

Brief Communication

Identification of a Dopamine Transporter Ligand That Blocks the Stimulant Effects of Cocaine

Rajeev I. Desai,¹ Theresa A. Kopajtic,¹ Mikhail Koffarnus,¹ Amy Hauck Newman,² and Jonathan L. Katz¹

¹Psychobiology and ²Medicinal Chemistry Sections, Medications Discovery Research Branch, National Institute on Drug Abuse, Intramural Research Program, Department of Health and Human Services, National Institutes of Health, Baltimore, Maryland 21224

There is a large unmet medical need for cocaine addiction treatments. Studies have indicated that the dopamine transporter (DAT) is the primary biological target of cocaine, and most drugs that have DAT affinity have behavioral effects like those of cocaine. However, analogs of bupropion have high DAT affinity and behavioral effects that show varying degrees of similarity to cocaine. We now report the discovery that a bupropion analog, JHW007, with high affinity for the DAT does not have cocaine-like behavioral effects and antagonizes the effects of cocaine. JHW007 occupied the DAT *in vivo* more slowly than did cocaine and had not reached an apparent plateau up to 270 min after injection. The *in vivo* binding of cocaine to the DAT suggested rate of DAT occupancy as an important contributor to its behavioral effects, and the slow association with the DAT may provide an explanation for JHW007 being relatively devoid of cocaine-like behavioral effects. The antagonism of cocaine suggests that DAT ligands with reduced cocaine-like activity can function as cocaine antagonists and suggests JHW007 as a lead for discovery of cocaine-abuse pharmacotherapeutics.

Key words: cocaine abuse; cocaine antagonist; dopamine transporter; *ex vivo* binding; JHW007; bupropion analogs

Introduction

Recent (1999–2002) annual estimates of the number of individuals using cocaine range from 2 to 3.2 million in the United States alone (ONDCP, 2001; SAMSA, 2002), which approximates 1% of the population. This prevalence is comparable with those for attention deficit hyperactivity disorder (Scahill and Schwab-Stone, 2000) or schizophrenia (Goldner et al., 2002), as two examples of disorders for which there are significant research investments. Although these estimates suggest more than adequate incentive for drug-discovery research, the currently proven treatments for cocaine addiction are exclusively behavioral.

Several studies indicate that the dopamine transporter (DAT) is the biological target for the effects of cocaine underlying its abuse liability. Binding at the DAT interferes with the reuptake of DA, producing an increase in extracellular DA concentrations (Javitch et al., 1984; Madras et al., 1989), and stimulant effects of cocaine are absent in mice lacking the DAT (Giros et al., 1996). Among the cocaine-like uptake inhibitors that also block serotonin and norepinephrine transport, it is affinity for the DAT that

correlates with potency in preclinical models of drug abuse (Kuhar et al., 1991), supporting the hypothesis that blockade of DA transport is the mechanism underlying the abuse liability of cocaine.

There are, however, some DA uptake inhibitors that do not share all of the behavioral effects of cocaine. Analogs of bupropion (BZT), for instance, have high affinity for the DAT and inhibit DA uptake *in vitro*. However, these drugs compared with cocaine have reduced effectiveness as behavioral stimulants and generally do not share subjective and reinforcing effects of cocaine in drug-discrimination and self-administration animal models (Newman et al., 1995; Katz et al., 1999; Woolverton et al., 2001). These findings suggest that some structural variants of drugs acting at the DAT may have reduced abuse liability compared with cocaine and a potential approach to the discovery of medications for cocaine abuse.

Previous studies of DA uptake inhibitors have indicated that DAT occupancy seems to be an important determinant of the cocaine-like behavioral effects of DA uptake inhibitors (Gatley et al., 1999). However, others have indicated that DAT occupancy is not related to behavioral activity in a simple way (Rothman et al., 1992; Vaugeois et al., 1993). To further study potential mechanisms for differences between cocaine and BZT analogs, we compared the *in vivo* binding to the DAT of cocaine and an analog of BZT, JHW007 (Agoston et al., 1997), and related those effects to behavioral activity. In the process, we found that JHW007 had *in vivo* affinity for the DAT and could antagonize the effects of cocaine, which are unprecedented findings among DAT ligands.

Materials and Methods

Subjects. Male Swiss-Webster mice (Taconic, Germantown, NY), weighing 25–40 g, were kept in a colony maintained at 21 ± 1°C under a 12 h

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Correspondence should be addressed to Dr. Jonathan L. Katz, National Institute on Drug Abuse, Intramural Research Program, National Institutes of Health, 5500 Nathan Shock Drive, Baltimore, MD 21224. E-mail: jkatz@intra.nida.nih.gov.

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light/dark cycle (lights on 7:00 A.M.). Experiments were conducted between 8:00 A.M. and 3:00 P.M., in a separate room.

Drugs. The drugs used in the present studies were as follows: (–)-cocaine hydrochloride (Sigma/Aldrich, St. Louis, MO), JHW007, *N*-(*n*-butyl)-(bis-fluorophenyl)methoxytropane, AHN2005, *N*-allyl-(bis-fluorophenyl)methoxytropane (Agoston et al., 1997), and [¹²⁵I]RTI-121 (specific activity, 2200 Ci/mmol; PerkinElmer Life Sciences, Boston, MA). All drug solutions were prepared fresh daily in sterile water (cocaine was dissolved in 0.9% NaCl). For *in vivo* studies, cocaine, JHW007, and AHN2005 were administered by the intraperitoneal route. [¹²⁵I]RTI-121 was administered intravenously in 0.2 ml of sterile water.

[³H]Dopamine uptake inhibition. Fresh rat striatum was homogenized in ice-cold buffer (5 mM HEPES and 0.32 M sucrose), using 10 strokes with a Teflon glass homogenizer before centrifugation (1000 × *g* for 10 min at 4°C). The supernatant was recentrifuged (10,000 × *g* for 10 min at 4°C), and the pellet was resuspended in ice-cold incubation buffer (in mM: 127 NaCl, 5 KCl, 1.3 NaH₂PO₄, 1.2 MgSO₄, 2.5 CaCl₂, 1.498 HEPES acid, 10 D-glucose, and 1.14 L-ascorbic acid, pH 7.4) and placed on ice for 15 min.

The synaptosomal tissue preparation was incubated in buffer at 37°C with 10 μM pargyline and JHW007. After 10 min, [³H]dopamine (0.5 nM) was added to each tube. After 5 min, the incubation was terminated by the addition of 3 ml of ice-cold buffer and rapid filtration through Whatman GF/B glass-fiber paper (presoaked in 0.1% polyethylenimine; Whatman, Clifton, NJ) using a cell harvester (Brandel, Gaithersburg, MD). Filters were then washed (3 ml) twice and transferred to scintillation vials, scintillation fluid (3 ml) was added, and the vials were left overnight. Radioactivity in the presence of 100 μM (–)-cocaine HCl was subtracted to determine specific uptake.

Locomotor activity. Mice were tested individually in acrylic chambers (40 cm³) equipped with light-sensitive detectors and corresponding infrared lights spaced 2.5 cm apart (Omnitech Electronics, Columbus, OH). Each light-beam interruption registered one horizontal activity count. Each dose or dose combination was injected intraperitoneally (*n* = 8), and mice were used once. Mice were given injections and immediately placed in the apparatus for 8 h, with activity counted every 10 min. For antagonism studies, JHW007, AHN2005 (10 mg/kg each), or vehicle was injected immediately before subjects were placed in the chamber, and saline or cocaine (5–60 mg/kg) was administered 270 min (JHW007) or 210 min (AHN2005) later, with these times based on maximum *in vivo* DAT occupancy (our unpublished data). Activity was monitored for an additional 90 min.

Ex vivo binding of [¹²⁵I]RTI-121. Displacement of [¹²⁵I]RTI-121 binding by cocaine (10–40 mg/kg), JHW007 (1–17 mg/kg), or vehicle was examined according to the methods of Scheffel et al. (1989). Subjects (*n* = 10) received 2 μCi of [¹²⁵I]RTI-121 in 0.2 ml of sterile water intravenously and were killed by cervical dislocation 2 h later. Intraperitoneal injections of vehicle or a displacer were administered at various times before the subjects were killed. When the subjects were killed, whole brains were removed rapidly; the striatum and cerebellum were dissected on ice, placed into separate plastic vials (Rohren Tubes; 55 × 12 mm), and weighed; and tissue radioactivity was measured (gamma counter; ICN Biomedicals, Huntsville, AL).

Cocaine discrimination. Mice were trained in two-lever operant-conditioning chambers (Med Associates, St. Albans, VT) with food reinforcement (20 mg pellets) to press one lever after cocaine injection and a second lever after saline injection. Lever presses were reinforced under a 10-response fixed-ratio (FR10) schedule.

Mice were placed inside the experimental chambers immediately after injection. The chamber was illuminated 5 min later, and responses on the appropriate lever were reinforced. Responses on the inappropriate lever reset the FR requirement. Sessions terminated after 20 food presentations or 15 min, whichever occurred first. Daily training sessions with cocaine and saline followed a mixed sequence.

Testing began when criteria were met of >85% of responses (over the session, and on the first FR) on the appropriate lever for four consecutive sessions. Substitution tests were conducted with different combinations of doses administered before sessions and two successive sessions of criterion performance between tests. For some tests, 10 mg/kg JHW007 was

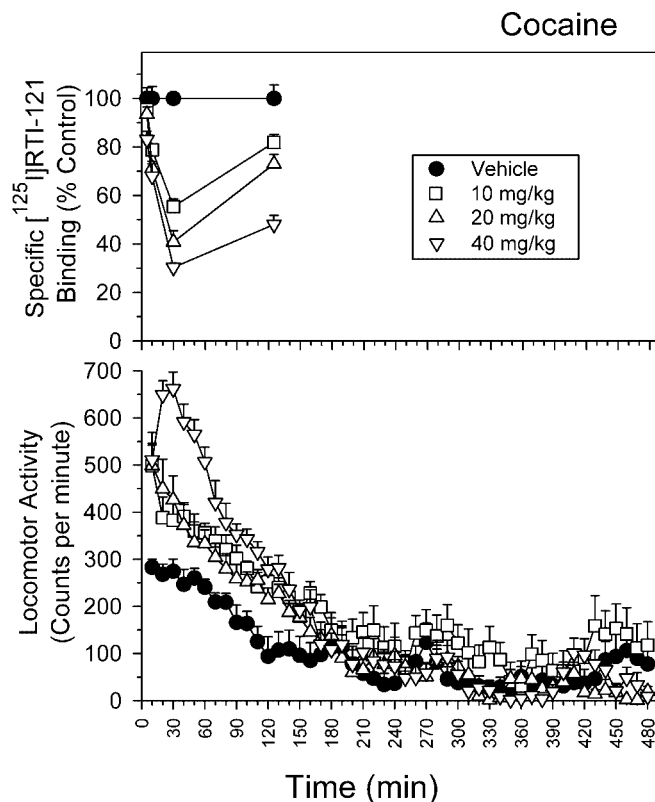


Figure 1. Time course of effects of cocaine on specific [¹²⁵I]RTI-121 binding (top) and locomotor activity in mice. *x*-axis, Time after cocaine injection; *y*-axis (top), specific [¹²⁵I]RTI-121 binding expressed as a percentage of vehicle control; *y*-axis (bottom), horizontal activity counts per minute. Each point represents data from 5–13 (top) or 8 (bottom) mice, with error bars representing +1 SEM.

administered at 270 min before cocaine. Test sessions were identical to training sessions, except that responding on either lever was reinforced.

Analysis of data. For *ex vivo* binding data, regional radioactivity levels (counts per minute) were divided by tissue weight, and a percentage of injected [¹²⁵I]RTI-121 dose per kilogram of body weight for each tissue was obtained. Estimates of specific-to-nonspecific binding were obtained [(striatum/cerebellum) – 1] based on the observation that DAT sites are highly concentrated in the striatum and relatively absent in the cerebellum (Scheffel et al., 1989). Specific binding after cocaine or JHW007 injection was expressed as a percentage of that obtained after vehicle injection. The displacement data and locomotor activity data were analyzed using two-way ANOVA and *post hoc* Tukey's tests. Dose–effect curves were analyzed using linear regression techniques to determine ED₅₀ values and their 95% confidence limits (CL). The percentage of DAT occupancy was calculated by subtracting the percentage of occupancy produced by each drug from that produced by the vehicle. Stimulation of locomotor activity during the ±10 min surrounding times at which binding was determined was calculated by subtracting the total counts after vehicle from the total counts after drug. The Pearson's product-moment correlation coefficient of these values was calculated.

Results

Among the concentrations examined, maximum displacement of [¹²⁵I]RTI-121 was obtained at 40 mg/kg cocaine at 30 min after injection. Less displacement was obtained at other times and doses (Fig. 1, top). Cocaine increased locomotor activity in mice in a dose- and time-dependent manner (Fig. 1, bottom) (10–40 mg/kg), with a maximum during the first 30 min. After this time, the amount of activity decreased progressively, reaching control levels at ~170 min after injection.

The cocaine-induced stimulation of activity was generally in

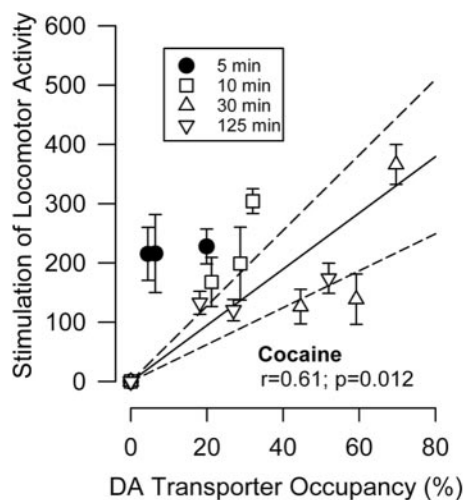


Figure 2. Relationship between occupancy of the DAT in the striatum by cocaine and its locomotor stimulant effects. *y*-axis, The amount of stimulation of horizontal locomotor activity expressed as counts after drug administration minus counts after vehicle administration; *x*-axis, DAT occupancy produced by cocaine (the inverse of specific [125 I]RTI-121 binding) expressed as a percentage of vehicle controls. The solid line represents the regression of stimulation of locomotor activity on the occupancy of the DAT with the line forced through the origin. The dashed lines represent the 95% confidence limits for the regression line. The error bars on points represent +1 SEM.

agreement with *in vivo* DAT occupancy (Fig. 2), and the correlation was significant ($r = 0.61$; $p = 0.012$). However, given the established relationship between DAT actions and behavioral effects (Kuhar et al., 1991), the correlation was poor, indicating that occupancy is only one determinant of these behavioral effects of cocaine. Figure 2 indicates that there was greater stimulation than predicted by DAT occupancy at 5 min after injection (filled circles). That this divergence occurred immediately after injection suggests that rate of occupancy also influences the behavioral effects of cocaine.

The BZT analog JHW007 had a relatively high DAT affinity (23.3 nM) (Agoston et al., 1997) and inhibited DA uptake ($IC_{50} = 24.6 \pm 1.97$ nM). The *ex vivo* displacement of [125 I]RTI-121 by JHW007 was dose related, with no plateau apparent at 270 min after injection (Fig. 3, top). In contrast to cocaine, JHW007 had a slow *in vivo* apparent association with the DAT; displacement of [125 I]RTI-121 by 17 mg/kg JHW007 occurred at a rate of $0.20 \pm 0.02\%/min$, which was significantly ($F_{(1,76)} = 39.67$; $p < 0.0001$) less than the $2.04 \pm 0.20\%/min$ (5–30 min) obtained with 40 mg/kg cocaine.

At doses from 1–10 mg/kg, JHW007 failed to produce a significant stimulation of locomotor activity throughout the 8 h observation period (Fig. 3, bottom) ($F_{(3,28)} = 1.25$; $p > 0.05$). The correlation of the stimulant effects and DAT occupancy of various doses and times after injection of JHW007 was not significant ($r = 0.002$; $p = 0.995$). Thus, JHW007 did not show a relationship between the DAT occupancy and behavioral stimulation like that observed with cocaine.

At 270 min after vehicle injection, cocaine produced a dose-related increase in activity, with a maximum at 40 mg/kg. The 60 mg/kg cocaine dose increased activity to a lesser extent (Fig. 4A, circles). Pretreatment with JHW007 (10 mg/kg) completely antagonized the effects of cocaine ($F_{(1,82)} = 41.953$; $p < 0.001$) (Fig. 4A, squares). JHW007 alone had no significant effects on activity (Fig. 4A, square). A second experiment confirmed these findings with a different shipment of mice (unconnected points at 40

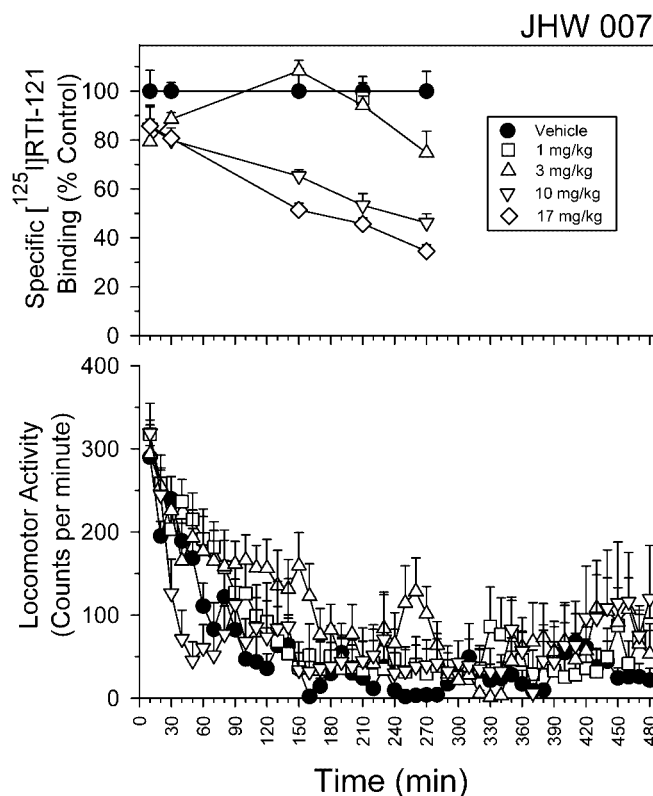


Figure 3. Time course of effects of JHW007 on specific [125 I]RTI-121 binding (top) and on locomotor activity in mice. *x*-axis, Time after JHW007 injection. Details are as in Figure 1, except binding data are from 5–10 mice (top).

mg/kg). In contrast, the *N*-allyl derivative of JHW007, AHN2005 (10 mg/kg), did not antagonize the effects of cocaine ($F_{(1,84)} = 0.426$; $p > 0.05$) (Fig. 4B, compare diamonds, circles), although there was a decrease in the effects of the highest doses of cocaine.

In cocaine discrimination, there was a dose-related increase in the percentage of responses emitted on the cocaine-appropriate lever, approximating 100% at the training dose ($ED_{50} = 3.31$ mg/kg; 95% CL = 2.39–4.59) (Fig. 4C). JHW007 shifted the cocaine dose–effect curve 3.07-fold (95% CL = 2.00–4.86) to the right, suggestive of a competitive antagonism. The following day (28.5 h after injection) (Fig. 4C, inverted triangle), the antagonist effects of JHW007 were absent.

Discussion

The DAT is considered the biological target responsible for the abuse liability of cocaine (Kuhar et al., 1991). Consequently, DAT ligands have been examined as potential cocaine-abuse treatments that have high affinity, selectivity, and, to decrease their own abuse liability, slow CNS penetration. Despite substantial effort, a compound that can function as a cocaine antagonist or substantially attenuate the behavioral effects of cocaine has not been reported. The fundamental obstacle has been that compounds that bind to the DAT most often have pharmacologies similar to cocaine. However, previous studies demonstrated that among analogs of BZT are compounds with affinity at the DAT and different magnitudes of cocaine-like effectiveness in behavioral assays (Agoston et al., 1997; Katz et al., 2004). These findings suggest that altering the chemical structure of drugs acting at the DAT may reduce intrinsic cocaine-like effects.

The present data show that JHW007 has a high *in vivo* affinity for the DAT. The time course for *in vivo* displacement revealed a

slower apparent association for JHW007 compared with cocaine. A previous pharmacokinetic comparison of cocaine and JHW007 in rats indicated that both compounds are highly permeable and detectable in the brain minutes after injection (Raje et al., 2003). Together, these studies demonstrate that JHW007 passes the blood–brain barrier and readily penetrates the brain, slowly achieving significant levels of *in vivo* DAT occupancy. Interestingly, JHW007 failed to produce appreciable cocaine-like locomotor stimulant effects, despite significant *in vivo* occupancy, consistent with previous studies indicating decreased efficacy of BZT analogs compared with cocaine (Katz et al., 1999, 2001; Woolverton et al., 2001).

Several studies have indicated a strong relationship between DAT binding and behavior for cocaine and other DA uptake inhibitors (Ritz et al., 1987; Bergman et al., 1989; Kuhar et al., 1991; Cline et al., 1992). However, in the present study, a given level of DAT occupancy by cocaine and JHW007 did not produce comparable behavioral effects. These findings indicate that the relationship between binding events at the DAT and behavioral effects may depend on several factors, including the compound being studied. Indeed, differences in chemical structure can lead to DAT inhibitors with different behavioral profiles, as illustrated by the present results with AHN2005. Several other studies have also reported complexities in the relationship between binding and the behavioral effects of DA uptake inhibitors. For example, Rothman et al. (1992) demonstrated in rats that comparable levels of locomotor stimulation were produced by several DA uptake inhibitors, but at different levels of DAT occupancy. Similar findings by Vaugeois et al. (1993), along with the present results, indicate that transduction of pharmacological actions from binding events at the DAT may involve intermediary steps that are modulated by the intrinsic nature of the ligand.

In the present study, the apparent association rate seemed to be a critical difference between JHW007 and cocaine. Immediately after injection the effects of cocaine were greater than that predicted solely by DAT occupancy. The 10-fold slower rate *in vivo* of DAT occupancy for JHW007 was accompanied by little or no locomotor stimulation. That cocaine-like behavioral effects are related to the rate of DAT occupancy is consistent with a recent report by Volkow et al. (2002) that concluded that rate of DAT occupancy plays a critical role in the subjective response to cocaine in human subjects.

The rate of DAT occupancy alone cannot account for all of the present effects. Pharmacokinetic studies of JHW007 indicate that levels in the brain decline slowly within 30 min after injection, making the continued slow decrease in [¹²⁵I]RTI-121 binding counter intuitive. A reconciliation of these results might be obtained by examining the effects of JHW007 on the number of DAT sites available for [¹²⁵I]RTI-121 binding. Regulation of DAT membrane surface expression levels has been demonstrated (Blakely and Bauman, 2000), and that some drugs can regulate this expression suggests a potential mechanism for the present effects of JHW007.

It is also possible that JHW007 acts at sites other than the DAT, and those sites may contribute to its unique activity. We

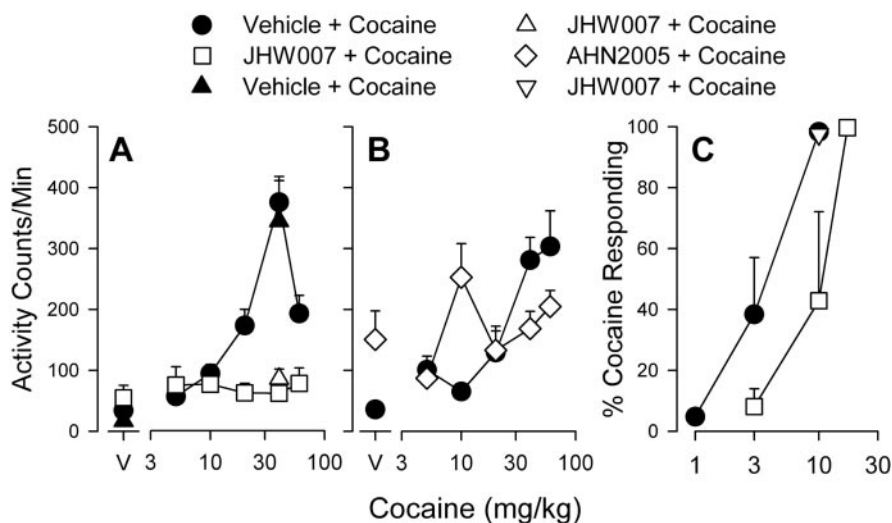


Figure 4. Interactions of cocaine and JHW007 or AHN2005. *A, B*, *y*-axis, Locomotor activity counts; *x*-axis, treatment condition, vehicle (V), or dose of cocaine. Each point represents the average effect determined in eight mice, except $n = 6$ for combination of 10 mg/kg JHW007 plus 60 mg/kg cocaine. The unconnected points above 40 mg/kg cocaine show a replicate determined several months later in a separate shipment of mice. *C*, *y*-axis, Percentage of responses on the cocaine-appropriate lever after drug; *x*-axis, dose of cocaine. Circles, Effects of cocaine alone; squares, effects of cocaine administered 4.5 h after treatment with 10 mg/kg JHW007; triangles, effects of cocaine administered 28.5 h after treatment with JHW007. Each cocaine point represents the average effect determined in five to six mice; for drug combinations, each point represents the effects of three to four mice. The error bars represent ± 1 SEM.

have previously demonstrated that JHW007 has a high selectivity for the DAT relative to other monoamine transporters (Agoston et al., 1997) and have also evaluated the binding of JHW007 to other targets (Katz et al., 2004). Among the binding sites examined, JHW007 had a high affinity for σ -binding sites, and actions at these sites have been noted to influence the effects of cocaine (Menkel et al., 1991). Although the effects of σ ligands are generally not as profound as those presently obtained with JHW007 (Katz et al., 2003), the possibility that antagonism of the behavioral effects of cocaine by JHW007 was attributable to actions at σ receptors deserves further examination. JHW007 also has affinity for H1 histamine receptors, although actions at these sites do not appear to alter the behavioral effects of cocaine (Campbell et al., 2002).

It has been suggested that compounds with potential as therapeutic agents in the treatment of cocaine abuse might be found among those with a relatively high ratio of potency for the inhibition of DA transport and DAT affinity. Using this strategy, Simoni et al. (1993) examined a 7-methoxylated tropane analog that exhibited an approximate fourfold separation of these effects and produced a two-fold antagonism of the inhibition of DA uptake produced by cocaine. Subsequent studies have identified compounds with ratio values of ~ 1.0 that attenuated the increases in locomotor activity produced by cocaine (Zhao et al., 2000). Other compounds have been identified that had a ratio value >1 , but without cocaine-antagonist effects (Wang et al., 2000; Xu et al., 2002). Along similar lines, the DA uptake inhibitor GBR 12909 antagonized the increase in extracellular DA concentration after administration of cocaine (Rothman et al., 1991) and produced selective decreases in self-administration of cocaine (Glowa et al., 1995). Although there are many differences among the results of all of these studies, a general conclusion is that not all DA uptake inhibitors produce effects identical to those of cocaine and that, under certain conditions, antagonist effects may be obtained.

The ratio of the IC_{50} value of JHW007 for the inhibition of DA uptake and its *in vitro* affinity for the DAT is 1.06. This finding is consistent with the literature summarized above that suggests that

antagonist effects of DA uptake inhibitors are not derived from differential activity within these assays. Nonetheless, the antagonism of cocaine by JHW007 and previous findings that not all DA uptake inhibitors produce effects identical to those of cocaine suggest that the DAT can function more like a receptor, at which compounds can have varying degrees of effectiveness. However, a second-messenger signaling system similar to that observed for G-protein-coupled receptors is not likely involved, and the molecular events responsible for variations in transduction of binding events to pharmacological actions remain to be determined.

In summary, JHW007 is a novel drug that has high affinity and selectivity for the DAT *in vitro* (Agoston et al., 1997; Katz et al., 2004). It passes the blood–brain barrier and readily penetrates the brain (Raje et al., 2003), slowly achieving significant levels of DAT occupancy without appreciable cocaine-like stimulant or subjective effects. Most importantly, JHW007 antagonizes the locomotor stimulant and subjective effects of cocaine. The present results in conjunction with others suggest that the rate of DAT occupancy is an important component of cocaine-like actions and potential for abuse. Furthermore, this study suggests *in vivo* association as a critical feature contributing to the effects of DAT ligands and suggests that JHW007 has the attributes necessary to serve as a lead candidate for the treatment of cocaine abuse.

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