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ORIGINAL INVESTIGATION

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Relationships among dopamine transporter affinities and cocaine-like discriminative-stimulus effects

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Abstract *Rationale:* The discriminative-stimulus effects of cocaine have been reported to be mediated by indirect agonist actions initiated by the blockade of dopamine uptake, and the potencies of drugs that have discriminative-stimulus effects like cocaine are directly related to their dopamine transporter binding affinities. The binding to the dopamine transporter by cocaine and many of its analogs has been reported to fit better using a two-site model than a one-site model. *Objectives:* The present study examined the relationship among binding affinities of dopamine uptake inhibitors at these two sites and their potencies to produce discriminative-stimulus effects. *Methods:* The inhibition constants (K_i values) were derived for unlabeled dopamine uptake inhibitors for displacement of [^{3}H]WIN 35,428 from rat caudate putamen membranes. These K_i values were related to the ED_{50} values obtained in rats trained to discriminate 10 mg/kg cocaine from saline injections under a fixed-ratio 20 schedule of food reinforcement. *Results:* Among the dopamine uptake inhibitors studied, the binding data for eight compounds (WIN 35,428, nomifensine, WIN 35,981, WIN 35,065-2, methylphenidate, cocaine, cocaethylene, and bupropion) were better fit by a two-site model than a one-site model. The data for the remaining eleven compounds (RTI-31, RTI-55, RTI-121, RTI-32, LU19-005, BTCP, GBR12909, GBR12935, mazindol, LU17-133, and EXP561) were better fit by a one-site model. Of the drugs that were fit best by a two-site model, there was a higher correlation among the K_i values for the high-affinity site and the ED_{50} values (R^2=0.655; P=0.015) than there was for the low-affinity site (R^2=0.543; P=0.037). Of the remaining drugs, there was a high correlation among the K_i values and the ED_{50} values for the discriminative-stimulus effects (R^2=0.523; P=0.012). *Conclusions:* These data suggest that the discriminative-stimulus effects of cocaine are more closely related to actions mediated by high-affinity binding to the dopamine transporter than they are to actions mediated by the low-affinity site. The further assessment of the respective contributions of high- and low-affinity binding to the behavioral effects of cocaine will be greatly enhanced with the development of pharmacological tools that have a high degree of selectivity for one of these components.

Key words Cocaine · Binding · Dopamine transporter · Behavior · Discriminative-stimulus effect · High-affinity site · Low-affinity site

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Introduction

A number of studies have indicated that the pharmacological effects of cocaine are related to its actions at the dopamine transporter. For example, several studies by Heikkila and his colleagues (Heikkila et al. 1979a, 1979b, 1981, Heikkila and Manzino 1984) examined stimulation of locomotor activity and rotation after unilateral degeneration of dopaminergic neurons by treatment with 6-hydroxydopamine (6-OHDA). In these studies, the behavioral effects of cocaine and phenyltropane congeners, analogs of mazindol, and a series of dialkylpiperazines (GBR12909 and its analogs) were related to their dopamine uptake inhibiting effects. In related studies, Kuhar and colleagues (Ritz et al. 1987; Cline et al. 1992a, 1992b) have shown that the potencies of cocaine and several of its analogs to produce different behavioral effects, including reinforcing effects, were directly related to their affinities for displacing ligands labeling the dopamine transporter. Further, the relationship between behavioral potency and dopamine trans-

porter affinity was stronger than either the relationship between behavioral potency and affinity for the norepinephrine or serotonin (5-HT) transporters (Ritz et al. 1987). Similar results were reported by others (Bergman et al. 1989; Spealman et al. 1989). Finally, a chronic stimulation of locomotor behavior is observed in dopamine transporter knockout mice (Giros et al. 1996). The similarity of this genetically engineered behavioral outcome to the pharmacological effects of cocaine suggests that the dopamine transporter is the primary target through which cocaine exerts its behavioral effects.

Heterogeneity of binding of cocaine and many of its congeners to the dopamine transporter has been shown in several studies. For example, Madras et al. (1989a) showed that saturable binding of cocaine was better fit by a two-site model than a one-site model. Others have demonstrated multiple-site binding for cocaine analogs (Madras et al. 1989b; Izenwasser et al. 1993) and the existence of two binding sites on the cloned (Boja et al. 1992; Pristupa et al. 1994; Eshleman et al. 1995) and solubilized (Gracz and Madras 1995) dopamine transporter. It has also been suggested that mazindol and GBR12935 may bind to a different site on the dopamine transporter than cocaine (Berger et al. 1990), and that there are differences in the binding domains of the GBR12935 analog, DEEP, or the benztropine analog, GAII-34, and cocaine (Vaughan et al. 1999). These studies suggest that the heterogeneity of dopamine transporter binding may be related to the existence of two sites on, or states of, a single protein.

In addition, functional assays of drug actions at the dopamine transporter have indicated some heterogeneity in the inhibition of dopamine transport produced by cocaine. Most often, inhibition of dopamine uptake is assessed by measuring accumulation of radiolabeled dopamine in synaptosomal preparations. Under these conditions, the inhibition of uptake by cocaine and other drugs is monophasic (Coyle and Snyder 1969). However, when the uptake is assessed in a chopped tissue preparation, in which the tissue is left in a relatively more intact state relative to synaptosomes, the inhibition of dopamine uptake produced by cocaine and some of its analogs can be resolved into two components (Izenwasser et al. 1992).

Currently, the relevance of heterogeneity of these in vitro actions to the behavioral effects of dopamine uptake inhibitors is not known (Katz et al. 1997). The purpose of the present paper was to examine the significance of multiple binding sites on the dopamine transporter and the discriminative-stimulus effects of uptake inhibitors. The discriminative-stimulus effect of cocaine provides an assessment of the subjective effects of cocaine, which likely play an important role in its abuse. A selection of structurally different dopamine uptake inhibitors were assessed for their displacement of [^{3}H]WIN35,428 binding to rat caudate putamen, and the data were modeled to determine high- and low-affinity binding constants. In addition, rats were trained to discriminate cocaine from saline injections. Once a stable discrimination was acquired, the potencies of uptake inhibitors as substitutes for cocaine were assessed. As in the study by Ritz et al. (1987), these in vivo potencies were related to binding affinities; however, in the present study, the binding affinities compared were those for the high- and low-affinity binding components labeled by the dopamine transporter ligand WIN35,428.

Materials and methods

Dopamine transporter binding

Details of the procedures used have been published previously (Izenwasser et al. 1993). In brief, male Sprague-Dawley rats (200–250 g, Taconic, Germantown, N.Y.) were decapitated and their brains removed to an ice-cooled dish for dissection of the caudate putamen. The tissue was homogenized in 30 volumes ice-cold modified Krebs-Hepes buffer (15 mM Hepes, 127 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.3 mM NaH_2PO_4, 10 mM D-glucose, with pH adjusted to 7.4) using a Brinkman polytron at setting 5. The tissue was then centrifuged at 20,000 *g* for 10 min at 4°C. The resulting pellet was then washed two more times by resuspension in ice-cold buffer and centrifugation at 20,000 *g* for 10 min at 4°C. Fresh homogenates were used in all experiments.

Binding assays were conducted in modified Krebs-Hepes buffer on ice. The total volume in each tube was 0.5 ml, and after all additions the final concentration of membrane was 0.5% (w/v), corresponding to 200–300 µg of protein/sample. Membrane suspensions were preincubated for 5 min in the presence or absence of the compound being tested. [^{3}H]WIN35,428 (final concentration 1.5 nM) was added and the incubation continued for 1 h on ice. The incubation was terminated by the addition of 3 ml of ice-cold buffer and rapid filtration through Whatman GF/B glass-fiber filter paper [presoaked in 0.1% bovine serum albumin (BSA) in water to reduce non-specific binding] using a Brandel Cell Harvester (Gaithersburg, Md.). The filters were washed with three additional 3-ml washes and transferred to scintillation vials. Absolute ethanol (0.5 ml) and Beckman Ready Value Scintillation Cocktail (2.75 ml) were added to the vials, which were counted the next day at an efficiency of about 36%. Under these assay conditions, an average experiment yielded approximately 6,000 dpm total binding per sample and approximately 250 dpm non-specific binding, defined as binding in the presence of 100 µM cocaine. Each compound was tested with concentrations ranging from 0.01 nM to 100 µM for competition against binding of [^{3}H]WIN35,428, in three independent experiments, each performed in triplicate.

Displacement data were analyzed using the nonlinear least-squares curve-fitting computer program LIGAND (Munson and Rodbard 1980). Non-specific binding was less than 5% of total binding. Data from replicate experiments were modeled together to produce a set of parameter estimates and the associated standard errors of these estimates. In each case, the data were fit to both a one-site and a two-site model, and the fits were compared according to the *F* test. The K_i values reported are the inhibition constants derived for the unlabeled ligands.

Binding constants are reported from one-site models ($K_{0.5}$ values) unless a two-site model was considered a significantly better fit (as described above, at $P \leq 0.05$. When a two-site model was preferred, data are expressed as K_{hi} and K_{lo} values, representing affinities for the high- and low-affinity sites, respectively, labeled by [^{3}H]WIN35,428. Some of these values were originally presented in our previously published paper (Izenwasser et al. 1994).

Behavioral studies

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington Mass.) weighing 310–385 g were individually housed under a 12 h/12 h light/dark cycle (lights on 0700 hours). The rats had unrestricted access to water in their home cages and were fed 15 g Purina rodent chow daily, 30 min after testing.

Testing of rats was conducted in two-lever operant-conditioning chambers (BRS/LVE, model RTC-022, Laurel, Md.) individually contained within light- and sound-attenuating compartments. White noise was present throughout testing to mask any extraneous sounds. Ambient illumination was provided by a lamp in the top center of the front panel. Levers were set 17 cm apart, with stimulus lamps above the levers. A downward force on the lever of 0.4 N through 1 mm produced an audible click and was recorded as a response. Each reinforced response produced one 45-mg pellet (BioServe, Frenchtown N.J., USA) into a centrally located food tray.

Rats were initially trained to press both levers under a fixed-ratio (FR) schedule of food reinforcement. Responding on each lever was trained separately in random order, with the active lever on a given training session indicated by illumination of the lamps directly above it. Rats were then trained to discriminate intraperitoneal (i.p.) injections of cocaine (10 mg/kg) from i.p. injections of saline. Following cocaine injection, responses on only one lever were reinforced; following saline injection, responses on the other lever were reinforced. The assignment of cocaine- and saline-appropriate levers was counterbalanced across rats. Immediately after injection, rats were placed inside the experimental chambers, with all lamps extinguished; during this time, responses had no scheduled consequences. Five minutes later, all lamps were illuminated and responses on the appropriate lever were reinforced according to a FR schedule. The FR value was increased to 20 (FR20) over several training sessions. Responses on the inappropriate lever reset the FR response requirement on the appropriate lever. Each food presentation was followed by a 20-s time-out period, during which all lamps were off and responding had no scheduled consequences. Sessions ended after 20 food presentations or 15 min, whichever occurred first. Training sessions for which cocaine (C) and saline (S) injections were administered were conducted in a ...SCCS... sequence and continued until subjects attained criterion performance on four successive sessions (two saline and two cocaine). The criteria were at least 85% appropriate responding overall and during the first FR of the session. When testing was initiated, test sessions were conducted after consecutive SC or CS training sessions.

On test sessions, different doses of cocaine or doses of other dopamine uptake inhibitors were substituted for cocaine or saline to produce dose–effect curves. A test session was conducted for a given subject if it attained criterion performance on both of the immediately preceding saline and cocaine training sessions. The criteria were at least 85% appropriate responding overall and during the first FR of the session. Test sessions were identical to training sessions, with the exception that 20 consecutive responses on either lever were reinforced.

The drugs tested were: (–)-cocaine HCl (Sigma Chemical Company, St. Louis, Mo.); WIN35,428 naphthalenedisulfonate, GBR12909 diHCl, GBR12935 diHCl, zimelidine diHCl (Research Biochemicals Inc., Wayland, Mass.); WIN35,981 tartrate, WIN35,065-2 tartrate, RTI-31 tartrate, RTI-32 tartrate, methylphenidate HCl, cocaethylene fumerate (National Institute on Drug Abuse, Rockville, Md.); RTI-55 tartrate, RTI-121 HCl (courtesy of F.I. Carroll at Research Triangle Institute, Research Triangle Park, N.C.); LU19-005 HCl, LU17-133 diHCl (Lundbeck A/S, Sweden), BTCP HCl (courtesy of J.M. Kamenka, CNRS, Montpellier C'dex 1, France); EXP 561 HCl (courtesy S.W. Tam, Du Pont/Merck Pharmaceutical Co., Wilmington, Del.); mazindol (Sandoz Pharmaceutical Corp., Hanover, N.J.); nomifensine maleate (Hoechst-Roussel Pharmaceuticals Inc., Somerville, N.J.); benztropine mesylate (Aldrich Chemical Co., Milwaukee, Wis.); bupropion HCl (Burroughs Wellcome Co.); nisoxetine HCl and tomoxetine HCl (Eli Lilly and Company, Indianapolis Ind.).

For behavioral studies, all drugs were dissolved in sterile water with the exceptions of cocaine and WIN35,428, which were dissolved in 0.9% saline, and mazindol, which was dissolved in lactic acid and diluted with pH adjusted to the appropriate concentration in 0.9% NaCl. The drugs were administered i.p. in a volume of 1 ml/kg body weight. All drugs were injected immediately before placing subjects in the experimental chambers (5 min before testing), with the exceptions of nisoxetine (40 min before testing), zimelidine, LU19-005, LU17-133 (each 30 min before testing), benztropine, EXP561 (each 15 min before testing), and RTI-121 (30 min before testing). These pre-test intervals were selected for CNS accessibility at the time of testing.

On-line experimental control and data collection were accomplished using MED Associates (Med Associates, St. Albans, Vt.) or SKED (State Systems, Inc., Kalamazoo, Minn.) software. For each subject, the overall response rate and the percentage of responses occurring on the cocaine-appropriate lever were calculated. The mean values were calculated for each measure at each drug dose tested. Data from any rat that failed to emit 20 responses on either lever (a rate of at least 0.02 responses per second) was not included in the calculation of mean cocaine-appropriate responding at that dose. If less than half of the subjects met the response rate requirement, no mean value was calculated for percentage of cocaine-appropriate responding at that dose. Standard analysis of variance (ANOVA) and linear regression techniques were used to calculate ED_{50} values and their 95% confidence limits (Snedecor and Cochran 1967). A significance level of $P<0.05$ was assumed throughout.

Correlations of the ED_{50} values for the behavioral studies and the various K_i values were performed in order to assess the relationship between these effects. For three of the drugs, the ED_{50} values from our previous studies that used identical behavioral methods were used (Cline et al. 1992a; Katz et al. 1992). Data for these compounds are included in the tables but not in Fig. 1. To assess the reliability of these behavioral effects across studies, the effects of RTI-32 and cocaine were determined for this study, and the resulting ED_{50} values were found to be virtually identical to those found in the previous studies.

Results

The majority of the compounds tested produced a dose-dependent increase in the percentage of responses on the cocaine-appropriate lever (Fig. 1, top panels) and also produced dose-related decreases in rates of responding (bottom panels). The norepinephrine uptake inhibitor, nisoxetine, and the 5-HT uptake inhibitor, zimelidine, did not fully substitute for cocaine (Table 1). The lack of substitution for cocaine by these two drugs occurred across a range of doses, from those having no effect on to those decreasing response rates. In addition, two of the dopamine uptake inhibitors failed to produce cocaine-appropriate responding. As has been reported previously, benztropine did not fully substitute for cocaine

Table 1 Effects and dose ranges tested of drugs that did not fully substitute for the discriminative-stimulus effects of cocaine

Drug	Doses tested (mg/kg)	Maximal % drug responding	Maximal decrease in response rate
Zimelidine	5.6–30	22.9 (17)[a]	89.78
Nisoxetine	10–30	31.9 (30)	48.28
Benztropine	3–17	36.01 (5.6)	76.93

[a] Parenthetical values represent the dose at which maximal effects were obtained

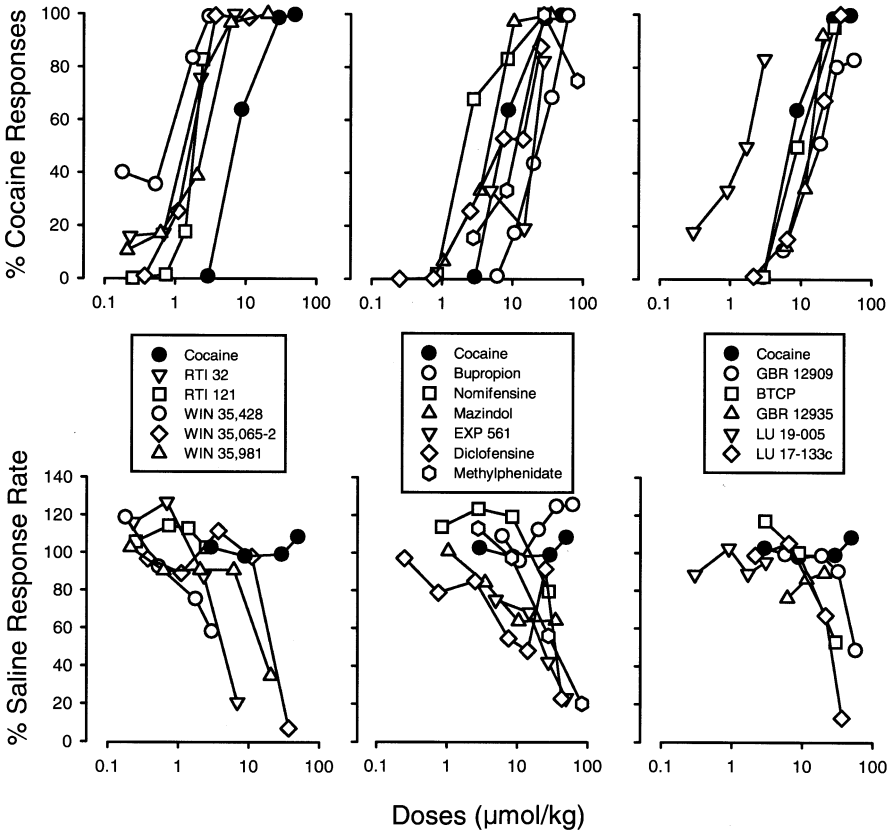

Fig. 1 The substitution for 10 mg/kg cocaine of various doses of cocaine and several other drugs with affinity for the dopamine transporter. *Top panel*: the percentage of all responses occurring on the cocaine-appropriate lever as a function of dose of the drug administered, i.p. Each point represents the mean of at least three and typically six subjects. *Bottom panel*: rate of responding (as a percentage of control response rate) occurring on the cocaine-appropriate lever as a function of dose of the drug administered. The saline control response rates averaged 1.49±0.05 (SEM) responses per second. Each point represents the mean of typically six subjects. Note that all of the drugs fully substituted for cocaine with varying potencies relative to cocaine. The ED_{50} values obtained with these drugs are shown in Table 2

Table 2 Affinities for competition against [^{3}H]WIN35,428 binding to rat caudate putamen membranes and ED_{50} values in substitution for cocaine in rats trained to discriminate cocaine. For binding, a two-site model is reported only if the fit was considered significantly better than with the one-site model ($P \leq 0.05$). Actual P values for reported two-site models did not exceed 0.018. Binding data represent values obtained when data from at least three independent experiments, each performed in triplicate, were combined for modeling. The ED_{50} values represent the dose that would produce 50% substitution in rats trained to discriminate 10 mg/kg cocaine from saline. These values were determined using linear regression. *No Subst.* the compound did not fully substitute for the cocaine training stimulus, *NS* non-significant linear regression

Compound	Affinity (nM)			ED_{50} value (µmol/kg)	
	$K_{0.5}$	K_{hi}	K_{lo}	Drug discrimination	Response rate
RTI-31	2			0.69	0.45
RTI-55	2.1			0.55	NS
RTI-121	4			0.7	NS
RTI-32	5			1.2	4.84
LU19-005	8			1.28	NS
BTCP	10			9.53	38.88
GBR12909	12			17.29	44.03
GBR12935	13			12.04	NS
Mazindol	16			4.39	75.37
WIN35,428	22	7	126	0.78	NS
LU17-133	25			12.13	21.75
Nomifensine	43	12	197	2.96	172.63
WIN35,981	81	19	192	1.64	29.5
EXP561	84			20.17	20.05
WIN35,065-2	87	33	314	1.37	18.61
Methylphenidate	185	107	12266	10.93	33.15
Cocaine	189	32	388	8.94	NS
Cocaethylene	309	211	12422	9.02	NS
Bupropion	373	118	1890	21.69	NS
Benztropine	118			No Subst.	NS
Zimelidine	11200			No Subst.	36.02
Nisoxetine	563			No Subst.	NS

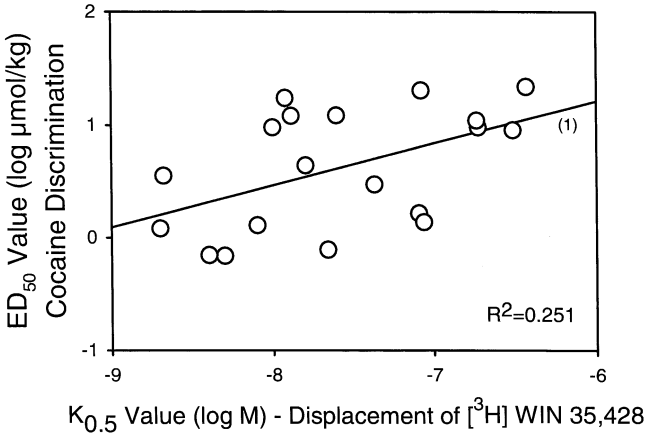

Fig. 2 The relationship between potency in producing cocaine-like discriminative-stimulus effects and affinity for the dopamine transporter. Potency was measured as ED_{50} value in substitution for 10 mg/kg cocaine in the drug discrimination procedure; affinity was assessed as K_i values determined using a one-site binding model ($K_{0.5}$ values) for competition against binding of [^{3}H]WIN35,428. The regression line represents the relation of ED_{50} value and $K_{0.5}$ value (r=0.251; P=0.029) and corresponds to row 1 of Table 3

(Colpaert et al. 1979; Acri et al. 1996; Katz et al. 1999) across a range of behaviorally active doses (Table 1).

The ED_{50} values for those compounds that fully substituted for cocaine are shown in Table 2. Among the compounds active in substituting for cocaine, the ED_{50} values ranged from the most potent 0.55 µmol/kg of RTI-55 to the least potent bupropion at 21.69 µmol/kg.

The $K_{0.5}$ values ranged over an approximately 200-fold range, from 2 nM for the most active compound (RTI-31) to 373 nM for the least active (bupropion). Only eight of the compounds modeled better for two sites than they did for one site. The K_{hi} values for these compounds varied over an approximate 30-fold range, from 7 nM (for WIN35,428) to 211 nM (for cocaethylene). The K_{lo} values for these compounds varied over an approximate 100-fold range, from 126 nM (for WIN35,428) to 12,422 nM (for cocaethylene).

The relationship of log ED_{50} value for cocaine substitution in the drug discrimination experiment with log $K_{0.5}$ values (regardless of whether the displacement data were better fit using a one- or two-site model) is shown in Fig. 2. In addition, the results of the regression analysis are shown in Table 3 (row 1). As can be seen, there was a significant relationship between potency in substituting for cocaine and the $K_{0.5}$ value (R^2=0.251, P=0.029). The slope of the line was 0.374.

The relationship between ED_{50} values from the drug discrimination experiment and the $K_{0.5}$ values for only those compounds better fit using a one-site model than a two-site model was also determined (Table 3; row 2). The regression line for this analysis (Fig. 3A; regression line 2, open points) was again significant, with an R^2 value of 0.523 (P=0.012). The slope of this regression line was considerably steeper than that for the $K_{0.5}$ data for all of the compounds (Table 3; compare rows 1 and 2).

The results of the regression analysis of ED_{50} values from the drug discrimination experiment on the K_{hi} values (for only those compounds better fit by a two-site model) are also shown in Table 3 (row 3) and the regression line is shown in Fig. 3A (line 3, filled points). The regression was again significant, with an R^2 value of 0.655 (P=0.015). Further, the slope of this regression line was considerably steeper than that for the $K_{0.5}$ data for all of the compounds. The slope and intercept for this regression line were comparable with those for the regression of the $K_{0.5}$ values for those compounds modeling better for a single site (Table 3; row 2).

The relationship between behavioral ED_{50} and K_{hi} values (for those compounds better fit using a two-site model) along with the $K_{0.5}$ values (one-site model) is shown in Fig. 3A (line 5, open and filled points). The results of the regression analysis are shown in Table 2 (row 5). As can be seen, the regression analysis was again significant, with an R^2 value of 0.408. The slope of this regression line was considerably steeper than that for the $K_{0.5}$ data for all of the compounds (compare Fig. 3A line 5 to Fig. 2 and, in Table 3, compare rows 1 and 5). The inclusion of the $K_{0.5}$ values did not increase the amount of variance accounted for by the regression; however, the chance likelihood of the regression (P value) was reduced. As can be seen in Fig. 3A, the relationship between ED_{50} and K_{hi} values (line 3) was comparable with

Table 3 Relationships of binding affinities and potency to produce discriminative-stimulus effects. Results of various regression analyses of affinities for competition against [^{3}H]WIN35,428 binding to rat caudate putamen membranes and ED_{50} values in substitution for cocaine in rats trained to discriminate cocaine from saline

	Values used for regression		R^2 value	P value for regression	Slope	n
	Compounds modeling better for two sites	Compounds modeling better for one site				
1	$K_{0.5}$	$K_{0.5}$	0.251	0.029*	0.37	19
2	None	$K_{0.5}$	0.523	0.012*	0.86	11
3	K_{hi}	None	0.655	0.015*	0.81	8
4	K_{lo}	None	0.543	0.037*	0.47	8
5	K_{hi}	$K_{0.5}$	0.408	0.003*	0.59	19
6	K_{lo}	$K_{0.5}$	0.146	0.107	0.17	19

*Significant linear regression (P<0.05)

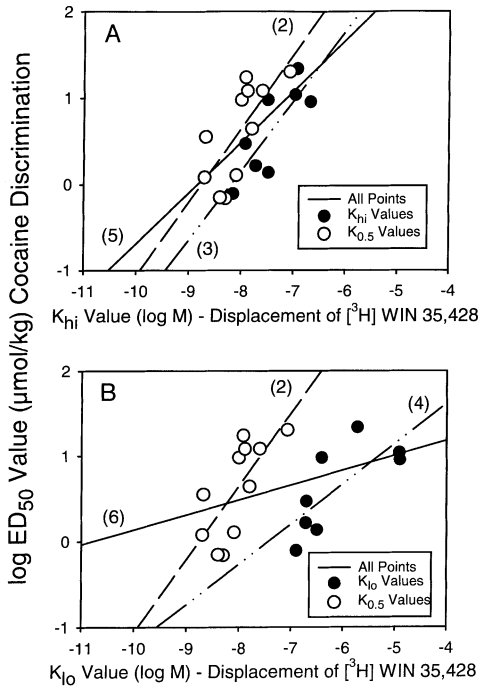

Fig. 3A, B Comparisons of the relationships between potency in producing cocaine-like discriminative-stimulus effects and affinities for the dopamine transporter for compounds that modeled statistically better for one or two sites. Potency was measured as ED_{50} value in substitution for 10 mg/kg cocaine in the drug discrimination procedure; affinity was assessed as K_{hi} and K_{lo} values for compounds that were fit better using a two-site model, and $K_{0.5}$ values for compounds that were fit better using a one-site model. Affinities were determined in competition against binding of [^{3}H]WIN35,428. **A** *Open points* and regression *line 2* (corresponding to row 2 of Table 3) show the relationship between the ED_{50} value and the $K_{0.5}$ value for those compounds better fit by a one-site model. *Filled points* and regression *line 3* (corresponding to row 3 of Table 3) show the relationship between the ED_{50} value and the K_{hi} value for those compounds that were fit better by a two-site model. Regression *line 5* (corresponding to row 5 of Table 3) shows the relationship between the ED_{50} value and the $K_{0.5}$ and K_{hi} values for all of the compounds shown in this panel. **B** *Open points* and regression *line 2* (corresponding to row 2 of Table 3) show the relationship between the ED_{50} value and the $K_{0.5}$ value for those compounds that were fit better using a one-site model (same data as in **A**). *Filled points* and regression *line 4* (corresponding to row 4 of Table 3) show the relationship between the ED_{50} value and the K_{lo} value for those compounds fit better using a two-site model. Regression *line 6* (corresponding to row 6 of Table 3) shows the relationship between the ED_{50} value and the $K_{0.5}$ and K_{lo} values for all of the compounds shown in this panel. Note that the data points generally fall along a single regression line when the ED_{50} value is related to K_{hi} and $K_{0.5}$ values. In contrast, two distinctive regression lines are obtained when ED_{50} values are related to K_{lo} and $K_{0.5}$ values

that for ED_{50} and $K_{0.5}$ values (line 2) and the analysis combining these two sets of data (Fig. 3A; compare lines 3 and 5, and the corresponding rows in Table 3).

The relationship between ED_{50} values from the drug discrimination experiment and the K_{lo} values (for only those compounds better fit by a two-site model) are shown in Fig. 3B (line 4; see also Table 3, row 4). The regression was again significant, with an R^2 value of 0.543 (P=0.037). The slope of the line for this regression was less steep than that for the K_{hi} data (compare with Table 3, row 3) and between that and the slope for the regression on $K_{0.5}$ values for all compounds (Table 3, row 1).

The relationship between behavioral ED_{50} and K_{lo} values (for those compounds better fitted using a two-site model) along with the $K_{0.5}$ values (one-site model) is shown in Fig. 3B (line 6, open and filled points). The results of the regression analysis are shown in Table 3 (row 6). As can be seen, the regression analysis was not significant, with an R^2 value of 0.146 (P=0.107). The slope of this common regression line was considerably less steep than that for the others (Table 3, compare row 6 with others). Further, the regression line for these data (Fig. 3B, line 6) was not representative of either of the two sets of data (compare open and filled points).

Many, but not all, of the drugs decreased response rates at some of the doses tested; the exceptions being RTI-55, RTI-121, LU19-005, GBR12935, WIN35,428, cocaine, cocaethylene, bupropion, and benztropine (Fig. 1, lower panels). The decreases in response rates produced by the drugs generally occurred at doses that were active in producing cocaine-appropriate responding; however, these two effects were not closely related in that the drugs listed above that did not affect response rate still substituted for cocaine. Further, the ED_{50} values (Table 2) for the two effects were not well correlated among the 11 compounds that shared these effects (for log transforms: R^2=0.280; P=0.94). Moreover, there was not a high or significant correlation of log ED_{50} values for decreases in response rates and log $K_{0.5}$ values, and parceling compounds on the basis of results of modeling the binding data did not improve the regression analysis results (Table 4).

Discussion

A number of studies have assessed the relationship between pharmacological effects of cocaine and its actions at the dopamine transporter (Kuhar et al. 1992). Studies examining the correlation among dopamine uptake inhibitors between dopamine transporter binding affinities and potencies for producing various pharmacological effects, including effects on behavior, have indicated that there is a strong relationship. For example, Ritz et al. (1987) reported that the potencies of a number of dopamine uptake inhibitors in maintaining drug self-administration were more closely related to affinities for the dopamine transporter than to affinities at other monoamine transporters. A number of other studies have established similar relationships for reinforcing effects of dopamine uptake inhibitors (Bergman et al. 1989), behavioral stimulant effects (Heikkila et al. 1979a, 1979b, 1981, Heikkila and Manzino 1984; Spealman et al. 1989; Cline et al. 1992a; though see Rothman et al. 1992; Vaugueois et al. 1993; Izenwasser et al. 1994), and, to a somewhat lesser extent, for discriminative-stimulus effects (Balster et al. 1991; Cline et al. 1992b). Consistent with those findings, in the present study, all of the structurally di-

Table 4 Relationships of binding affinities and potency to decrease response rates. Results of various regression analyses of affinities for competition against [^{3}H]WIN35,428 binding to rat caudate putamen membranes and ED_{50} values for decreases in response rate in rats trained to discriminate cocaine from saline

	Values used for regression		R^2 value	*P* value for regression	Slope	*n*
	Compounds modeling better for two sites	Compounds modeling better for one site				
1	$K_{0.5}$	$K_{0.5}$	0.234	0.131	0.54	11
2	None	$K_{0.5}$	0.25	0.253	0.74	7
3	K_{hi}	None	0.302	0.451	0.57	4
4	K_{lo}	None	0.054	0.767	0.11	4
5	K_{hi}	$K_{0.5}$	0.162	0.22	0.55	11
6	K_{lo}	$K_{0.5}$	0.169	0.209	0.26	11

verse monoamine uptake inhibitors substituted for the discriminative-stimulus effects of cocaine in a dose-related manner. The cocaine-like discriminative effects of these drugs appeared to be related to their activity at the dopamine transporter, because this effect was not shared by drugs that were relatively selective for the norepinephrine and 5-HT transporters (see also Baker et al. 1993).

The binding to the dopamine transporter by cocaine and many of its analogs has been reported as fitting a two-site model better than a one-site model. Two binding sites have been reported in rat caudate putamen (Schoemaker et al. 1985; Izenwasser et al. 1993) and human caudate (Little et al. 1993) and putamen (Schoemaker et al. 1985; Staley et al. 1994). In monkey brain, the binding of both [^{3}H]cocaine and the more potent cocaine analog [^{3}H]WIN35,428, fit a two-site binding model better than a one-site model (Madras et al. 1989a, 1989b). However, in mouse (Reith, et al. 1980) and rabbit (Kirifides et al. 1992) brain, only a single site has been observed. It is currently unclear whether these two binding sites represent two distinct binding sites or two conformational states of a single site (but see Staley et al. 1994 and below). Further, the pharmacological significance of actions mediated by these two sites is not known. It is known, however, that both components exist on the dopamine transporter because [^{3}H]WIN35,428 binds to two sites on the cloned rat (Boja et al. 1992) and expressed human (Pristupa et al. 1994; Eshleman et al. 1995) dopamine transporter, and in solubilized dopamine transporter from non-human primate striatum (Gracz and Madras 1995).

Because the behavioral relevance of these two sites was not clear, the present study compared the relationships among the binding of dopamine uptake inhibitors at these two sites with their potencies for producing cocaine-like discriminative-stimulus effects. First, the ED_{50} values for this behavioral effect were correlated with the calculated one-site model affinities ($K_{0.5}$ values) for all of the compounds. Subsequently, the correlations were determined for either the high- or low-affinity sites. Because a best fit to a one-site model may reflect a lack of sufficient statistical power to resolve two similar affinities of the displacer at the label's two sites, the correlations were also determined by combining either the high-affinity (K_{hi}) or low-affinity (K_{lo}) values for drugs that modeled better for two sites with the affinities ($K_{0.5}$ values) of the remaining compounds that modeled best for one site. All of the drugs, whether fit best to a one- or two-site model, had the effect examined, substituting for cocaine in the behavioral assay. Therefore, assuming actions at the dopamine transporter are responsible for the behavioral effect (see above), at least one of the two dopamine transporter sites mediates the discriminative effect.

Although there was a significant relationship between one-site affinities and behavioral potency, there was a stronger relationship among those affinities and behavioral potency when the only compounds included were those better fit using a one-site than a two-site model. Among those compounds better fit by a two-site model, the K_{hi} values accounted for more of the variance in ED_{50} values than did the K_{lo} values. In addition, the slopes of regression lines were greatest for the regression of K_{hi} and ED_{50} values compared with that for K_{lo} and ED_{50} values (Fig. 3; Table 3), indicating that differences in K_{hi} values produce greater changes in the ED_{50} value than do comparable differences in K_{lo} values. Comparing all of these relationships (Table 3), the strongest was that between behavioral potency in the cocaine-discrimination procedure and binding to the high-affinity site. These data suggest that the discriminative-stimulus effects of cocaine are more closely related to actions mediated by high-affinity binding to the dopamine transporter than they are to actions mediated by the low-affinity site.

The present finding that the behavioral effects of dopamine-uptake inhibitors are more closely related to binding at the high-affinity site than the low-affinity site is further evidence to support the biological relevance of these two sites, which are arrived at through mathematical modeling. However, there are several caveats to any conclusion reached solely on the basis of the present results. First, there was a limited number of drugs studied, and the data from only eight of these fit a two-site model better than a one-site model. Possibly more effective comparisons could have been made if there were equal

numbers of compounds for the various correlations. With the relatively small number of compounds, a single compound could have unduly influenced the regression line, although, by visual inspection, no single point appeared to significantly alter outcome. Obviously, a stronger statement could have been made if there had been a wider range of potencies of the drugs examined. Finally, and most importantly, the assessment of the contribution of high- and low-affinity binding to the behavioral effects of cocaine would be greatly enhanced with the development of pharmacological tools that have a high degree of selectivity for one of these components over the other (Husbands et al. 1997).

Several previous studies also suggest a biological significance for the distinction between the high- and low-affinity sites. For example, Staley et al. (1994) found an apparent selective upregulation of the high- over the low-affinity [^{3}H]WIN35,428 binding site on the dopamine transporter in victims of cocaine overdose. This upregulation was in relation to that obtained in drug-free age-matched control subjects. Interestingly, the upregulation of the high-affinity site was not accompanied by a compensatory decrease in the B_{max} for the low affinity site, suggesting that the two sites, though likely identical proteins, are not inter-convertible states (Staley et al. 1994).

Pristupa et al. (1994) found a correlation of potency for uptake inhibition and K_i values for displacement of [^{3}H]WIN35,428 in COS-7 cells. Further, among the drugs studied that modeled better for two- than one-site binding, the K_{hi} values fit the regression line better than the K_{lo} values. In addition, amfonelic acid displaced [^{3}H]WIN35,428 in COS cells with high affinity, but only partially, while fully inhibiting dopamine uptake. These data suggest that the high-affinity site is related to DA uptake inhibition (Pristupa et al. 1994).

In contrast to that which occurs in synaptosomes, dopamine-uptake inhibition by cocaine in a chopped tissue preparation exhibits both high- and low-affinity components (Izenwasser et al. 1992). Previously, we showed that meperidine interacts with this high-affinity component with some selectivity; there was a greater separation of potency for the high and low affinity components than that obtained with cocaine. Thus, meperidine was used as a probe to assess the behavioral effects of selective activation of this high-affinity component (Izenwasser et al. 1996). In monkeys trained to discriminate cocaine from saline, meperidine (in the presence of naltrexone to block its opioid effects) generalized to cocaine. This result suggested that the discriminative effects of cocaine are related to the high-affinity component of the inhibition of dopamine uptake (Izenwasser et al. 1996).

Thus, a number of results taken together are consistent with the results of the present study, suggesting different pharmacological effects of actions mediated by the high- and low-affinity sites on the dopamine transporter. Certainly, these findings collectively are not definitive, and there remain numerous questions about the roles of these sites in terms of the actions of uptake inhibitors, how those actions may be transduced into pharmacological effects, and how they are related to behavioral outcomes. Further outstanding questions involve the relationship between these two sites and the regulation of the sites through various processes, in particular chronic cocaine exposure. Nonetheless, the current results and those in the literature suggest that the high-affinity site identified by [^{3}H]WIN35,428 is intimately and selectively involved in the covert behavioral effects of dopamine-uptake inhibitors that likely play a significant role in their liability for abuse.

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