lever at low rates when saline was initially made available (Figure 3, open circles). Monkeys 97R0111 and 96R0679, however, did not press a lever; therefore, the baiting procedure was used for the first five sessions. Monkeys took a mean of between 0.7 (96R0679) and 12.7 (M1389) injections/session over the last three sessions of saline availability (Figure 3). When 0.0015 mg/kg/injection of RTI 31 was substituted for saline, monkeys M1389 and 96R0661 self-administered RTI 31 for an average of 18.3 and 21.3 injections/session, respectively. Further, the responses on the inactive lever in those two monkeys diminished from 5 (96R0661) or 84.7 (M1389) to 2.3 and 2 injections/session, respectively, during this period. The self-administration of RTI 31 by those two monkeys increased to an average of 75.3 (96R0661) and 84.7 (M1389) injections/session when the dose of RTI 31 was reduced to 0.0003 mg/kg/injection. Total intake/session of RTI 31 was similar across doses. Monkey M1389 self-administered 0.032 and

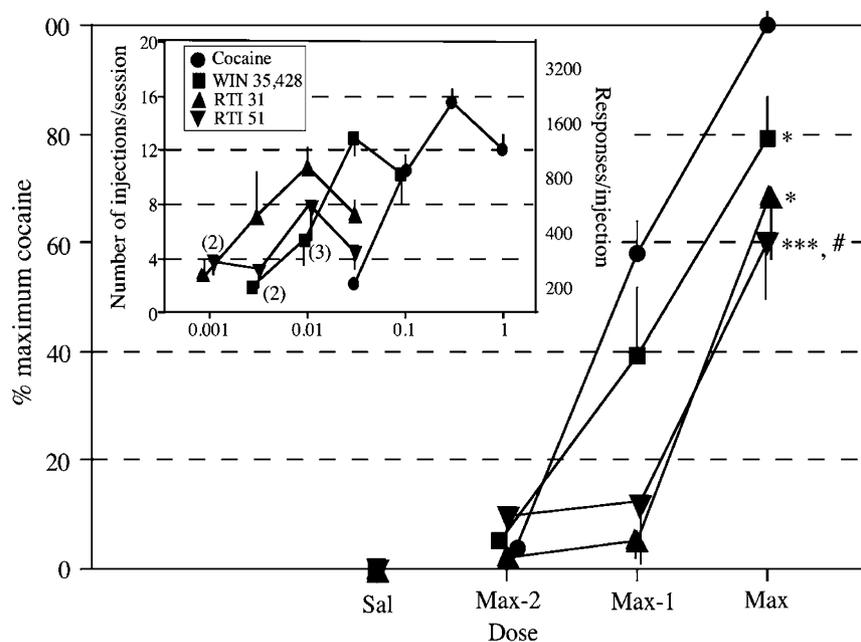


Figure 2 Self-administration of cocaine, WIN 35428, RTI 51, and RTI 31 under a PR schedule of reinforcement. Injections/session of each drug were normalized to the percentage of the maximum cocaine injections in each monkey and collapsed across the monkeys as to a dose that maintained the maximum injections ($n = 5$ or 4 for RTI 51). Vertical bars indicate SEM values. *** $p < 0.001$ compared with cocaine. * $p < 0.05$ compared with cocaine. # $p < 0.05$ compared with WIN 35428. Sal: saline; Max: the dose that maintained the maximum injections in each animal; Max-1: a half-log lower dose than Max; Max-2: a half-log lower dose than Max-1. Inset: Dose-response functions of cocaine, WIN 35428, RTI 51, and RTI 31 in four monkeys under a PR schedule. Since the fifth monkey was tested under a different PR schedule, the data in that monkey were not included. The drugs were tested in different dose ranges in monkeys because of different sensitivities to the drugs. Thus, data points are the mean of four or less monkeys. When the data point was pooled from less than four monkeys, the number of subjects is indicated in parenthesis.

Table 1 Potency Comparisons among the Drugs in Self-Administration under a PR Schedule of Reinforcement and in Dopamine Transporter Binding

	Drug				Potency ratio
	RTI 31	RTI 51	WIN 35428	Cocaine	
Self-administration ($\mu\text{mol/kg/inj.}$)	0.005 (± 0.002)	0.013 (± 0.006)	0.03 (± 0.006)	0.32 (± 0.01)	1:2.6:6:64
<i>In vivo</i> DAT binding ($\mu\text{mol/kg/rat}$)	0.08 (0.02–0.2)	0.08 (0.02–0.2)	0.39 (0.16–0.9)	8.8 [#] (7.75–10.0)	1:1:5:110
<i>In vitro</i> DAT binding (nM)	1.96 (1.49–1.58)	1.55 (0.9–2.6)	29 (24–35)	265 (213–328)	1:0.8:15:135

$N = 5$ or 4 (RTI 51) for self-administration by rhesus monkeys under a PR schedule; $n = 3$ for *in vivo* DAT binding in rats; and $n = 3$ *in vitro* DAT binding in rhesus monkey brain. Self-administration data and *in vivo* binding data are ED₅₀ doses (\pm SEM or 95% CI, respectively). *In vitro* binding data are K_i concentrations (95% CI). [#]Data have previously been published (Woolverton et al., 2002).

0.025 mg/kg/session of RTI 31 when 0.0015 and 0.0003 mg/kg/injection of RTI 31 were made available, while monkey 96R0661 self-administered 0.028 and 0.023 mg/kg/session at these doses.

Although there was some initial self-administration of 0.0015 mg/kg/injection of RTI 31 by 97R0111 and 96R0679, stable self-administration above saline levels was not maintained in these monkeys (Figure 3). When the dose of RTI 31 was increased to 0.01 mg/kg/injection, stable self-administration was maintained above saline level with an average of 5.7 (97R0111) or 7.7 (96R0679) injections/session. When 0.0015 mg/kg/injection was again made available, self-administration of 36.3 (97R0111) and 42

(96R0679) injections/session was maintained. In monkey 97R0111, the number of injections/session increased further to an average of 117 as the dose of RTI 31 was further decreased to 0.0003 mg/kg/injection, but the number of injections/session was not maintained in monkey 96R0679. As with the other two monkeys, the total intake of reinforcing doses of RTI 31 were similar across the doses (ie 97R0111: 0.057 mg/kg/session at 0.01 mg/kg/injection and 0.055 mg/kg/session at 0.0015 mg/kg/injection; 96R0679: 0.077 mg/kg/session at 0.01 mg/kg/injection and 0.063 mg/kg/session at 0.0015 mg/kg/injection). When saline was available at the end of the study, the number of injections decreased to the initial saline level in all monkeys (Figure 3).

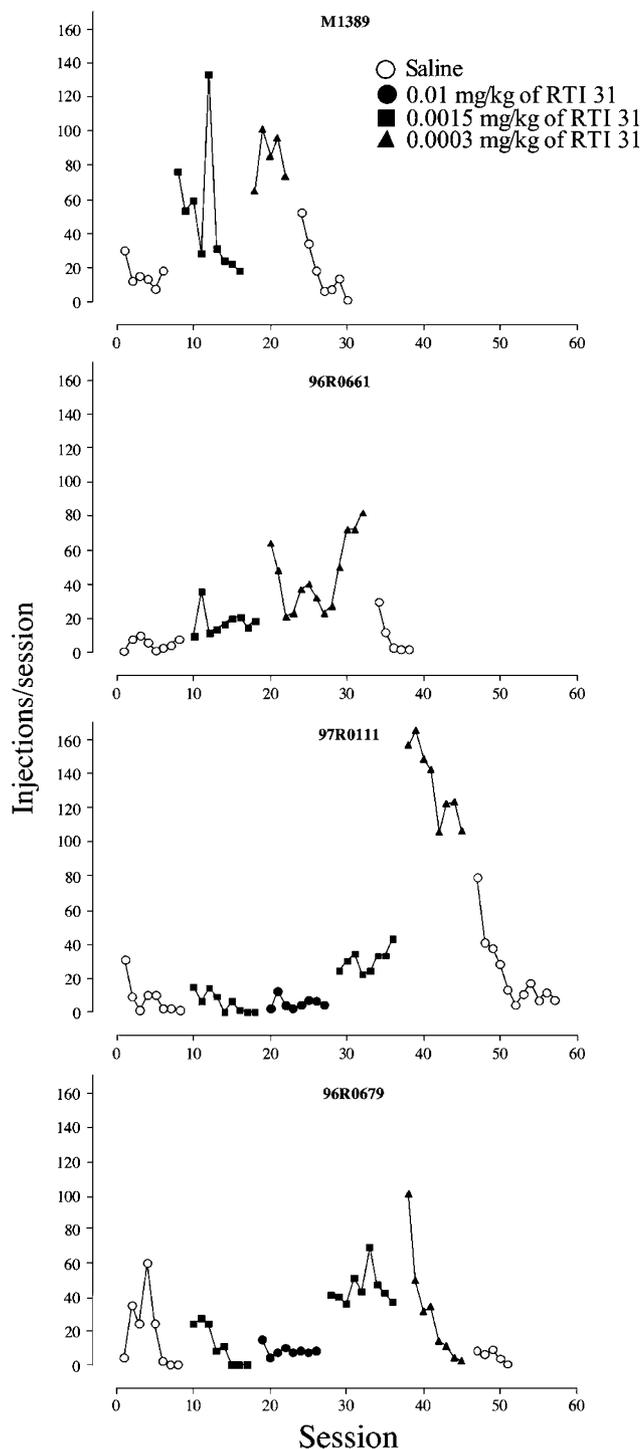


Figure 3 Self-administration of RTI 31 by individual monkeys under a FR 1 schedule of reinforcement. Abscissa: consecutive sessions; ordinate: injections/session. Sessions were 4 h long.

In Vivo Binding

The striatum/cerebellum ratio of *in vivo* [^3H]WIN 35428 binding was approximately 5.8. All the compounds displaced [^3H]WIN 35428 binding in a dose-dependent manner (Figure 4). The order of potency was RTI 31 = RTI 51 > WIN 35428 > cocaine (Table 1).

When compared to cocaine, RTI 31 and RTI 51 were approximately 100-fold more potent, whereas WIN 35428 was about 23-fold more potent than cocaine.

In the kinetic experiments, cocaine displaced radioligand binding within 2 min of the injection (Figure 5a). WIN 35428, RTI 31, and RTI 51, on the other hand, took between 20 min (WIN 35428) and 30 min (RTI 51) after the injection to decrease radioligand binding. All the compounds displaced the [^3H]WIN 35428 binding between 38 and 51% at maximum. The slopes of the best fit lines for [^3H]WIN 35428 displacement were 0.017, 0.012, 0.0074, and 0.0077 for cocaine, WIN 35428, RTI 31, and RTI 51, respectively (Figure 5b). The estimated T_{25} 's were 5.8, 22.3, 30.7, and 44.1 min for cocaine, WIN 35428, RTI 31, and RTI 51, respectively. The T_{50} 's were 14.3, 34.7, 50.4, and 63.1 min for cocaine, WIN 35428, RTI 31, and RTI 51, respectively. There was a significant difference among the slopes for [^3H]WIN 35428 displacement. Specifically, pairwise *post hoc* comparisons revealed that the slope for cocaine significantly differed from all the others, whereas no other differences were statistically significant.

In Vitro Binding

Cocaine and the cocaine analogs displaced the binding of all the radioligands at monoamine transporters in a concentration-dependent manner (data not shown). The data for all the compounds were fit best to a one-site model for all three transporters. The order of DAT affinity was RTI 31 = RTI 51 > WIN 35428 > cocaine (Table 2). RTI 31, RTI 51, and WIN 35428 were, respectively, about 133-, 133- and nine-fold more potent than cocaine at the DAT, 15-, 123-, and three-fold at the NET, and 13-, 64- and 1.3-fold at the 5-HTT. The affinity ratios of the compounds at the 5-HTT relative to the DAT differed within an order of magnitude across the compounds. On the other hand, the affinity ratios of the compounds at the NET relative to the DAT varied within a range of 5–82.

Relationship between Reinforcing Effects, Pharmacokinetics, and Pharmacodynamics

The potency relationship among the drugs for *in vivo* DAT binding was significantly correlated with potency as a reinforcer ($r^2 = 0.99$, $p = 0.004$). The correlation between *in vitro* binding affinity at the DAT and potency as a reinforcer also achieved statistical significance ($r^2 = 0.98$, $p = 0.009$). Binding affinities of the drugs at the DAT *in vivo* were correlated with those *in vitro* ($r^2 = 0.94$, $p = 0.03$). Potency as a reinforcer was not correlated with any other measure of *in vitro* binding at monoamine transporters including binding affinities at the NET, 5-HTT, NET/DAT, or 5-HTT/DAT.

The maximum number of injections under the PR schedule was significantly correlated with the postinjection time of T_{25} and T_{50} in *in vivo* DAT binding (Figure 6) and with the slopes of the linear regression lines for *in vivo* DAT binding (Figure 5b; $r^2 = 0.95$, $p = 0.03$). With regard to *in vitro* binding affinities at monoamine transporters, the maximum number of injections was not correlated with any of binding affinities at the NET, 5-HTT, 5-HTT/DAT, or NET/DAT. Interestingly, the negative correlation between

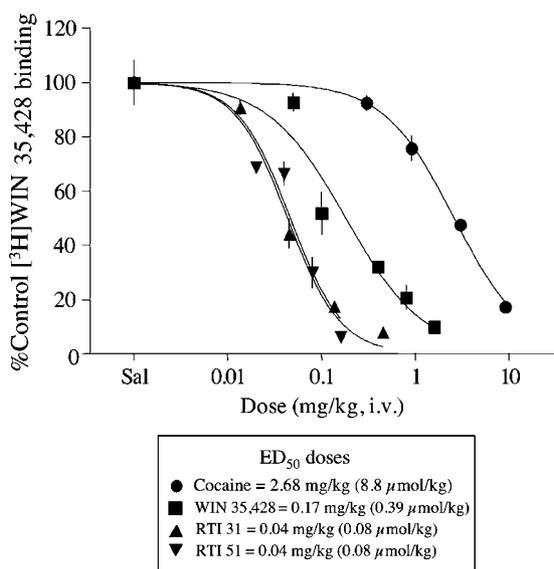


Figure 4 Dose–response function of *in vivo* DAT binding of cocaine, WIN 35428, RTI 31, and RTI 51 as a function of dose in rats ($n=3$). Cocaine data were redrawn from Woolverton *et al* (2002). Data of [³H]WIN 35428 binding ratio at striatum/cerebellum were transformed to the percent of those after saline treatment. Various doses of a drug were injected first, then [³H]WIN 35428 was injected. All the rats were killed 45 min after [³H]WIN 35428 injection.

the maximum number of injections and binding affinities at the DAT *in vitro* and between the maximum number of injections and binding affinities at the DAT *in vivo* was found significant ($r^2 = 0.97$, $p = 0.02$; $r^2 = 0.96$, $p = 0.02$).

DISCUSSION

As has been reported for DAT ligands (Bergman *et al*, 1989; Ritz *et al*, 1987), cocaine, WIN 35428, RTI 31, and RTI 51 functioned as positive reinforcers. WIN 35428 has been previously found to function as a positive reinforcer in squirrel monkeys and rats responding under a second-order and a fixed-ratio schedule, respectively (Spealman *et al*, 1991; Norman *et al*, 2004, respectively). Self-administration of RTI 31 and RTI 51 has not been reported previously. The significant correlation between DAT binding affinity and potency as a reinforcer further supports the notion that

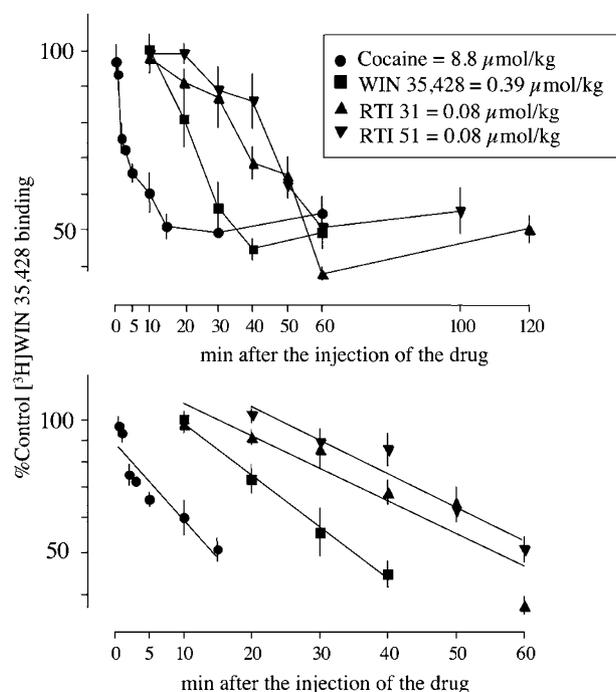


Figure 5 (a) *In vivo* DAT binding of ED₅₀ doses of cocaine, WIN 35428, RTI 31, and RTI 51 as a function of time in rats ($n=3-6$). Cocaine data were redrawn from Woolverton *et al* (2002). [³H]WIN 35428 was first injected to occupy the DAT and the ED₅₀ doses of the drugs were injected 45 min after [³H]WIN 35428 injection. Rats were killed at indicated time points after the ED₅₀ doses of the drugs were injected. (b) *In vivo* DAT binding of the cocaine and analogs. A linear regression line was obtained for each drug from the latest time point when the [³H]WIN 35428 binding was not reduced below 100% of the control binding (ie mean + SEM \geq 100) to the time point of the maximum displacement. The ordinate is in log scale.

action at the DAT contributes to the reinforcing effect of cocaine-like compounds (see Wise, 1978; Woolverton and Johnson, 1992). Besides the reinforcing effect, cocaine-like discriminative stimulus effects have been reported for WIN 35428 and RTI 31 in pigeons and rats, respectively (Jarbe, 1981; Balster *et al*, 1991). The potency relationship between cocaine and RTI 31 as discriminative stimuli was comparable to the relationship as positive reinforcers, that is, approximately 30-fold, implying the involvement of DA in the discriminative stimulus effect of cocaine and RTI 31 (Balster *et al*, 1991). All of the tested compounds have also

Table 2 Inhibition of Radioligand Binding to Monoamine Transporters in Rhesus Monkey Brain Tissues

	K _i (nM)			Ratio (DAT : NET : SERT)
	[³ H]WIN 35428	[³ H]Nisoxetine	[³ H]Paroxetine	
RTI 51	1.55 (0.9–2.6)	11 (7.2–16.7)	3.3 (1.9–5.8)	1 : 6 : 2
RTI 31	1.96 (1.49–1.58)	161 (105–247)	4.55 (3.2–6.4)	1 : 82 : 2.3
WIN 35428	29 (24–35)	491 (315–765)	153 (87–269)	1 : 17 : 5
Cocaine	265 (213–328)	1241 (512–3009)	192 (138–265)	1 : 5 : 0.7

Data are the mean and 95% CI (within parentheses) of three experiments ($n=3$), each in duplicate determinations. The caudate/putamen was used for the dopamine transporter binding; the cerebellum, for the norepinephrine transporter binding; and the frontal cortex, for the serotonin transporter binding.

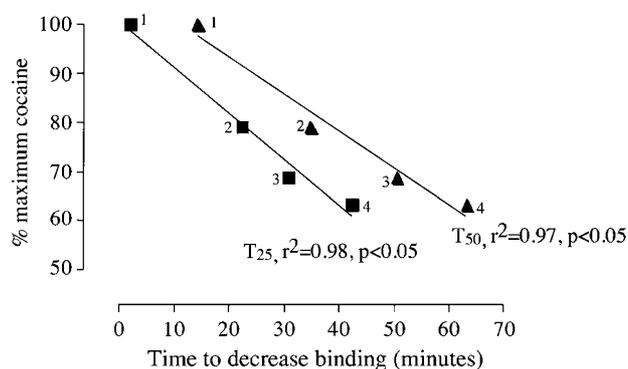


Figure 6 Correlation between the maximum self-administration by rhesus monkeys under a PR schedule and binding onsets at the DAT in rats. T_{25} and T_{50} represent the postinjection time when a drug displaced 25 and 50% of control [^3H]WIN 35428 binding, respectively, in *in vivo* binding assays. 1, Cocaine; 2, WIN 35428; 3, RTI 31; and 4, RTI 51.

been shown to increase locomotor activity in rats or mice (de Saint Hilaire *et al*, 1995; Kimmel *et al*, 2001; Tolliver and Carney, 1995). Together, these results suggest that these cocaine analogs would have psychostimulant-like abuse potential.

By progressively increasing response requirement, a PR schedule measures the maximum amount of behavior that a subject emits for a reinforcer, thereby allowing a quantitative estimate of the reinforcing strength or efficacy (see Depoortere *et al*, 1993; Stafford *et al*, 1998). When measured under the present PR schedule, the order of the maximum number of injections was cocaine > WIN 35428 > RTI 31 \geq RTI 51. Taking into account a fast onset of cocaine in the CNS, slower onset of WIN 35428, RTI 31, and RTI 51 were hypothesized to contribute to their diminished reinforcing efficacy relative to cocaine. In the *in vivo* DAT binding experiments in rats, onset of DAT binding obtained as T_{25} or T_{50} was in the order of cocaine > WIN 35428 > RTI 31 > RTI 51, comparable to the order of reinforcing efficacy of cocaine > WIN 35428 > RTI 31 \geq RTI 51. Rate of binding was, moreover, negatively correlated with reinforcing efficacy, supporting the hypothesis. Onset of *in vivo* DAT binding includes various pharmacokinetic factors including distribution to the CNS and association rate with the DAT. The slopes of linear regression lines for *in vivo* DAT binding may represent rate of DAT binding at the site of action. The significantly steeper slope for cocaine than those for the cocaine analogs in *in vivo* DAT binding suggested a faster binding rate of cocaine at the DAT than that of the cocaine analogs. A faster association rate of cocaine with the DAT than that of WIN 35428 has previously been suggested in that the equipotent concentrations of [^3H]cocaine and [^3H]WIN 35428 achieved the equilibrium binding at the DAT *in vitro* in 20 and 90 min, respectively (Madras *et al*, 1989a, b).

Under the present PR schedule, all the drugs maintained responding with biphasic dose-response functions. Although a 1-h time out between trials and 30 min of a limited hold for an injection was used to minimize a drug accumulation, the descending limbs suggest that drug accumulation over a session at the highest doses disrupted

ongoing responding, thereby influencing the maximum number of injections at those doses. Spealman *et al* (1977) reported that doses of cocaine and WIN 35428 in the range of those tested herein could disrupt ongoing schedule-controlled responding in squirrel monkeys. Similar results were described with cocaine and WIN 35428 in rats responding for food presentation (D'Mello *et al*, 1981). The *in vivo* binding data, in fact, suggest that these cocaine analogs have long duration of action at the DAT relative to cocaine, consistent with previous reports (Spealman *et al*, 1977; Kimmel *et al*, 2001). Biphasic dose-response functions are common in drug self-administration, even with short-acting drugs and when long TO periods programmed after injections (Griffiths *et al*, 1979; Panlilio and Schindler, 2000; Risner and Goldberg, 1983). The mechanism for these nonspecific effects has not been clearly established, and may include motor disruptions, satiation, or aversive effects of high doses. That dose-response functions were parallel over the full range of doses argue against the conclusion that these effects differentially influenced estimates of relative reinforcing effect of the drugs under the present PR condition. Nevertheless, the present results should be considered with the caveat that drug accumulation may have influenced the maximum number of injections.

The order of onset of *in vivo* DAT binding in the present experiment was different from the previous report describing the order of onset of DAT binding as cocaine > RTI 51 > RTI 31 = WIN 35428, in mice (Stathis *et al*, 1995). Although the exact reason(s) is not clear, different ED_{50} doses were used in the two studies, perhaps leading to different conclusions. The ED_{50} 's of RTI 51, RTI 31, and cocaine were higher in Stathis *et al*'s (1995) study than in the present study by two to four times, while the values of WIN 35428 were similar. In the present study, to determine ED_{50} 's, we established optimal pretreatment time between each test drug and [^3H]WIN 35428 injection (ie 3–10 min) to allow enough time for a test drug to bind at the DAT. In the Stathis *et al* (1995) study, however, a standard 5-min pretreatment time was used for all drugs. The fact that the pretreatment time for WIN 35428 in the present study was 5 min and the ED_{50} value was comparable to that in Stathis *et al*'s study (1995) supports this conclusion.

Previous research raised the possibility of a positive relationship between DAT affinity and efficacy as a reinforcer. For instance, local anesthetics with weaker affinities at the DAT maintained lower break points than cocaine under a PR schedule (Wilcox *et al*, 2000; Woolverton, 1995). Similarly, a piperidine cocaine analog, which has slightly weaker affinity at the DAT than cocaine, was a weaker reinforcer than cocaine (Woolverton *et al*, 2002). However, when a correlation between efficacy as a reinforcer and potency at the DAT was made among seven monoamine transporter inhibitors, no relationship was found (Lile *et al*, 2003). Interestingly, the present study found a negative relationship between binding affinities at the DAT *in vivo* and reinforcing efficacy and between binding affinities at the DAT *in vitro* and reinforcing efficacy. Thus, the relationship between potency at the DAT and efficacy as a reinforcer among DAT ligands remains equivocal.

The self-administration of the cocaine analogs achieved at relatively high response requirements under the PR schedule may be surprising considering the slow onset of binding, particularly for RTI 31 and RTI 51. It is possible that the extensive histories of these monkeys in the self-administration of cocaine and other stimulants influenced the self-administration of these compounds. Nader and Mach (1996) reported that 7-OH-DPAT, a D3 DA receptor agonist, reliably maintained self-administration in drug-experienced monkeys but failed to do so in drug-naïve monkeys. It has been proposed that similar discriminative stimulus effects between a baseline drug and a test drug may contribute to an increased likelihood of self-administration of the test drug (Young and Woods, 1981). The observation that naïve monkeys acquired dose-responsive self-administration of RTI 31 suggests that the reinforcing effect of RTI 31 under the PR schedule was not determined by previous history of stimulant self-administration. This demonstration that a slow-onset drug such as RTI 31 established and maintained self-administration in naïve monkeys is consistent with the suggestion that although slow-onset kinetics can reduce reinforcing efficacy, there may be a limit to this effect (Woolverton and Wang, 2004). The observations that various classes of abused drugs can function as positive reinforcers via the oral route, where onset is relatively slow, provides support for this conclusion (see review Meisch, 2001).

Besides onset of action, pharmacodynamic actions may influence reinforcing efficacy. Serotonergic activity of stimulants has been proposed to influence relative reinforcing efficacy of compounds with mixed monoamine actions (Roberts *et al*, 1999; Wee *et al*, 2005). It, however, seems unlikely that differences in the serotonergic activity of the drugs in the present study contributed to the differences in relative reinforcing efficacy observed under the PR schedule. In an *in vitro* binding assay using monkey brain tissue, we found that the affinity ratios of 5-HTT/DAT were within an order of magnitude across compounds. Further, the relative potency of the drugs at the 5-HTT/DAT or at the 5-HTT alone was not related to reinforcing efficacy of the compounds.

Cocaine binds at sodium channels, dopamine, muscarinic, and sigma receptors in addition to monoamine transporters (see reviews Ritz and George, 1997; Matsumoto *et al*, 2003; Smith *et al*, 2004). Among those sites, the interaction with muscarinic receptors and sigma receptors were found to be related to the toxicity, seizure, or lethality of cocaine (Matsumoto *et al*, 2004; Ritz and George, 1993). Interaction with the sodium channels was also implicated to work against the reinforcing effect of local anesthetics (Wilcox *et al*, 1999). The potencies of WIN 35428, RTI 31, and RTI 51 at sodium channels and muscarinic and sigma receptors were not determined in the present study. In another study, WIN 35428 was reported to have five- to six-fold higher potency in binding at muscarinic M1 and M2 receptors and similar affinity at sigma receptors to that of cocaine (Ritz and George, 1993). Thus, considering the structural similarity, the cocaine analogs may have good affinities at those sites. Wilcox *et al* (2000) suggested that the 'therapeutic index' or the ratio between reinforcing dose and toxic/adverse dose determines reinforcing efficacy of drugs. Therefore, the likely increased affinities

of the cocaine analogs at muscarinic or sigma receptors might have narrowed their 'therapeutic indices', thereby limiting the maximum self-administration of the drugs, particularly at high doses, but it is still speculative.

Taken together, the present data strengthen the conclusion that a slower onset or rate of DAT binding is associated with diminished efficacy as a reinforcer among DAT ligands. Our findings also support the conclusion that although onset of action is a determinant of reinforcing efficacy, there may be limits to this effect. Drugs with quite slow onsets of action can function as efficacious reinforcers.

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