2-Isoxazol-3-Phenyltropane Derivatives of Cocaine: Molecular and Atypical System Effects at the Dopamine Transporter

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ABSTRACT

The present study examined RTI-371 [3β -(4-methylphenyl)-2 β -[3-(4-chlorophenyl)-isoxazol-5-yl]tropane], a phenyltropane cocaine analog with effects distinct from cocaine, and assessed potential mechanisms for those effects by comparison with its constitutional isomer, RTI-336 [3β -(4-chlorophenyl)-2 β -[3-(4-methylphenyl)-isoxazol-5-yl]tropane]. In mice, RTI-371 was less effective than cocaine and RTI-336 in stimulating locomotion, and incompletely substituted ($\sim 60\%$ maximum at 5 minutes or 1 hour after injection) in a cocaine (10 mg/kg i.p.) saline discrimination procedure; RTI-336 completely substituted. In contrast to RTI-336, RTI-371 was not self-administered, and its pretreatment (1.0–10 mg/kg i.p.) dose-dependently decreased maximal cocaine self-administration more potently than food-maintained responding. RTI-336 pretreatment dose-dependently left-shifted the cocaine self-administration dose-effect curve. Both RTI-336 and RTI-371 displaced [^3H]WIN35,428 [^3H](–)-3 β -(4-fluorophenyl)-tropan-2 β -carboxylic acid methyl ester tartrate] binding

to striatal dopamine transporters (DATs) with K_i values of 10.8 and 7.81 nM, respectively, and had lower affinities at serotonin or norepinephrine transporters, or muscarinic and σ receptors. The relative low affinity at these sites suggests the DAT as the primary target of RTI-371 with minimal contributions from these other targets. In biochemical assays probing the outward-facing DAT conformation, both RTI-371 and RTI-336 had effects similar to cocaine, suggesting little contribution of DAT conformation to the unique pharmacology of RTI-371. The locomotor-stimulant effects of RTI-371 (3.0–30 mg/kg i.p.) were comparable in wild-type and knockout cannabinoid CB₁ receptor (CB₁R) mice, indicating that previously reported CB₁ allosteric effects do not decrease cocaine-like effects of RTI-371. DAT occupancy in vivo was most rapid with cocaine and least with RTI-371. The slow apparent association rate may allow compensatory actions that in turn dampen cocaine-like stimulation, and give RTI-371 its unique pharmacologic profile.

Introduction

The dopamine transporter (DAT) is considered the primary biologic target of cocaine, and most stimulants that block dopamine (DA) uptake have behavioral effects and abuse liability similar to cocaine (Taylor and Ho, 1978; Ritz et al., 1987; Bergman et al., 1989). Further, the DAT affinities of standard uptake inhibitors are directly related to their potencies for producing behavioral-stimulant effects, subjective

effects (determined by drug-discrimination procedures), and self-administration (Ritz et al., 1987; Bergman et al., 1989; Katz et al., 2000).

In contrast to the standard DAT inhibitors, several compounds with high affinity for the DAT do not have cocaine-like behavioral effects and abuse liability and, for that reason, have been categorized as atypical DAT inhibitors. For example, analogs of benztropine (BZT) potentially bind the DAT but are not self-administered and are less effective than cocaine in producing several characteristic stimulant effects (Tanda et al., 2009b). Further, several of these atypical DAT inhibitors, including the BZT analog JHW007 [(*N*-butyl)-3 α -[bis(4-fluorophenyl)methoxy]tropane], antagonize various effects of cocaine, including locomotor stimulation (Desai et al., 2005b), increases in nucleus accumbens DA concentrations (Tanda et al., 2009a), and self-administration (Hiranita et al., 2009).

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ABBREVIATIONS: ANOVA, analysis of variance; BZT, benztropine; CB₁R, cannabinoid CB₁ receptor; DA, dopamine; DAT, dopamine transporter; EXT, extinction; FR, fixed ratio; IC₅₀, half-maximal inhibitory concentration; IRP, Intramural Research Program; JHW007, (*N*-butyl)-3 α -[bis(4-fluorophenyl)methoxy]tropane; KO, knockout; LED, light-emitting diode; NET, norepinephrine transporter; OWW, original wet weight; PEO₂, polyethylene oxide; PBS, phosphate-buffered saline; PBSCM, PBS with CaCl₂ and MgCl₂; RTI-113, 3 β -(4-chlorophenyl)-2 β -carboxylic-acid-phenyl-ester; RTI-336, 3 β -(4-chlorophenyl)-2 β -[3-(4-methylphenyl) isoxazol-5-yl]tropane; RTI-371, 3 β -(4-methylphenyl)-2 β -[3-(4-chlorophenyl) isoxazol-5-yl]tropane; SERT, serotonin transporter; TM, transmembrane domain; TO, timeout; σ_1 R, σ_1 receptor; σ_2 R, σ_2 receptor; WIN 35,428, (–)-3 β -(4-fluorophenyl)-tropan-2 β -carboxylic acid methyl ester tartrate; WT, wild type.

Several hypotheses have been suggested for mechanisms underlying these atypical effects. It was initially suggested that differences from standard DAT inhibitors were due to off-target actions of the atypical compounds. The parent compound BZT is a well-known antimuscarinic and is used therapeutically for those effects (Lees, 2005). Further, BZT also has histaminic H_1 antagonist effects (Richelson, 1981; McKearney, 1982). However, neither of those actions appears to be involved in the atypical effects of the BZT analogs, as ligands selective for these particular off-target sites do not block the effects of cocaine (Campbell et al., 2005; Tanda and Katz, 2007). Other potential targets have been identified through broad screens of receptor binding. Several BZT analogs have affinity for the σR (Katz et al., 2004; Li et al., 2011; Hiranita et al., 2014), and recent studies have indicated that σR antagonist activity coupled with DAT inhibition can block the self-administration of cocaine (Hiranita et al., 2011). Therefore, atypical DAT inhibitors may act through these dual actions.

Desai et al. (2005a,b) suggested that slow DAT-association rates promote the atypical effects of DAT inhibitors. In those studies, the rate of DAT occupancy in vivo for several atypical DAT inhibitors was less than that for cocaine. Additionally, Loland et al. (2008) suggested that the conformational state of the DAT leads to atypical DAT-inhibitor effects. Cocaine binding has been proposed to stabilize an outward-facing DAT conformation, whereas atypical DAT inhibitors stabilize an inward-facing conformation (Reith et al., 2001; Loland et al., 2008; Hong and Amara, 2010). Loland et al. (2008) found that compounds with cocaine-like behavioral effects were about 100-fold less potent in mutant DAT (Y335A) with an inward-facing conformation compared with wild-type (WT) DAT, which has an outward-facing conformational equilibrium. In contrast, the potencies of atypical DAT inhibitors with behavioral effects unlike cocaine were relatively tolerant to DAT conformation, linking different modes of DAT interaction with cocaine-like or atypical behavioral effects.

Previous studies suggested that the isoxazol phenyltropane derivative RTI-371 [3β -(4-methylphenyl)- 2β -[3-(4-chlorophenyl)isoxazol-5-yl]tropane] (Fig. 1) has atypical DAT-inhibitor effects. For example, RTI-371 failed to stimulate locomotor activity in mice across a range of behaviorally active doses and did not substitute in rats trained to discriminate cocaine from saline (Carroll et al., 2004). Further, a preliminary report indicated that RTI-371 blocked cocaine-induced locomotor stimulation (Navarro et al., 2005). Finally, Navarro et al. (2009) suggested that the antagonism of cocaine by RTI-371 was due at least partly to positive-allosteric modulation of the cannabinoid CB_1 receptor (CB_1R), as both RTI-371 and the BZT analog JHW007 had this activity.

Because RTI-371 is structurally different from BZT analogs, studies of its mechanisms may shed light on the necessary or sufficient actions leading to atypical DAT-inhibitor effects. Thus, we further assessed the effects of RTI-371 and compared it with its constitutional isomer, RTI-336 [3β -(4-chlorophenyl)- 2β -[3-(4-methylphenyl)isoxazol-5-yl]tropane] (Fig. 1), a compound selected for preclinical development (Carroll et al., 2006b) and clinical trials (ClinicalTrials.gov) as a potential antagonist of stimulant effects. RTI-336 stimulated locomotion and substituted for cocaine in rats trained to discriminate cocaine from saline (Carroll et al., 2004), and it was self-administered in primates at rates greater than those

Fig. 1. Chemical structures of cocaine and the isoxazol phenyltropane derivatives used in this study. The isoxazol phenyltropanes are constitutional isomers having the same molecular formula but with the exchanged points of attachment of methyl and chloride atoms.

obtained with vehicle but less than those with cocaine. Additionally, pretreatment with RTI-336 decreased maximal rates of cocaine self-administration (Howell et al., 2007).

Materials and Methods

Subjects. At the start of all in vivo experiments, male mice and rats (Taconic Farms, Germantown, NY or Charles River Laboratories, Wilmington, MA) were acclimated to the animal colony for a minimum of 1 week. During this time, food and tap water were freely available. Swiss-Webster or CB_1R mutant mice (Zimmer et al., 1999) bred at the National Institute on Drug Abuse (NIDA) Intramural Research Program (IRP) for in vivo binding or locomotor-activity assessments were housed in groups of three to four per cage with ad libitum access to food and water except during testing. Swiss-Webster mice for chronic behavioral studies were housed individually and were fed daily at least 30 minutes after testing with about 3 g of standard laboratory chow that maintained them at their individual weights throughout the study. Sprague-Dawley rats for behavioral studies were maintained at approximately 320 g by adjusting their daily food ration. The humidity- and temperature-controlled colony rooms were maintained on a 12-hour light/dark cycle with lights on at 7:00 AM. The animals were cared for in accordance with the National Institutes of Health *Guidelines of the Animal Care and Use Program* as executed by the NIDA IRP Animal Program, which is fully accredited by the

Association for Assessment and Accreditation of Laboratory Animal Care International.

Monoamine Transporter Binding Assays. Tissue was dissected and homogenized in buffer using a Brinkman Polytron (at setting 6 for 20 seconds) and was subsequently centrifuged at 20,000g (50,000g for the NET assay) for 10 minutes at 4°C. The resulting pellet was resuspended in buffer, recentrifuged, and suspended in buffer again to a concentration of 10, 80, or 15 mg/ml (original wet weight, OWW) for DAT, NET, or SERT binding assays, respectively. Incubations were conducted in assay tubes containing 0.50 ml of buffer and 0.50 nM (1.4 nM for SERT assay) radioligand, tissue, and various concentrations of inhibitors. See Table 1 for further details.

M₁ Muscarinic Receptor Binding Assay. Tissue was thawed in ice-cold buffer and homogenized with a Brinkmann Instruments (Westbury, NY) Polytron at setting 6 for 20 seconds. The homogenate was centrifuged at 1000g for 10 minutes at 4°C. The resulting supernatant was then centrifuged at 10,000g for 20 minutes at 4°C. The resulting pellet was resuspended in a buffer volume of 200 mg/ml OWW. The ligand binding assays were conducted in tubes containing 0.50 ml of buffer, 3.0 nM radioligand, and 20 mg of tissue, OWW. See Table 1 for further details.

σ_1 and σ_2 Receptor Binding Assay. Guinea pig brain was used because of the relatively higher density of σ_1 R and σ_2 R in that tissue compared with rats (Nguyen et al., 1996). The tissue was thawed on ice, homogenized (with a glass and Teflon apparatus) in buffer, and subsequently centrifuged at 800g for 10 minutes at 4°C. The supernatant was collected into a clean centrifuge tube, and the remaining pellet was resuspended by vortex in 10 ml of buffer (tissue) then respun at 800g for 10 minutes at 4°C. The supernatants were pooled and spun at 50,000g for 15 minutes at 4°C. The remaining pellet was resuspended 80 mg/ml, OWW, in buffer and vortexed. The tissue suspension was incubated at 25°C (water bath) for 15 minutes, and then respun at 50,000g for 15 minutes. The supernatant was poured off, and the pellet was gently resuspended in buffer to 80 mg/ml, OWW. Incubations were conducted in polypropylene assay tubes containing 0.50 ml of buffer, 1.4 nM radioligand [and 200 nM (+)-pentazocine for σ_2 binding], tissue, and various concentrations of inhibitors. See Table 1 for further details.

The reactions in all binding assays were started with the addition of tissue and terminated by rapid filtration through Whatman GF/B filters (GE Healthcare Bio-Sciences, Pittsburgh, PA) (presoaked in

polyethylenimine in water, all at 0.050%, except at 0.30% for the SERT binding assay) using a Brandel Cell Harvester (Brandel Instruments Gaithersburg, MD). The filters were washed twice with 5.0 ml of ice-cold buffer, transferred to scintillation vials to which Beckman Ready Safe scintillation cocktail (3.0 ml; Beckman Coulter Instruments, Fullerton, CA) was added, and the vials were counted the next day using a Beckman LS6000 liquid scintillation counter at 50% efficiency. The assays were typically conducted as three or more independent experiments, each performed with triplicate tubes.

The IC₅₀ values for the displacement of radioligands were computed using a nonlinear, least-squares regression analysis for competitive binding (GraphPad Prism, San Diego, CA). Inhibition constants (K_i values) were calculated using the concentration of radioligand used in the assay, and the Cheng-Prusoff equation and the historical value for the K_d value of the radioligand were determined in this laboratory.

Ligand-Induced DAT conformation. Detailed methods for using maleimide-polyethylene oxide-biotin (maleimide-PEO₂-biotin; Pierce Biotechnology, Rockford, IL) surface biotinylation were described previously (Hong and Amara, 2010). In brief, human embryonic kidney 293 (HEK293) cells stably transfected with WT or T316C/C306A human DAT were seeded into polyethylenimine-coated six-well plates and cultured to confluence. The cells were washed with ice-cold phosphate-buffered saline (PBS) with 0.10 mM CaCl₂ and 1.0 mM MgCl₂, pH 7.1 (PBSCM) and incubated with DAT inhibitors in PBSCM for 20 minutes at 4°C. The cells were then further incubated with 1.0 mg/ml maleimide-PEO₂-biotin in the presence of DAT inhibitors for 30 minutes at 4°C, followed by quenching with 100 mM cysteine in PBSCM for 15 minutes at 4°C. The cells were then washed, harvested, and lysed in lysis buffer (10 mM Tris, 150 mM NaCl, 1.0 mM EDTA, 1.0% Triton X-100, protease inhibitors, pH 7.5) for 60 minutes at 4°C, followed by a 20-minute centrifugation of 18,000g. The lysates were incubated with 60 μ l of NeutrAvidin agarose beads (Pierce Biotechnology) overnight at 4°C. After the beads were washed three times with lysis buffer, the biotinylated proteins were eluted with sodium dodecyl sulfate (SDS) sample buffer, separated by polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and probed with MAB369 antibodies (Chemicon, Temecula, CA). The mean densities of the chemiluminescent DAT bands were quantified using the National Institutes of Health ImageJ software (<http://rsbweb.nih.gov/ij/>) and were normalized to percentage of vehicle.

TABLE 1

Specific conditions used for studies of displacement of radioligands by RTI-336 and RTI-371 at different binding sites

Assay	Radiolabel	Tissue	Incubation Buffer	Incubation	Nonspecific Binding
DAT	0.50 nM [³ H]WIN 35,428 (PerkinElmer Life and Analytical Sciences)	1.0 mg/tube, frozen striatum (from male Sprague-Dawley rats brains supplied on ice from Bioreclamation, Hicksville, NY)	Modified sucrose phosphate buffer (0.320 M sucrose, 7.74 mM Na ₂ HPO ₄ , 2.26 mM NaH ₂ PO ₄ , pH adjusted to 7.4)	120 min on ice	100 μ M Cocaine HCl
NET	0.50 nM [³ H]Nisoxetine (PerkinElmer Life and Analytical Sciences)	40 mg/tube, frozen frontal cortex (Bioreclamation)	50 mM Tris buffer (120 mM NaCl and 5.0 mM KCl, adjusted to pH 7.4)	180 min on ice	1.0 μ M Desipramine
SERT	1.4 nM [³ H]Citalopram (PerkinElmer Life and Analytical Sciences)	1.5 mg/tube, frozen rat midbrain (Bioreclamation)	50 mM Tris buffer (as for NET)	60 min at room temperature (~25°C)	10 μ M Fluoxetine
Muscarinic M ₁	3.0 nM [³ H]Pirenzepine (PerkinElmer Life and Analytical Sciences)	20 mg/tube, rat brains excluding cerebellum (Bioreclamation)	0.50 ml of buffer (10 mM Tris-HCl, 5.0 mM MgCl ₂)	60 min at 37°C in a water bath	100 μ M Quinuclidinyl benzilate
σ_1	3.0 nM [³ H](+)-Pentazocine (PerkinElmer Life and Analytical Sciences)	8.0 mg/tube, frozen guinea-pig brains excluding cerebellum (Pel Freez Biologicals, Rogers, AR)	10 mM Tris-HCl with 0.32 M sucrose, pH 7.4	120 min at room temperature	10 μ M Haloperidol
σ_2	3.0 nM [³ H]1,3-Di- <i>o</i> -tolylguanidine (PerkinElmer Life and Analytical Sciences), with 200 nM (+)-pentazocine	8.0 mg/tube, frozen guinea-pig brains excluding cerebellum (Pel Freez Biologicals)	10 mM Tris-HCl with 0.32 M sucrose, pH 7.4	120 min at room temperature	100 μ M Haloperidol

In Vivo Binding. Swiss-Webster mice weighing 30 to 50 g at the time of testing were injected intravenously with 2.0 μ Ci of [125 I]RTI-121 (specific activity = 2200 Ci/mmol; PerkinElmer Life and Analytical Sciences, Waltham, MA) and then killed by cervical dislocation 120 minutes later. Vehicle or drugs were administered intraperitoneally at 10, 30, 45, or 60 minutes before the mice were killed and their brains rapidly removed. The striatum and cerebellum were dissected on ice, placed into separate plastic vials (55 $\times$ 12 mm; Röhren Tubes; Sarstedt, Aktiengesellschaft & Co., Nümbrecht, Germany) and weighed, and the tissue radioactivity was measured using an automated gamma counter (10/600 PLUS; MP Biomedicals, Irvine, CA).

RTI-336, RTI-371, and [125 I]RTI-121 were dissolved in sterile water; cocaine was dissolved in sterile saline. Cocaine, RTI-336, and RTI-371 were administered a volume of 10 ml/kg i.p. [125 I]RTI-121 was administered (tail vein) a volume of 0.20 ml/mouse i.v.

Regional radioactivity levels were divided by tissue mass (counts per minute per milligram of tissue). Specific binding was calculated as counts per minute per milligram of tissue in striatum divided by counts per minute per milligram cerebellum minus 1 ([Striatum/Cerebellum] - 1), as previously described elsewhere (Scheffel et al., 1991; Desai et al., 2005a,b). This calculation is based upon the observation that the DAT is highly concentrated in the striatum and is relatively absent in the cerebellum, yielding an assessment of specific to nonspecific binding (Scheffel et al., 1991). Specific binding values were then converted to the percentage of specific binding after vehicle injection. Data were analyzed using two-way analysis of variance (ANOVA) followed by Tukey's post hoc tests.

The apparent rate of occupancy for each drug was calculated by determining the slope of a linearly fitted line for displacement of [125 I]RTI-121 over time. The data for cocaine at 60 minutes after injection were excluded from the analysis because the displacement was not different between 45 and 60 minutes after injection. The apparent rates of occupancy were compared across drugs at the highest doses of each to minimize the contributions of uptake and distribution.

Locomotor Activity Studies. Swiss-Webster mice weighing 30 to 35 g were placed singly in clear acrylic chambers (43.2 $\times$ 43.2 $\times$ 29.8 cm) for the assessment of horizontal locomotor activity. In addition, for some studies, CB $_1$ R KO and WT mice were used. All mutant mice had a C57BL/6J genetic background and were bred within the NIDA IRP. The acrylic chambers fit within monitors (Med Associates, St. Albans, VT), which were equipped with detectors sensitive to infrared light which were spaced 2.5 cm apart along two perpendicular walls. Mounted on the opposing walls and directed at the detectors were infrared light sources. One activity count was registered each time the subject interrupted a single light beam. White noise was present in the room throughout testing to mask extraneous sounds. Ambient illumination was provided by overhead illumination of the laboratory room. Mice were injected (in volumes of 1.0 ml/100 g i.p.) and immediately placed in the apparatus, with activity counts totaled each 10 minutes over a 240-minute period. Effects are shown for the first 30 minutes of each hour after injection. Locomotor counts were further analyzed as a function of dose using ANOVA.

Cocaine Discrimination Studies. Swiss-Webster mice weighing 30 to 35 g served as subjects. Subjects were trained daily (Mon–Fri) in two-lever operant-conditioning chambers (14.0 $\times$ 15.2 $\times$ 12.7 cm, model ENV 307A; Med Associates, St. Albans, VT) that were housed within light- and sound-attenuating enclosures equipped with ventilation fans. White noise was present throughout testing to mask extraneous sounds. Ambient illumination was by a lamp in the top center of the front panel (house light). On the front wall of each chamber were two response levers, 7.0 cm apart, with a pair of lamps (light-emitting diodes; LEDs) above each. A downward displacement of either lever with a force of 0.02 N through approximately 1.0 mm produced an audible click of a relay mounted behind the front wall of the chamber and was counted as a response. Food pellets (20 mg; BioServe, Frenchtown, NJ) were dispensed into a food tray centered between the levers. Online experimental control and data collection

were accomplished with computers running Med Associates software and interfacing equipment.

Subjects were trained to press both levers under a 10-response fixed ratio (FR 10) schedule of food reinforcement and to discriminate cocaine (10 mg/kg i.p.) from saline injections. After cocaine injection, responses on one of the levers were reinforced; after saline injection, responses on the other lever were reinforced. The assignment of cocaine- and saline-appropriate levers was counterbalanced across subjects. Immediately after injection, subjects were placed in the experimental chambers with house light and LEDs extinguished and no scheduled consequences for any responses, the time-out (TO) period. After the TO, the house light and LEDs were illuminated, and responses on the appropriate lever were reinforced upon completion of the FR. Responses on the inappropriate lever reset the FR response requirement. Each food presentation was followed by a 20-second TO period during which all lights were off, and responding had no scheduled consequences. Sessions ended after 20 food presentations or 15 minutes. Training sessions with cocaine (C) and saline (S) injections were conducted daily 5 days per week in a double alternation sequence (e.g., SCCS . . .). Test sessions were initiated once performances had reached the criteria of at least 85% appropriate responding (throughout and during the first FR 10 of the session) over four consecutive sessions. Test sessions were conducted with different doses of cocaine administered 5 minutes (all drugs) or 60 minutes (RTI-336, RTI-371) before sessions. After a test session, the subjects were required to meet the above performance criteria over two consecutive (cocaine and saline) training sessions before another test session. Test sessions were identical to training sessions, with the exception that 10 consecutive responses on either lever were reinforced.

The percentage of responses on the cocaine lever was calculated by dividing the number of responses on the cocaine-appropriate lever by the total number of responses emitted (excluding those emitted during TO periods). The rate of responding was calculated by dividing the total number of responses by the session time (excluding TO periods) and is expressed as the percentage of saline (control) rate of responding. These data are shown as mean values ($\pm$ S.E.M.) for groups of subjects at each drug dose. If fewer than half of the subjects responded at a particular dose, the data sample was judged as insufficient, and no mean value was calculated for the percentage of cocaine-appropriate responding at that dose. Greater than 20% cocaine-appropriate responding was considered a difference from saline, and greater than 85% cocaine-appropriate responding was considered full substitution for cocaine.

Cocaine Self-Administration Studies. Drug self-administration studies were conducted with male Sprague-Dawley rats in experimental chambers (25.5 $\times$ 32.0 $\times$ 26.5 cm, modified ENV-203; Med Associates) that were placed within sound-attenuating cubicles equipped with ventilation fans. White noise was present throughout testing to mask extraneous sounds. Ambient illumination was by a lamp in the top center of the front panel (house light). On the front wall of each chamber were two response levers, 4.0 cm above the grid floor and 5.0 cm from the midline, with three LEDs positioned in a row 10 cm above each. A downward displacement of either lever with a force of 0.20 N through about 1.0 mm produced an audible click of a relay mounted behind the front wall of the chamber and was counted as a response. Food pellets were delivered to a tray mounted behind a 5.0 $\times$ 5.0 cm opening (2.0 cm above the floor at midline) in the front panel. A syringe driver (Model 22; Harvard Apparatus, Holliston, MA) mounted above each chamber delivered injections of specified volumes and durations from a 10-ml syringe. The syringe was connected by Tygon tubing to a single-channel fluid swivel (375 Series Single Channel Swivels; Instech Laboratories, Plymouth Meeting, PA) mounted on a balance arm above the chamber. Tygon tubing from the swivel through a hole in the top of the chamber was connected to the subject's catheter and completed the connection to the subject. The tubing was protected by a surrounding metal spring.

Eighteen rats were initially trained with food reinforcement (45-mg food pellets; Bio-Serv, Frenchtown, NJ) to press the right lever under an FR 5-response schedule of reinforcement. Each fifth response produced a food pellet, which was followed by a 20-second TO period during which responses had no scheduled consequences (other than feedback clicks). Sessions ended after delivery of 30 food pellets or 20 minutes.

Once subjects obtained 30 food pellets within each of three consecutive sessions, they were divided into two groups. One group ($N = 6$) continued with food reinforcement, whereas subjects in the other group ($N = 12$) were surgically implanted with a chronic indwelling catheter in the right external jugular vein that exited at the midscapular region of the subject's back. Catheter surgery was performed under a mixture of ketamine (60.0 mg/kg i.p.) and xylazine (12.0 mg/kg i.p.) anesthesia. Thereafter, catheters were infused daily with 0.1 ml of a sterile saline solution containing heparin (30.0 IU/ml) and penicillin G potassium (250,000 IU/ml) to minimize the formation of clots, fibroids, and infection. Subjects recovered from surgery for at least 7 days before experiments resumed.

Cocaine self-administration (with 1.0 mg/kg per injection) sessions lasting 120 minutes were then conducted daily until performances showed no discernible daily trends. During these sessions, illumination of the LEDs above the right lever signaled the availability of injections, and completion of an FR 5 turned off the LEDs, produced a 0.20-second infusion, and initiated a 20-second TO period. After the TO, LEDs were again illuminated, and the next five responses produced another injection. All responses during TO periods were recorded but had no scheduled consequences. These responses were infrequent.

Once three consecutive 120-minute sessions had occurred with at least 30 injections, the final conditions were applied, which consisted of dividing the session into five sequential 20-minute components, each preceded by a 2.0-minute TO period. The components differed only by the infusion volumes and durations so that cocaine unit doses were available in the following ascending order: no injection (extinction), 0.03, 0.10, 0.32, and 1.0 mg/kg per injection. Infusion volumes (and durations) were, respectively, 0 μ l (0 seconds), 5.6 μ l (0.32 seconds), 18.0 μ l (1.0 seconds), 56.0 μ l (3.2 seconds), and 180 μ l (10 seconds), based on a body weight of 0.32 kg. An injection of cocaine at the available dose was delivered independently of responding at the beginning of each component. Training continued until 1) at least 5.0 mg/kg cocaine was self-administered within a session with less than 20% variation in the total number of cocaine injections across sessions; 2) the maximal response rates were maintained at a dose that varied by no more than one-half log unit over sessions; and 3) the maximum response rates were greater than 3-fold higher than those maintained during extinction. The effects of substitution of RTI-336 or RTI-371 (intravenous), or their pre-session treatments (intraperitoneal) on cocaine self-administration were separated by a minimum of 2 days. All of the tests of pre-session treatments were conducted with a mixed order of doses.

Subjects studied with food reinforcement were also trained under the five-component procedure after obtaining 30 food pellets within three consecutive sessions. All conditions were identical to those used with cocaine self-administration except that different numbers of food pellets were available in each component (0 to 4 pellets in the first to fifth components) rather than different doses of cocaine. Because the response rates with food reinforcement were initially higher than those maintained by cocaine, and because the conditions of the experiment including the control response rates can influence the effects of drug treatments (Dews, 1958), to make response rates more comparable the subjects studied with food reinforcement were given their daily ration of food (Harlan Rodent Chow, 15 g; Harlan Laboratories, Indianapolis, IN) 150 minutes before sessions.

The response rates were calculated by dividing the total responses by the elapsed time during components, excluding responses and time during TO periods. The statistical significance of effects on response rates was assessed by ANOVA, with post hoc Bonferroni t tests.

A two-way repeated-measures ANOVA was used to assess the effects of successive response rates during drug self-administration (factors were component, or dose, and treatment: drug or saline). A one-way repeated-measures ANOVA was used to assess the effects of drug substitution on successive response rates during drug self-administration (see data shown in Fig. 8A). A two-way repeated-measures ANOVA was also used to assess effects of pre-session treatment with RTI-336 or RTI-371 on self-administration, with drug pretreatment dose and component (no injection and cocaine dose) as factors (see Fig. 8, B and C). The ED_{50} values for the effects of pretreatments were calculated by linear regression (Snedecor and Cochran, 1967) of response rates on dose of pretreatment drug using rates from the fourth component of the session, when maximal response rates were maintained by cocaine injections or food presentation (see Fig. 8, D and E).

Drugs and Reagents. The drugs used in this study were as follows: (–)-cocaine HCl (Sigma-Aldrich, St. Louis, MO), RTI-336, RTI-371 (Fig. 1; synthesized in the Medicinal Chemistry Section of the NIDA IRP according to procedures described in Carroll et al., 2004). Desipramine, fluoxetine, and haloperidol (Sigma-Aldrich) were used for in vitro radioligand binding assays, and benztropine mesylate (Sigma-Aldrich) and JHW007 (NIDA IRP Medicinal Chemistry Section) were used in studies of ligand-induced DAT conformational change. Self-administration of the test drugs was assessed with intravenous delivery of injections. Pre-session treatments of the test drugs were assessed with the drugs injected intraperitoneally immediately before sessions. In vivo doses are expressed as milligrams of the salt form per kilogram body weight. All drug solutions were prepared fresh daily in 0.9% NaCl (USP; Hospira Inc., Lake Forest, IL).

Results

Radioligand Binding Assays. Both RTI-336 and RTI-371 displaced [3 H]WIN 35,428 binding to the DAT in rat striata with similar nanomolar affinities (Table 2), which were comparable to their IC_{50} values in previously reported results (Carroll et al., 2004). Each of the compounds was selective for the DAT over the other monoamine transporters, with RTI-336 having 2690- or 101-fold greater affinity for the DAT than the SERT or NET, respectively, and RTI-371 having 6430- or 113-fold greater affinity for the DAT than the SERT or NET, respectively. Both compounds had micromolar affinity for σ_1 R, with RTI-336 having a 3-fold greater affinity than RTI-371 at this site, and for muscarinic M_1 receptors. Both compounds had an affinity for σ_2 R that was, respectively, 34-fold and 45-fold less than their affinities for the DAT (Table 2).

Ligand-Induced DAT Conformation. Figure 2A shows a homology-based structural model of human DAT based on *Drosophila* DAT X-ray crystal structure (Penmatsa et al., 2013). Sequence aligned amino acid residues of human DAT were threaded onto the peptide backbone structure of *Drosophila* DAT using the NIH Cn3D software. To compare the effects of RTI-336 and RTI-371 on DAT conformation, we used a previously established biochemical method (Hong and Amara, 2010) to probe the cysteine accessibility in DAT using maleimide- PEO_2 -biotin, a membrane impermeant biotin derivative that was shown to react selectively with the sulfhydryl side chain of cysteine 306, a residue located on the apex of transmembrane domain 6a (TM6a; Fig. 2A). Saturating concentrations (>100 -fold K_i values) of cocaine (100 μ M), RTI-336 (10 μ M), and RTI-371 (10 μ M) all enhanced the labeling of WT DAT by maleimide- PEO_2 -biotin (Fig. 2B), suggesting binding of these drugs induced a similar conformational state of DAT that altered the reactivity of cysteine 306.

TABLE 2
Inhibition of the radioligands by RTI-336 and RTI-371
All values are in nanomolars with 95% confidence limits in parentheses.

Compound	DAT K_i Value	SERT K_i Value	NET K_i Value	M_1 K_i Value	σ_1R K_i Value	σ_2R K_i Value
Cocaine	76.6* (72.6–80.5)	289 (257–325)	2550 (2060–3150)	61,100 (41,900–89,100)	5,190* (3800–7060)	19,300* (16,000–23,300)
RTI-336	10.8 (8.27–14.0)	29,000 (16,300–51,600)	1090 (736–1620)	1,340 (1170–1530)	5,180 (3060–8780)	365 (21.4–6220)
RTI-371	7.81 (6.93–8.79)	50,200 (20,400–124,000)	880 (505–1530)	11,000 (8850–13,600)	15,800 (10,100–24,900)	353 (187–665)

*These data were reported originally in Garcés-Ramírez et al. (2011) and were collected using procedures identical to those used in this article.

Effects of cocaine or BZT analogs on DAT conformation were further assessed using a substituted-cysteine DAT mutant T316C/C306A that exhibited functional [³H]DA uptake in transfected cells (Fig. 2C). The changes of cysteine 306 to alanine and threonine 316 to cysteine yield a sole cysteine residue in the extracellular vestibule of DAT in an outward-facing conformation, with reactivity to maleimide-PEO₂-biotin becoming a reporter of DAT conformational status (Hong and Amara, 2010). Incubation with cocaine (100 μM), RTI-336 (1.0 μM), and RTI-371 (1.0 μM) significantly increased DAT labeling (Fig. 2D), indicating that the side chain accessibility of residue 316 in the middle of TM6a was altered in a similar way by the binding of these drugs to DAT. In contrast, reduced DAT labeling occurred with incubation with BZT (100 μM) and its derivative JHW007 (1.0 μM), suggesting that these inhibitors, which have structures distinct from cocaine, induced different DAT conformational changes, as previously reported (Loland et al., 2008).

In Vivo Binding. Cocaine produced a dose- and time-related displacement of [¹²⁵I]RTI-121 binding in striatum (Fig. 3A). The ANOVA indicated significant effects of dose ($F_{3,34} = 106$; $P < 0.001$), time ($F_{3,34} = 24.0$; $P < 0.001$), and their interaction ($F_{9,34} = 3.05$; $P = 0.009$). Maximal displacement of radiolabel was obtained at 40 mg/kg at 45 minutes after injection, with no further displacement at 60

minutes after injection. At that dose displacement during the first 45 minutes occurred at a rate of 1.22%/min.

RTI-336 also displaced [¹²⁵I]RTI-121 binding in striatum in a dose- and time-dependent manner [Fig. 3B; dose ($F_{2,12} = 188$; $P < 0.001$), time ($F_{2,12} = 18.0$; $P < 0.001$), and dose × time ($F_{4,12} = 5.89$; $P = 0.007$)]. Maximal displacement of radiolabel was obtained at 56 mg/kg at 60 minutes after injection, with longer times not tested. The slopes of the lines fitted to the time course of displacement were generally less steep than those obtained with cocaine. At the 56 mg/kg dose, displacement by RTI-336 occurred at a rate of 0.722%/min.

The binding of [¹²⁵I]RTI-121 was also displaced by RTI-371 in a dose- and time-related manner (Fig. 3C). Significant effects of the RTI-371 dose ($F_{4,27} = 42.5$, $P < 0.001$) and time ($F_{2,27} = 5.47$, $P = 0.01$) but not their interaction ($F_{8,27} = 1.29$; $P = 0.291$) were obtained. Post hoc tests indicated that displacement of [¹²⁵I]RTI-121 was significantly different from vehicle for each dose across all time points, though there were no differences among the doses of RTI-371 within individual time points. Maximum displacement of [¹²⁵I]RTI-121 was obtained with 56 mg/kg at the 60-minute time point. The slopes of the lines fitted to the time course of displacement by RTI-371 were generally the least steep of all of the drugs tested though the slopes were most steep at the highest doses. At the 56 mg/kg dose, displacement occurred at a rate of

Fig. 2. RTI-336 and RTI-371 modulate the conformation of DAT in a similar manner. (A) Homology-based structural model of human DAT. Based on sequence alignment, amino acid residues of human DAT are threaded onto the peptide backbone structure of *Drosophila* DAT using the NIH Cn3D software, with C306 and T316 on TM6a highlighted. (B) Binding of RTI-336 and RTI-371 produces effects similar to those of cocaine on the cysteine accessibility of WT DAT. Representative immunoblot from two experiments. (C) Representative curve of [³H]DA uptake by the substituted cysteine mutant of T316C/C306A DAT in stably transfected human embryonic kidney 293 (HEK293) cells (mean ± S.D. of triplicate samples). Ordinates: [³H]DA uptake in picomole per minute per well. Abscissae: DA concentration in μM. (D) Opposite effects on the cysteine accessibility of DAT T316C/C306A by cocaine analogs compared with BZT analogs. DAT cysteine accessibility is expressed as percentage of vehicle for each of the drugs tested ($N = 3$). Quantified DAT band densities (mean ± S.E.M.) were analyzed by one-way ANOVA with post hoc Dunnett's test. ** $P < 0.01$; * $P < 0.05$ compared with vehicle. A representative blot is shown below the averages shown in the histogram.

Fig. 3. In vivo displacement of [125 I]RTI-121 in the striatum at various times following intraperitoneal injections of vehicle, cocaine, RTI-336, and RTI-371. Ordinates: specific [125 I]RTI-121 binding expressed as percentage of respective vehicle binding. Abscissae: time in minutes. Vehicle (●), 3.0 mg/kg (○), 10 mg/kg (△), 20 mg/kg (▽), 30 mg/kg (□), 40 mg/kg (◇), 56 mg/kg (open hexagons). Each point represents the mean $\pm$ S.E.M. ($N = 2-5$). (A) Cocaine. (B) RTI-336. (C) RTI-371.

0.320%/min, which was approximately one-half or one-fourth of the rates with RTI-336 or cocaine, respectively.

Locomotor Activity. Cocaine, as has been demonstrated previously elsewhere (Dews, 1953), dose-dependently increased horizontal ambulatory activity with a maximum mean of 428 counts/minute during the first 30 minutes of the session at 40 mg/kg (Fig. 4A, filled symbols). The effects were diminished at later times after injection. Two-way repeated-measures ANOVA indicated a statistically significant effect of cocaine dose ($F_{4,100} = 38.6$; $P < 0.001$), pretreatment time ($F_{3,100} = 266$; $P < 0.001$), and an interaction of the two factors on activity counts ($F_{12,100} = 6.40$; $P < 0.001$).

Dose-dependent increases in locomotor activity were also produced by RTI-336. Maximal activity of 363 counts/min

produced by RTI-336 was obtained at 30 mg/kg 60–90 minutes after injection and was less than the maximal activity produced by cocaine (compare Fig. 4, A and B). Two-way repeated-measures ANOVA indicated a significant effect of RTI-336 dose ($F_{4,100} = 39.0$; $P < 0.001$), pretreatment time ($F_{3,100} = 12.2$; $P < 0.001$), and an interaction of the two factors on activity counts ($F_{12,100} = 5.09$; $P < 0.001$).

RTI-371 was less effective in stimulating activity than was RTI-336, and was ineffective in the first 30 minutes after injection (Fig. 4C). At later times over the course of the observation period, RTI-371 increased activity to a maximum mean of 266 counts/min (100 mg/kg at 60–90 minutes after injection). Two-way repeated measures ANOVA indicated a significant effect of RTI-371 dose ($F_{5,120} = 6.81$; $P < 0.001$), pretreatment time ($F_{3,120} = 3.92$; $P = 0.010$), and an interaction of the two factors on activity counts ($F_{15,120} = 5.01$; $P < 0.001$).

RTI-371 also increased horizontal ambulatory activity in CB₁R KO and WT mice from 60 to 90 minutes after injection. The maximum mean ambulatory counts were of 100 and 74.4 counts/min during that time period at 30 mg/kg for WT and KO mice, respectively (Fig. 5B, compare open and filled symbols). RTI-371 also stimulated activity from 120 to 150 minutes after injection, but was less effective in doing so (Fig. 5C). A three-way ANOVA indicated significant effects of dose ($F_{3,144} = 3.22$; $P = 0.025$), time ($F_{3,144} = 3.62$; $P = 0.015$), and the dose $\times$ time interaction (RTI-371: $F_{9,144} = 3.66$; $P < 0.001$; RTI-371: $F_{3,144} = 0.715$; $P = 0.545$), although no effect of genotype ($F_{1,144} = 0.123$; $P = 0.726$).

Cocaine Discrimination. As has been shown previously (Middaugh et al., 1998), cocaine produced dose-related increases in the percentage of cocaine-appropriate responses in mice trained to discriminate cocaine (10 mg/kg) from saline (Fig. 6A and B, filled symbols). In addition, RTI-336 closely approximated those effects, with a maximum substitution for cocaine averaging 87% at 5 or 60 minutes after injection, and with a greater potency at the later time (Fig. 6A, open symbols). Across the range of doses that produced these effects, RTI-336 produced dose-related decreases in rates of responding that did not substantially differ at the different times after injection (Fig. 6C).

In contrast to those effects, RTI-371 produced a level of substitution greater than that produced by saline, although less than that produced by cocaine (Fig. 6B, compare open and filled symbols). The maximum mean substitution for cocaine was 60% or 68% obtained at 5 or 60 minutes after injection, respectively, although there were no significant differences in maximal effects at these two time points. The lack of full substitution was a result of some subjects showing full substitution at some doses and other subjects showing substitution that was not greater than 33% drug-appropriate responding at any dose tested. There were differences in the dose-related decreases in rates of responding produced by RTI-371 with the decreases at 5 minutes after injection occurring at lower doses than at 60 minutes after injection (Fig. 6D).

Self-Administration. Performances maintained by cocaine were characteristic of performances maintained under FR schedules by conventional reinforcers as well as cocaine injections; a brief pause was followed by a sequence of five responses made in rapid sequence producing the injection (Fig. 7A). Responses were emitted on the inactive lever only occasionally (vertical marks on the line below cumulative

Fig. 4. Effects of cocaine, RTI-336, and RTI-371 on locomotor activity in naive Swiss-Webster mice. Ordinates: activity counts per minute. Abscissae: drug dose in mg/kg (log scale). Veh: vehicle. 0–30 minutes (●), 60–90 minutes (○), 120–150 minutes (△), and 180–210 minutes (▽). Each point represents the mean $\pm$ S.E.M. ($N = 6$). All these drugs were administered intraperitoneally immediately before sessions. (A) Cocaine. (B) RTI-336. (C) RTI-371.

curve), or during the 2-minute TO periods between components (lower event line displaced upward). In the first component, no injections were delivered, and generally few FR sequences of five responses were emitted. In subsequent components, response rates increased with increasing cocaine dose. The highest rate of responding was typically at a dose

Fig. 5. Effects of injections of RTI-371 alone on locomotor activity in naive CB_1R WT [$CB_1 (+/+)$] and KO [$CB_1 (-/-)$] mice. Ordinates: activity counts per minute. Abscissae: RTI-371 dose in mg/kg (log scale). Veh: vehicle. CB_1R WT (+/+) mice (●), CB_1R KO (-/-) mice (○). Each point represents the mean $\pm$ S.E.M. ($N = 5$ in CB_1R WT mice, and $N = 6$ in CB_1 KO mice). RTI-371 (3.0, 10, and 30 mg/kg) was administered intraperitoneally immediately before sessions. (A) 0–30 minutes. (B) 60–90 minutes. (C) 120–150 minutes. (D) 180–210 minutes.

per injection of 0.32 mg/kg during the fourth component. When saline injections were available throughout the session (Fig. 7B), responses were typically not emitted at rates greater than those maintained in the first component when no injections were delivered.

The average response rates were a bitonic function of cocaine dose, with a maximum of 0.205 ± 0.067 responses/second at 0.32 mg/kg per injection (Figs. 7A and 8A). Response rates maintained by cocaine were significantly greater than the rates during the first-component (0.052 ± 0.015), or those obtained (0.056 ± 0.014 responses/second) with vehicle injections throughout the session (Figs. 7B and 8A, ○). Two-way repeated measures ANOVA indicated significant differences in response rates between cocaine or saline self-administration ($F_{1,20} = 8.64$; $P = 0.032$), and of component/dose ($F_{4,20} = 6.55$; $P = 0.002$), with an interaction of the two factors ($F_{4,20} = 8.32$; $P < 0.001$). Post hoc comparisons indicated that 0.32 mg/kg per injection of cocaine maintained response rates greater than those obtained in the first component ($t = 5.72$; $P < 0.001$) and greater than those obtained in the same component with saline ($t = 5.09$, $P < 0.001$; Fig. 8A, ○).

Average response rates were also a bitonic function of RTI-336 dose, with a maximum of 0.107 ± 0.028 responses/second at 0.32 mg/kg per injection (Figs. 7C and 8A, △). At the doses of RTI-336 that maintained the highest response rates (0.1 and 0.32 mg/kg per injection), the temporal patterns of

Fig. 6. Substitution of RTI-336 and RTI-371 in Swiss-Webster mice trained to discriminate intraperitoneal injections of cocaine (10 mg/kg) from saline under an FR 10-response schedule of food reinforcement. Abscissae: drug dose in mg/kg (log scale). (A and B) Ordinates: percentage of responses on the cocaine-appropriate lever. (C and D) Ordinates: percentage of control response rate after saline administration. Each point represents the mean $\pm$ S.E.M. ($N = 6-11$). The percentage of responses emitted on the cocaine-appropriate lever was considered unreliable and not plotted if fewer than half of the subjects responded at that dose. Cocaine was administered intraperitoneally at 5 minutes before sessions. RTI-336 and RTI-371 were administered intraperitoneally at 5 or 60 minutes before sessions. (●) Cocaine administered 5 minutes before sessions; (○) RTI-336 or RTI-371 administered 5 minutes before sessions; (△) RTI-336 or RTI-371 administered 60 minutes before sessions. (A and C) RTI-336 substitution for cocaine. (B and D) RTI-371 substitution for cocaine.

responding were similar to those maintained by the 0.1 mg/kg per injection dose of cocaine, though the responses were emitted at a lower rate, primarily due to longer pauses before the sequence of five responses was initiated (Fig. 7C). Response rates ($F_{4,20} = 10.0$; $P < 0.001$) were significantly affected by dose, and post hoc tests indicated that rates maintained by 0.32 mg/kg per injection were significantly greater than those in extinction (EXT, $t = 4.56$; $P < 0.001$).

With RTI-371 injections available for self-administration, the response rates were low throughout the experimental session and not different from those obtained when saline injections were available (compare Fig. 7, B–D). The average response rates with RTI-371 substitution were low and comparable to those obtained with vehicle across all doses per injection studied (Fig. 8A, compare ∇ with $\circ$). Statistical analysis indicated no difference between RTI-371 and saline substitution. Further, the response rates obtained with RTI-371 were well below those maintained by cocaine (Fig. 8A, compare ∇ with $\bullet$). Post hoc tests indicated no significant differences between any RTI-371 dose/injection compared with the no injection component (EXT).

Pre-session treatments with RTI-336 enhanced the effects of lower doses of cocaine (Fig. 7E). At the 10.0 mg/kg pretreatment dose of RTI-336, response rates obtained with 0.03 and 0.1 mg/kg per injection of cocaine approached those maintained by 0.32 and 1.0 mg/kg per injection of cocaine after saline pretreatment (Fig. 7A). The cocaine self-administration dose-effect curve was dose-dependently shifted leftward by RTI-336, without affecting maximum response rates (Fig. 8B). Doses of 3.2 and 10 mg/kg RTI-336 shifted the cocaine self-administration dose-effect curve approximately 3- and 10-fold to the left, respectively (Fig. 8B). In addition, at the 10 mg/kg dose, RTI-336 pretreatment increased response rates during extinction (Fig. 8B, downward triangle above EXT). A two-way repeated-measures ANOVA supported these descriptions with a significant effect of cocaine dose ($F_{4,60} = 13.7$; $P < 0.001$), pre-session RTI-336 treatment ($F_{3,60} = 10.8$; $P < 0.001$), and a significant interaction of the two ($F_{12,60} = 12.9$; $P < 0.001$).

In contrast to the effects of RTI-336, its constitutional isomer RTI-371 only produced decreases in response rates maintained by cocaine (compare Fig. 7, A–F). The representative performance (Fig. 7F) shows that RTI-371 increased the pauses before initiation of responding, and that responding often ceased before the end of the component. In contrast to the leftward shifts produced by RTI-336, RTI-371 produced an insurmountable antagonism of cocaine self-administration (Fig. 8C). At the 10 mg/kg dose of RTI-371, no dose of cocaine maintained responding at levels above those maintained by saline (Figs. 7F and 8C; compare downward triangles in Fig. 8C to open circles in Fig. 8A). The ANOVA of effects of RTI-371 on response rates supported these observations, with a significant effect of cocaine dose ($F_{4,60} = 13.9$; $P < 0.001$), pre-session treatment ($F_{3,60} = 12.9$; $P < 0.001$), and their interaction ($F_{12,60} = 11.7$; $P < 0.001$).

A comparison of the effects of the phenyltropane analogs on responding maintained by cocaine or food reinforcement was conducted at the 0.32 mg/kg per injection dose of cocaine that maintained maximal response rates (Fig. 8, D and E). Rates of responding maintained by food reinforcement in the fourth component of the session averaged 0.620 responses/second, whereas those maintained by 0.32 mg/kg per injection of cocaine (also in the fourth component) averaged 0.255 responses/second. These values were significantly different ($F_{1,10} = 8.89$; $P = 0.014$). Both compounds dose-dependently decreased rates of responding maintained by cocaine (Fig. 8, D and E, filled symbols), and the ED_{50} values were below those for decreases in rates of responding maintained by food reinforcement with 95% confidence limits that did not overlap (Table 3). Two-way ANOVA indicated significant effects of dose ($F_{2,20} = 10.0$; $P < 0.001$ or $F_{2,20} = 24.0$; $P < 0.001$) and reinforcer ($F_{1,20} = 10.7$; $P = 0.008$ or $F_{1,20} = 5.18$; $P = 0.046$) for either RTI-336 or RTI-371, respectively. Additionally, at 1.0 mg/kg RTI-336 increased rates of responding maintained by food reinforcement, an effect not seen with RTI-371 (Fig. 8, D and E).

Discussion

Our present study compared the effects of two 3-substituted-phenyltropane DAT inhibitors and found RTI-371 to have several effects that differed substantially from those of the standard DAT inhibitor, cocaine. As initially reported (Carroll

Fig. 7. Representative cumulative records showing patterns of self-administration in real time maintained by intravenous cocaine injection under the FR five-response schedule, and those obtained when saline, RTI-336, or RTI-371 was substituted for cocaine. Ordinates: cumulative responses; abscissae: time. The five 20-minute self-administration components of each session are indicated by the lower event line displaced down. The preceding 2-minute TO periods are indicated by the lower event line displaced up. In the first component, each fifth response turned off the LEDs for 20 seconds but did not activate the infusion pump (EXT, extinction), whereas in subsequent components the injections were also delivered with each fifth response (diagonal marks on the cumulative record) with doses (in mg/kg per injection) indicated. Vertical marks on the line below the cumulative curve indicate responses on the left (inactive) lever. The cumulative curve reset to the baseline at the end of the 20-minute component. (A) Cocaine (intravenous) with saline (intraperitoneal) 5 minutes before. (B) Saline substitution for cocaine (intravenous) with saline (intraperitoneal) 5 minutes before. (C) RTI-336 substitution for cocaine (intravenous) with saline (intraperitoneal) 5 minutes before. (D) RTI-371 substitution for cocaine (intravenous) with saline (intraperitoneal) 5 minutes before. (E) Cocaine (intravenous) with RTI-336 (10 mg/kg i.p.) 5 minutes before. (F) Cocaine (intravenous) with RTI-371 (10 mg/kg i.p.) 5 minutes before.

et al., 2004), RTI-371 neither increased locomotion nor produced cocaine-like discriminative-stimulus effects. Further, RTI-371 was not self-administered at rates above vehicle levels, and produced a dose-dependent insurmountable antagonism of cocaine self-administration. These unique effects were obtained despite high affinity and selectivity for the DAT among the monoamine transporters, a profile that often conveys abuse liability (Ritz et al., 1987). Effects similar to those of RTI-371 have been obtained in the past with analogs of BZT (Hiranita et al., 2009, 2014; Li et al., 2013), which also have high-affinity DAT binding, though the present findings are unique for phenyltropane DAT inhibitors. A previous study had indicated that RTI-371 blocked locomotor stimulation produced by cocaine (Navarro et al., 2005), so the present antagonism of cocaine self-administration is not unprecedented.

In contrast, RTI-336, the constitutional isomer, had effects resembling those of cocaine and enhanced cocaine self-administration. Previous studies had found that RTI-336 dose-dependently decreased cocaine self-administration in rats (Haile et al., 2005; Carroll et al., 2006a) and primates (Howell et al., 2007). Those outcomes may appear to contradict the enhanced cocaine self-administration in our study, evidenced by a leftward shift in the cocaine dose-effect curve. However, the cocaine doses in the previous studies with rats (≥ 0.25 mg/kg per injection) were comparable to our present doses on the descending limb of the bitonic cocaine dose-effect curve. In the study with primates, RTI-336 dose-dependently decreased the self-administration of two different doses of cocaine as well as decreased the response rates maintained by food reinforcement. Both doses of cocaine in that study maintained the highest rates of responding, and the results with rats and primates are consistent with a

leftward shift in the cocaine self-administration dose-effect curve. As shown in our study, a leftward shift in the dose-effect curve is accompanied by decreased response rates at the highest cocaine doses, an effect previously reported for other indirect DA agonists (Schenk, 2002; Hiranita et al., 2011; Li et al., 2013). The stimulation of locomotion (Carroll et al., 2004, 2006a; Haile et al., 2005) and the cocaine-like discriminative-stimulus effects of RTI-336 obtained in our study and previous studies (Carroll et al., 2004, 2006a; Haile et al., 2005; Kimmel et al., 2008) are consistent with the cocaine-like effects of RTI-336 adding with effects of cocaine and enhancing cocaine self-administration.

As effects of RTI-371 resemble those of several BZT analogs, which are considered atypical DAT inhibitors, it was of interest to investigate several potential mechanisms for the differences between RTI-371 and standard DAT inhibitors. Finding a mechanism common for the structurally divergent RTI-371 and BZT analogs should provide a compelling hypothesis for what makes these compounds different from cocaine. Alternatively, mechanisms for effects shared between RTI-371 and RTI-336, which is more similar to cocaine, are less likely as potential explanations for the differences from cocaine.

The decrease in the maximum self-administration of cocaine produced by RTI-371 could be obtained with drugs that produce generalized disruptions in operant behavior through any number of mechanisms. For example, Howell et al. (2000) found that 3β -(4-chlorophenyl)-2 β -carboxylic-acid-phenyl-ester (RTI-113) similarly decreased rates of responding maintained by cocaine as well as those maintained by other reinforcers. However, the decreases in cocaine self-administration produced by RTI-371 in our study were selective, as they were obtained at a dose that had no effect on comparable responding maintained by food reinforcement.

Fig. 8. Effects of RTI-336 and RTI-371 in rats trained to self-administer cocaine (0.032–1.0 mg/kg per injection). (A) Substitution of saline, RTI-336, and RTI-371 for cocaine. Ordinates: responses per second; abscissae: dose of each substituted drug in milligram per kilogram per injection or component. Each point represents the mean $\pm$ S.E.M. ($N = 6$). EXT, extinction. (B and C) Effects of pre-session treatments with RTI-336 or RTI-371 on responding maintained by cocaine injection. Ordinates: responses per second; abscissa: cocaine injection dose in milligram per kilogram per injection. Each point represents the mean $\pm$ S.E.M. ($N = 6$). RTI-336 and RTI-371 were administered intraperitoneally at 5 minutes before sessions. (D and E) Comparison of effects of pre-session treatments with RTI-336 and RTI-371 on responding maintained by cocaine injection (0.32 mg/kg per injection) or food presentation (fourth component, each). Ordinates: response rates as percentage of control response rates (sessions before drug tests), which averaged $0.255 (\pm 0.068)$ and $0.620 (\pm 0.102)$ responses/second, respectively, for cocaine- and food-maintained responding; abscissae: milligram per kilogram of test compounds administered intraperitoneally, log scale. The control rates maintained by food presentation were significantly higher than those maintained by cocaine injections ($F_{1,10} = 8.89$; $P = 0.014$, one-way ANOVA). Cocaine injections (●). Food presentation (◇). inj, injection.

Previous studies indicated that dual inhibitory actions at the DAT and σR could selectively decrease stimulant self-administration (Hiranita et al., 2011; 2014). Specifically, decreases in cocaine self-administration, like those produced by RTI-371, were obtained with rimcazone analogs, σR antagonists with DAT affinity (Husbands et al., 1999). RTI-371 had an affinity for $\sigma_1 R$ and $\sigma_2 R$, respectively, 2020- and 45-fold lower than for the DAT. In contrast, differences in the affinity of rimcazone at the DAT and at $\sigma_1 R$ or $\sigma_2 R$ were,

respectively, less than 10- or 2.5-fold (Husbands et al., 1999; Hiranita et al., 2011). Further, RTI-336 had a ratio of $\sigma_1 R$ or $\sigma_2 R$ to DAT affinity similar to that of RTI-371. Although minimal requirements of affinity for σR and the DAT for antagonism of cocaine self-administration are currently unclear, the ratios of affinity at σR for RTI-336 and RTI-371 are similar and are substantially lower than those for rimcazone and its analogs, casting doubt on a role for σR in the present effects.

Navarro et al. (2009) proposed that unique effects of RTI-371 and the BZT analog JHW007 were due at least in part to positive allosteric modulation of CB₁R. If a positive allosteric modulation of CB₁R indeed modified the otherwise stimulant effects of RTI-371, greater stimulant effects in CB₁R KO mice compared with WT mice would be expected. Across the range of doses studied, the effects of RTI-371 did not differ in the mice with different genotypes, indicating that positive allosteric modulation of CB₁R is unlikely as a factor in the unique effects of RTI-371.

Cocaine binding has been proposed to stabilize an outward facing DAT conformation, and affinities of cocaine-like

TABLE 3

ED₅₀ values for phenyltropanes in decreasing rates of responding maintained by cocaine injection (0.32 mg/kg per injection) or food presentation (three pellets/presentation)
ED₅₀ values reported in milligram per kilogram with 95% confidence limits in parentheses.

Treatment	Cocaine	Food Presentation	Relative Potency
RTI-336	3.92 (3.42–4.53)	10.9 (6.77–24.5)	0.255* (0.101–0.510)
RTI-371	3.33 (3.06–3.63)	8.21 (5.30–15.6)	0.377 (0.181–0.703)

*Value is only an estimate as there were significant effects of preparations and a significant deviation from parallel.

inhibitors are relatively intolerant to DAT conformational change (Reith et al., 2001; Loland et al., 2008; Hong and Amara, 2010). For example, Loland et al. (2008) showed that cocaine-like drugs were about two orders of magnitude less potent in blocking DA uptake in mutant DAT with an inward-facing conformation compared with WT DAT. In contrast, the potencies of BZT analogs were relatively tolerant to DAT conformation change, suggesting a link between modes of interaction with DAT and behavioral effects. Intriguingly, the cysteine-accessibility profiles of RTI-371 and RTI-336 were both like cocaine, suggesting stabilization of an outward-facing DAT, and different from that of BZT and JHW007, which had effects like those previously reported (Loland et al., 2008). Thus, RTI-371 and RTI-336 with distinct behavioral effects had similar effects in stabilizing DAT conformation. Thus, the correlation between DAT inhibitor-induced conformational change and behavioral profile appears to be more complicated than previously recognized.

Maximal response rates maintained by RTI-336 in the present self-administration study were lower than those maintained by cocaine, as reported previously (Howell et al., 2007; Kimmel et al., 2007; 2008; Czoty et al., 2010). Kimmel et al. (2008) showed a nonsignificant trend toward an inverse relation between the maximal number of injections obtained under a progressive-ratio schedule of reinforcement with several ^{11}C -labeled DA-uptake inhibitors, including RTI-336, and the time to maximal brain penetration determined by positron emission tomography (see also Czoty et al., 2010). As the authors note, the drugs studied also differed across a number of other parameters, including their selectivity for the DAT and their duration of action. However, the results suggest that brain penetration rate plays a role in the reinforcing effectiveness of DA-uptake inhibitors. In our study, the rate of displacement of radioligand from DAT in vivo was greatest for cocaine, less for RTI-336, and least for RTI-371.

Several previous studies (Stathis et al., 1995; Desai et al., 2005a,b; Tanda et al., 2009a) suggested that a slow rate of DAT occupancy will diminish not only abuse liability but other cocaine-like effects. However, a slow association rate alone does not account for the antagonism of cocaine effects unless the transduction of those effects also is diminished. It was suggested previously that a slow on-rate coupled with a desensitization process may account for the diminished cocaine-like effectiveness of BZT analogs (Tanda et al., 2009a; 2013). This hypothesized desensitization may involve post-synaptic DA receptors, which in turn would be less effective in transmitting DA-mediated signals.

In our study, increases in responding during extinction were obtained with RTI-336, but not with RTI-371. Increased rate of extinguished responding previously maintained by drugs has been labeled "reinstatement," and it was suggested that drugs producing such effects will induce relapse in a recovering addict. It should be noted that differences in effectiveness of these two compounds to increase extinguished responding corresponds to differences in maximal substitution for cocaine discriminative-stimulus effects (Katz and Higgins, 2003). If indeed such increases in responding are indications of potential to induce relapse, the present results indicate a low potential for this effect with RTI-371, and that not all compounds acting at the DAT will reinstate responding.

In summary, the 3-substituted phenyltropane analogs RTI-336 and RTI-371 were less effective than cocaine in producing several cocaine-like effects and selectively decreased cocaine-versus food-maintained responding. However, RTI-371 was most unlike cocaine, shifting the self-administration dose-effect curve down rather than leftward, and generally having effects atypical of standard DAT inhibitors. Several mechanisms proposed to account for atypical DAT inhibition (allosteric actions at CB_1R , antimuscarinic or σR off-target activity, DAT conformational status) were found to be inadequate to account for the unique actions of RTI-371. Together, our results broaden the structural requirements for atypical DAT-inhibitor effects, suggesting that drugs acting at the DAT can have distinctive mechanisms, and those features may contribute to the potential for these compounds as medications for stimulant abuse.

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Authorship Contributions

Participated in research design: Hiranita, Wilkinson, Hong, Kopajtic, Soto.

Conducted experiments: Hiranita, Wilkinson, Hong, Kopajtic.

Contributed new reagents or analytic tools: Hong, Zou, Lupica, Newman.

Performed data analysis: Hiranita, Wilkinson, Hong, Kopajtic, Soto, Katz.

Wrote or contributed to the writing of the manuscript: Hiranita, Wilkinson, Hong, Kopajtic, Soto, Lupica, Newman, Katz.

References

- Bergman J, Madras BK, Johnson SE, and Spealman RD (1989) Effects of cocaine and related drugs in nonhuman primates. III. Self-administration by squirrel monkeys. *J Pharmacol Exp Ther* **251**:150–155.
- Campbell VC, Kopajtic TA, Newman AH, and Katz JL (2005) Assessment of the influence of histaminergic actions on cocaine-like effects of 3 α -diphenylmethoxytropane analogs. *J Pharmacol Exp Ther* **315**:631–640.
- Carroll FI, Fox BS, Kuhar MJ, Howard JL, Pollard GT, and Schenk S (2006a) Effects of dopamine transporter selective 3-phenyltropane analogs on locomotor activity, drug discrimination, and cocaine self-administration after oral administration. *Eur J Pharmacol* **553**:149–156.
- Carroll FI, Howard JL, Howell LL, Fox BS, and Kuhar MJ (2006b) Development of the dopamine transporter selective RTI-336 as a pharmacotherapy for cocaine abuse. *AAPS J* **8**:E196–E203.
- Carroll FI, Pawlusch N, Kuhar MJ, Pollard GT, and Howard JL (2004) Synthesis, monoamine transporter binding properties, and behavioral pharmacology of a series of 3 β -(4-substituted phenyl)-2 β -(3'-substituted isoxazol-5-yl)tropanes. *J Med Chem* **47**:296–302.
- Czoty PW, Martelle JL, Carroll FI, and Nader MA (2010) Lower reinforcing strength of the phenyltropane cocaine analogs RTI-336 and RTI-177 compared to cocaine in nonhuman primates. *Pharmacol Biochem Behav* **96**:274–278.
- Desai RI, Kopajtic TA, French D, Newman AH, and Katz JL (2005a) Relationship between in vivo occupancy at the dopamine transporter and behavioral effects of cocaine, GBR 12909 [1-2-[bis-(4-fluorophenyl)methoxyethyl]-4-(3-phenylpropyl) piperazine], and benztropine analogs. *J Pharmacol Exp Ther* **315**:397–404.
- Desai RI, Kopajtic TA, Koffarnus M, Newman AH, and Katz JL (2005b) Identification of a dopamine transporter ligand that blocks the stimulant effects of cocaine. *J Neurosci* **25**:1889–1893.
- Dews PB (1953) The measurement of the influence of drugs on voluntary activity in mice. *Br Pharmacol Chemother* **8**:46–48.
- Dews PB (1958) Studies on behavior. IV. Stimulant actions of methamphetamine. *J Pharmacol Exp Ther* **122**:137–147.
- Garcés-Ramírez L, Green JL, Hiranita T, Kopajtic TA, Mereu M, Thomas AM, Mesangeau C, Narayanan S, McCurdy CR, and Katz JL, et al. (2011) Sigma receptor agonists: receptor binding and effects on mesolimbic dopamine neurotransmission assessed by microdialysis. *Biol Psychiatry* **69**:208–217.
- Haile CN, Zhang XY, Carroll FI, and Kosten TA (2005) Cocaine self-administration and locomotor activity are altered in Lewis and F344 inbred rats by RTI 336, a 3-phenyltropane analog that binds to the dopamine transporter. *Brain Res* **1055**:186–195.
- Hiranita T, Kohut SJ, Soto PL, Tanda G, Kopajtic TA, and Katz JL (2014) Preclinical efficacy of N-substituted benztropine analogs as antagonists of methamphetamine self-administration in rats. *J Pharmacol Exp Ther* **348**:174–191.
- Hiranita T, Soto PL, Kohut SJ, Kopajtic T, Cao J, Newman AH, Tanda G, and Katz JL (2011) Decreases in cocaine self-administration with dual inhibition of the dopamine transporter and σ receptors. *J Pharmacol Exp Ther* **339**:662–677.

- Hiranita T, Soto PL, Newman AH, and Katz JL (2009) Assessment of reinforcing effects of benztropine analogs and their effects on cocaine self-administration in rats: comparisons with monoamine uptake inhibitors. *J Pharmacol Exp Ther* **329**: 677–686.
- Hong WC and Amara SG (2010) Membrane cholesterol modulates the outward facing conformation of the dopamine transporter and alters cocaine binding. *J Biol Chem* **285**:32616–32626.
- Howell LL, Carroll FI, Votaw JR, Goodman MM, and Kimmel HL (2007) Effects of combined dopamine and serotonin transporter inhibitors on cocaine self-administration in rhesus monkeys. *J Pharmacol Exp Ther* **320**:757–765.
- Howell LL, Czoty PW, Kuhar MJ, and Carrol FI (2000) Comparative behavioral pharmacology of cocaine and the selective dopamine uptake inhibitor RTI-113 in the squirrel monkey. *J Pharmacol Exp Ther* **292**:521–529.
- Husbands SM, Izenwasser S, Kopajtic T, Bowen WD, Vilner BJ, Katz JL, and Newman AH (1999) Structure-activity relationships at the monoamine transporters and sigma receptors for a novel series of 9-[3-(cis-3, 5-dimethyl-1-piperazinyl)propyl]carbazole (rimcazole) analogues. *J Med Chem* **42**:4446–4455.
- Katz JL and Higgins ST (2003) The validity of the reinstatement model of craving and relapse to drug use. *Psychopharmacology (Berl)* **168**:21–30.
- Katz JL, Izenwasser S, and Terry P (2000) Relationships among dopamine transporter affinities and cocaine-like discriminative-stimulus effects. *Psychopharmacology (Berl)* **148**:90–98.
- Katz JL, Kopajtic TA, Agoston GE, and Newman AH (2004) Effects of N-substituted analogs of benztropine: diminished cocaine-like effects in dopamine transporter ligands. *J Pharmacol Exp Ther* **309**:650–660.
- Kimmel HL, Negus SS, Wilcox KM, Ewing SB, Stehouwer J, Goodman MM, Votaw JR, Mello NK, Carroll FI, and Howell LL (2008) Relationship between rate of drug uptake in brain and behavioral pharmacology of monoamine transporter inhibitors in rhesus monkeys. *Pharmacol Biochem Behav* **90**:453–462.
- Kimmel HL, O'Connor JA, Carroll FI, and Howell LL (2007) Faster onset and dopamine transporter selectivity predict stimulant and reinforcing effects of cocaine analogs in squirrel monkeys. *Pharmacol Biochem Behav* **86**:45–54.
- Lees A (2005) Alternatives to levodopa in the initial treatment of early Parkinson's disease. *Drugs Aging* **22**:731–740.
- Li L, Hiranita T, Hayashi S, Newman AH, and Katz JL (2013) The stereotypy-inducing effects of N-substituted benztropine analogs alone and in combination with cocaine do not account for their blockade of cocaine self-administration. *Psychopharmacology (Berl)* **225**:733–742.
- Li SM, Kopajtic TA, O'Callaghan MJ, Agoston GE, Cao J, Newman AH, and Katz JL (2011) N-substituted benztropine analogs: selective dopamine transporter ligands with a fast onset of action and minimal cocaine-like behavioral effects. *J Pharmacol Exp Ther* **336**:575–585.
- Loland CJ, Desai RI, Zou MF, Cao J, Grundt P, Gerstbrein K, Sitte HH, Newman AH, Katz JL, and Gether U (2008) Relationship between conformational changes in the dopamine transporter and cocaine-like subjective effects of uptake inhibitors. *Mol Pharmacol* **73**:813–823.
- McKearney JW (1982) Stimulant actions of histamine H1 antagonists on operant behavior in the squirrel monkey. *Psychopharmacology (Berl)* **77**:156–158.
- Middaugh LD, McGroarty KK, Groseclose CH, and Adinoff B (1998) Cocaine discrimination: relationship to local anesthetics and monoamine uptake inhibitors in C57BL/6 mice. *Psychopharmacology (Berl)* **136**:44–49.
- Navarro HA, Howard JL, Pollard GT, and Carroll FI (2005) The DAT-selective 3-phenyltropane, RTI-371, antagonizes the in vivo effects of cocaine. *67th Annual Meeting of the College on Problems of Drug Dependence* (<http://www.cpdd.vcu.edu/Media/Meetings/2005Abstracts/2005AbstractBook.pdf>).
- Navarro HA, Howard JL, Pollard GT, and Carroll FI (2009) Positive allosteric modulation of the human cannabinoid (CB) receptor by RTI-371, a selective inhibitor of the dopamine transporter. *Br J Pharmacol* **156**:1178–1184.
- Nguyen VH, Kassiou M, Johnston GA, and Christie MJ (1996) Comparison of binding parameters of sigma 1 and sigma 2 binding sites in rat and guinea pig brain membranes: novel subtype-selective trishomocubanes. *Eur J Pharmacol* **311**:233–240.
- Penmatsa A, Wang KH, and Gouaux E (2013) X-ray structure of dopamine transporter elucidates antidepressant mechanism. *Nature* **503**:85–90.
- Reith ME, Berfield JL, Wang LC, Ferrer JV, and Javitch JA (2001) The uptake inhibitors cocaine and benztropine differentially alter the conformation of the human dopamine transporter. *J Biol Chem* **276**:29012–29018.
- Richelson E (1981) Tricyclic antidepressants: interactions with histamine and muscarinic cholinergic receptors, in *Antidepressants: Neurochemical, Behavioral, and Clinical Perspectives* (Enna SJ, Malick JB, and Richelson E, eds) pp 53–73, Raven, New York.
- Ritz MC, Lamb RJ, Goldberg SR, and Kuhar MJ (1987) Cocaine receptors on dopamine transporters are related to self-administration of cocaine. *Science* **237**:1219–1223.
- Scheffel U, Pögun S, Stathis M, Boja JW, and Kuhar MJ (1991) In vivo labeling of cocaine binding sites on dopamine transporters with [³H]WIN 35,428. *J Pharmacol Exp Ther* **257**:954–958.
- Schenk S (2002) Effects of GBR 12909, WIN 35,428 and indatraline on cocaine self-administration and cocaine seeking in rats. *Psychopharmacology (Berl)* **160**: 263–270.
- Snedecor G and Cochran W eds (1967) *Statistical Methods*. 6th ed. Ames, Iowa State College.
- Stathis M, Scheffel U, Lever SZ, Boja JW, Carroll FI, and Kuhar MJ (1995) Rate of binding of various inhibitors at the dopamine transporter in vivo. *Psychopharmacology (Berl)* **119**:376–384.
- Tanda G and Katz JL (2007) Muscarinic preferential M₁ receptor antagonists enhance the discriminative-stimulus effects of cocaine in rats. *Pharmacol Biochem Behav* **87**:400–404.
- Tanda G, Li SM, Mereu M, Thomas AM, Ebbs AL, Chun LE, Tronci V, Green JL, Zou MF, and Kopajtic TA, et al. (2013) Relations between stimulation of mesolimbic dopamine and place conditioning in rats produced by cocaine or drugs that are tolerant to dopamine transporter conformational change. *Psychopharmacology (Berl)* **229**:307–321.
- Tanda G, Newman AH, Ebbs AL, Tronci V, Green JL, Tallarida RJ, and Katz JL (2009a) Combinations of cocaine with other dopamine uptake inhibitors: assessment of additivity. *J Pharmacol Exp Ther* **330**:802–809.
- Tanda G, Newman AH, and Katz JL (2009b) Discovery of drugs to treat cocaine dependence: behavioral and neurochemical effects of atypical dopamine transport inhibitors. *Adv Pharmacol* **57**:253–289.
- Taylor D and Ho BT (1978) Comparison of inhibition of monoamine uptake by cocaine, methylphenidate and amphetamine. *Res Commun Chem Pathol Pharmacol* **21**:67–75.
- Zimmer A, Zimmer AM, Hohmann AG, Herkenham M, and Bonner TI (1999) Increased mortality, hypoactivity, and hypoalgesia in cannabinoid CB1 receptor knockout mice. *Proc Natl Acad Sci USA* **96**:5780–5785.

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