JPET Fast Forward. Published on January 14, 2016 as DOI: 10.1124/jpet.115.230722
This article has not been copyedited and formatted. The final version may differ from this version.

2-Substituted 3ß-Aryltropane Cocaine Analogs Produce Atypical Effects Without Inducing Inward-Facing Dopamine Transporter Conformations

Weimin C. Hong, Theresa A. Kopajtic, Lifen Xu, Stacey A. Lomenzo, Bernandie Jean, Jeffry D. Madura, Christopher K. Surratt, Mark L. Trudell and Jonathan L. Katz

Department of Pharmaceutical Sciences, Butler University, Indianapolis, IN 46208 (WCH); Psychobiology Section (TAK, JLK), Intramural Research Program, Department of Health and Human Services, National Institute on Drug Abuse, National Institutes of Health, Baltimore, MD 21224; Chemistry Department (LX, SL, MLT), University of New Orleans, New Orleans, LA 70148; Department of Chemistry and Biochemistry (BJ, JDM) and Division of Pharmaceutical Sciences (CKS), Duquesne University, Pittsburgh, PA 15282

Running title: 2-Substituted Aryltropanes – Molecular Actions and Behavior

Correspondence: Jonathan L. Katz, Psychobiology Section, National Institute on Drug Abuse, Intramural Research Program, National Institutes of Health, 251 Bayview Blvd, Suite 200, Baltimore, MD 21224, USA

E-mail: jkatz@intra.nida.nih.gov

Phone: 443-740-2582

FAX: 443-740-2829

Document statistics

Pages: 39

Tables: 4

Figures: 7

References: 42

Abstract: 245 words

Introduction: 736 words

Discussion: 1499 words

Non-standard abbreviations: ANOVA: analysis of variance; BZT: benztropine; DA: dopamine; DAT: dopamine transporter; EXT: extinction; FR: fixed ratio; HEK293: human embryonic kidney 293; IC_{50}: half maximal inhibitory concentration; LED: light-emitting diode; NET: norepinephrine transporter; OWW: original wet weight; PEO: polyethylene oxide; PBS: phosphate buffered saline; PBSCM: PBS with $CaCl_2$ and $MgCl_2$; SERT: serotonin transporter; TO: timeout; σ1R: sigma1 receptor; σ2R: sigma2 receptor

Recommended section: Neuropharmacology

JPET Fast Forward. Published on January 14, 2016 as DOI: 10.1124/jpet.115.230722
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

Previous structure-activity relationship studies indicate that a series of cocaine analogs, 3ß-aryltropanes with 2ß-diarylmethoxy substituents, selectively bind to the dopamine transporter (DAT) with nanomolar affinities that are 10-fold greater than the affinities of their corresponding 2α-enantiomers. The present study compared these compounds to cocaine with respect to locomotor effects in mice, and assessed their ability to substitute for cocaine (10 mg/kg, ip) in rats trained to discriminate cocaine from saline. Despite nanomolar DAT affinity, only the 2β-Ph_2COCH_2-3ß-4-Cl-Ph analog fully substituted for cocaine-like discriminative effects. Whereas all of the 2β compounds increased locomotion, only the 2β-(4-ClPh)$PhCOCH_2$-3ß-4-Cl-Ph analog had cocaine-like efficacy. None of the 2α-substituted compounds produced either of these cocaine-like effects. To explore the molecular mechanisms of these drugs, their effects on DAT conformation were probed using a cysteine-accessibility assay. Previous reports indicate that cocaine binds with substantially higher affinity to DAT in outward (extracellular)- compared to inward-facing conformation, whereas atypical DAT inhibitors, such as benztropine, have more similar affinities to these conformations, and this is postulated to explain their divergent behavioral effects. All of the 2β- and 2α-substituted compounds tested altered cysteine accessibility of DAT in a manner similar to cocaine. Furthermore, molecular dynamics of *in silico* inhibitor-DAT complexes suggested that the 2-substituted compounds reach equilibrium in the binding pocket in a cocaine-like fashion. These behavioral, biochemical and computational results show that aryltropane analogs can bind to the DAT and stabilize outward-facing DAT conformations like cocaine, yet produce effects that differ from those of cocaine.

INTRODUCTION

Reuptake of dopamine (DA) by the dopamine transporter (DAT) regulates the temporal dynamics of DA synaptic neurotransmission (Rice and Cragg, 2008). Standard DAT inhibitors block dopamine (DA) uptake, and in laboratory animals stimulate locomotor activity, fully substitute for cocaine in subjects trained to discriminate cocaine from saline injections, and are self-administered (reviewed by Johanson and Fishman, 1989; Schmitt et al. 2013; Tanda et al., 2009). The relationship between DAT binding affinities and potencies for behavioral effects among DAT inhibitors is the basis for the hypothesis that inhibition of DA uptake mediated by actions at the DAT is the basis for the abuse liability of cocaine, and that compounds with affinity for the DAT will have behavioral effects like those of cocaine (Kuhar et al., 1991).

Contrary to the DAT hypothesis several groups of compounds with DAT affinity have *in vivo* or *in vitro* effects that are distinct from those of standard DAT inhibitors such as cocaine (reviewed by Reith et al., 2015). These "atypical" DAT inhibitors include benztropine (BZT) and its analogs, which consist of the tropane ring of cocaine and the diphenylether moiety common to the piperazine class of DA uptake inhibitors such as GBR 12909 (Van der Zee et al., 1980). BZT analogs selectively bind to the DAT over the other monoamine transporters and inhibit DA uptake *in vitro*, yet produce behavioral effects distinct from those of cocaine (Katz et al., 1999). BZT analogs are not self administered in laboratory animals and selectively decrease cocaine self administration when administered before experimental sessions (Hiranita et al., 2009; Woolverton et al., 2000; Woolverton et al., 2001).

Various mechanisms have been suggested for atypical actions of DAT inhibitors, including affinity for off-target sites such as the muscarinic M_1 receptors, which could interfere with the expression of a cocaine-like behavioral profile. However, several studies have

suggested that antagonist actions at M_1 sites contribute minimally if at all to the atypical effects of BZT analogs (see review by Reith et al., 2015). More recently, it has been suggested that compounds with sigma receptor (σR) antagonist effects along with their affinity for the DAT will exhibit atypical DAT inhibitory effects similar to BZT (Hiranita et al., 2011). A number of previous studies indicated that σR antagonists can block several effects of cocaine, including locomotor stimulation, sensitization, and place conditioning (see review by Katz et al., 2011), and it is noteworthy that BZT analogs also have affinity for σRs (Katz et al., 2004; Li et al., 2011).

Another potential mechanism for atypical actions of DAT inhibitors is the stabilization of cytosol-facing conformations resulting from ligand binding (see review by Reith et al., 2015). For example, Loland et al. (2008) proposed a correlation between conformational states stabilized with DAT binding and the behavioral effects of a diverse group of DAT ligands. Cocaine binds preferentially to an "outward-facing" conformation, in which the primary ligand binding pocket is accessible only from the DAT extracellular face. In contrast, BZT and several analogs can bind and stabilize an "inward-facing" conformation, in which the transporter is open to the cytoplasm. It was shown that compounds stabilizing the inward-facing DAT conformation, like BZT, did not fully substitute for cocaine in rats trained to discriminate cocaine from saline injections, and did not stimulate locomotor activity to the same degree as cocaine (Loland et al., 2008). However, a more recent study showed that RTI-371, a 2-isoxazol-3-phenyltropane cocaine derivative, has behavioral effects similar to the BZT analogs, but stabilizes an outward-facing DAT conformation like cocaine (Hiranita et al., 2014).

Madras et al., (2006) reported that substitutions at the C-2 position of the BZT tropane ring (Fig.1) decrease affinity for the M_1 muscarinic receptor. Additionally, analogs of cocaine

exhibit a high degree of tolerance for C-2 substitutions of the tropane ring, as substituents with considerable steric bulk typically do not interfere with cocaine-like pharmacological activity (Carroll et al., 1992; Xu et al., 2004). As the diphenylether moiety of the BZT analogs was shown to be critical for their deviation from typical cocaine-like activity, aryltropane analogs with diphenylether substituents at the C-2 position were of interest. Xu et al. (2004) predicted and confirmed that BZT analogs with C-2 substitutions have less affinity for M_1 receptors than the unsubstituted compounds but retain activity at DAT. In the present study, we report atypical behavioral effects of a series of 2-substituted 3ß-aryltropane cocaine analogs (Xu et al., 2004) and explored potential mechanism for their atypical effects.

METHODS

DAT Binding Assay. Tissue was dissected and homogenized in buffer using a Brinkman Polytron (at setting 6 for 20 sec) and subsequently centrifuged at 20,000 x *g* for 10 min at 4° C. The resulting pellet was re-suspended in buffer, re-centrifuged and suspended in buffer again to a concentration of 10 mg/ml (original wet weight, OWW). Incubations were conducted in assay tubes containing 0.50 ml of buffer, 0.50 nM of radioligand, tissue, and various concentrations of inhibitors. See Table 1 for details.

σ_1R and σ_2R Binding Assay. Guinea pig brain was used because of the relatively higher density of σRs in that tissue compared with rat (Nguyen et al., 1996). Tissue was thawed on ice, homogenized (with a glass and Teflon apparatus) in buffer, and subsequently centrifuged at 800 x *g* for 10 min at 4° C. The supernatant was collected into a clean centrifuge tube and the remaining pellet was resuspended by vortex in 10 ml buffer (tissue) and centrifuged at 800 x *g* for 10 min at 4° C. The supernatants were pooled and centrifuged at 50,000 x *g* for 15 min at 4°

C. The remaining pellet was resuspended 80 mg/ml, OWW, in buffer and vortexed. The tissue suspension was incubated at 25°C for 15 min, and then centrifuged at 50,000 x *g* for 15 min. The supernatant was decanted 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 of radioligand (and 200 nM of (+)-pentazocine for σ_2 binding), tissue, and various concentrations of inhibitors. See Table 1 for details.

The reactions in all binding assays were started with the addition of tissue and terminated by rapid filtration through Whatman GF/B filters (presoaked in 0.050% polyethylenimine) using a Brandel Cell Harvester (Brandel Instruments Gaithersburg, MD). The filters were washed twice with 5.0 ml cold buffer and transferred to scintillation vials, to which Beckman Ready Safe scintillation cocktail (3.0 ml) was added. The vials were assessed for radioactivity the next day using a Beckman LS6000 liquid scintillation counter (Beckman Coulter Instruments, Fullerton, CA) at 50% efficiency. Assays were typically conducted as three or more independent experiments, each performed with triplicate tubes.

The IC_{50} 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 Cheng-Prusoff equation (Cheng and Prusoff, 1973), with IC_{50} of inhibitors used in the assay and the K_d value of the radioligand previously determined in this laboratory.

Receptor Screen. An enantiomeric pair of compounds (LX-19 and LX-20), one with and one without cocaine-like locomotor-stimulant effects, were screened for their activity at various receptor sites by examining their competition with the appropriate radioligands (ProfilingScreen® procured from MDS Panlabs Pharmacology Services, Bothell, Washington).

The screen consisted of assays designed to assess the activity of the compounds at 35 mammalian receptors. Each compound was tested in each assay at a concentration of 10 μM. Vehicle and reference standards were assessed concurrently with each assay. Details of the procedures and targeted sites are provided in Table 2.

Estimating affinity of the compounds from radioligand displacement data at single concentrations follows a practice used by Kosterlitz et al. (1973) to estimate potencies from single concentrations of opioids inhibiting electrically-driven contractions of guinea pig ileum. For sites at which the compounds produced displacement between 10 and 90%, an IC_{50} value was derived from linear interpolation assuming a typical displacement curve spanning a 100-fold domain of concentrations. For sites at which the displacement was >90%, IC_{50} values were estimated to be <1 μM. The compound was considered inactive at sites for which the displacement by 10 μM was <10%. The Cheng-Prusoff equation (Cheng et al., 1973) was subsequently used to derive K_i values of compounds at a particular site from the estimated IC_{50} values, using K_d values of radioligands provided by the vendor.

Behavioral Studies. Male Swiss-Webster mice (Taconic Farms, Germantown, NY) for locomotor activity assessments and Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) used in cocaine discrimination studies were acclimated to the animal colony for at least one week before use. During this time, food and tap water were freely available. Mice were housed in groups of 3-4 per cage and rats were housed singly and maintained at approximately 320 *g* body weight by adjusting their daily food ration (~15g/day). The humidity- and temperature-controlled colony rooms were maintained on a 12:12-h light:dark cycle with lights on at 0700 hours. The animals were cared for in accordance with the guidelines of the National Institutes of Health Animal Care and Use Program and the National Institute on Drug

Abuse Intramural Research Program Animal Program, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Locomotor Activity. Subjects were placed individually in clear acrylic chambers (40 cm3) for the measurement of horizontal (locomotor/ambulatory) activity. The acrylic chambers were contained within monitors (Omnitech Electronics, Columbus, OH) equipped with infrared light sources mounted 2.5 cm apart along two perpendicular sides. Light-sensitive detectors were mounted on the opposite wall, in line with the light sources. One locomotor activity count was registered with each interruption of light to the detectors. Injections were administered i.p. immediately before the subjects were placed in the apparatus for 1 hr, with each drug dose studied in eight subjects. Locomotor activity counts were totaled separately in the first and second 30 minutes. Whether locomotor activity was stimulated above that obtained with vehicle was determined in each thirty-min portion of the session by analysis of variance (ANOVA) and post-hoc comparisons. Subjects were each used only once.

Cocaine Discrimination. Details of the appartatus and procedures are as described previously (Katz et al., 1999). Briefly, subjects were trained to press response levers under a 20-response fixed ratio (FR 20) schedule of food reinforcement. When saline was injected immediately before sessions, responses on only one lever were reinforced; with cocaine injections responses on only the other lever were reinforced. The assignment of saline and cocaine to right or left levers was counterbalanced across subjects. The session started with the illumination of the houselight and the LEDs 5-min after subjects were placed in the chamber. Only responses on the appropriate lever were reinforced and responses on the inappropriate lever reset the FR response requirement. Food presentations were followed by 20-sec time out periods during which all lights were off and responding had no scheduled consequences. Sessions ended

after 20 food presentations or 15 min after their start, whichever occurred first. Training sessions with saline (S) and cocaine (C) injections were conducted in a double-alternation sequence [e.g. ...SCCS...].

Testing was initiated when performances met or exceeded training criteria of at least 85% appropriate responding overall, and during the first FR 20 of the session over four consecutive sessions. Tests were conducted with different doses of cocaine, doses of the aryltropane analogs, or combinations of doses, after which training resumed. Subsequent testing was conducted only if the subject met the above criteria on both of the immediately preceding training sessions. Test sessions were identical to training sessions except that 20 consecutive responses on either lever were reinforced.

Overall response rate and the percentage of responses occurring on the cocaine-appropriate lever were calculated for each subject and averaged. If less than half of the subjects responded at a particular dose the mean value was not determined for percentage of cocaine appropriate responding. At least 80% cocaine- or saline-appropriate responding was considered similar to the training dose of cocaine or saline, respectively. Levels of cocaine appropriate responding between 20 and 80% were considered partial substitution.

Drugs. (-)-Cocaine HCl was purchased (Sigma, St. Louis, MO), and analogs of phenyltropane were synthesized at the University of New Orleans (Xu et al., 2004). Figure 1 shows reference compounds and structures of 2-substituted 3ß-aryltropane compounds used in this study. The substitutions examined in the present study were either on the diphenylether moiety or para substitutions on the 3-phenyl moiety. All drugs were dissolved in 0.9% NaCl, and administered i.p. at 1 ml/kg (rat) or 1 ml/100 g (mouse) body weight in doses as indicated.

Ligand-induced DAT cysteine accessibility. Human embryonic kidney 293 (HEK293) cells stably transfected with T316C/C306A human DAT were seeded into polyethylenimine-coated 6-well plates and cultured to confluency. Cells were washed with cold PBS with 0.10 mM $CaCl_2$ and 1.0 mM $MgCl_2$, pH 7.1 (PBSCM) and incubated with DAT inhibitors in PBSCM for 20 min at 4°C. Cells were then further incubated with 1.0 mg/ml maleimide-PEO_2-biotin (Pierce Biotechnology, Rockford, IL) in the presence of DAT inhibitors for 30 min at 4°C, followed by quenching with 100 mM cysteine in PBSCM for 15 min at 4°C. Cells were then washed, harvested and lysed in TNE lysis buffer (Tris 10 mM, NaCl 150 mM, EDTA 1.0 mM, 1.0% Triton X-100, protease inhibitors, pH 7.5) for 1 h at 4°C, followed by a 20-min centrifugation of 18,000 x *g*. The lysates were incubated with 60 µl NeutrAvidin agarose beads (Pierce Biotechnology) overnight at 4°C. After beads were washed three times with TNE buffer, biotinylated proteins were eluted with sodium dodecyl sulfate sample buffer, separated by polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and probed with rabbit antisera for DAT (Hong and Amara, 2010). Mean densities of chemiluminescent DAT bands were quantified using the NIH ImageJ software and normalized to percent of vehicle.

Molecular dynamics simulation of DAT– ligand interactions. Starting coordinates for the inward-open and outward-open rat DAT (rDAT) homology models were obtained from a previously developed homology model based on the 2A65 LeuT crystal structure (Yamashita et al., 2005). The rDAT model was subjected to 600 nanosecond (ns) of all-atom molecular dynamics (MD). The root-mean squared deviation (RMSD) was measured for backbone atoms of TMs 1-12 with reference to the outward-open crystal structure of dDAT (4M48) (Penmatsa et al, 2013) and the inward-open crystal structure of LeuT (3TT3) (Krishnamurthy and Gouaux,

2012). Cocaine was docked into the outward-open rDAT homology model and BZT was docked into the inward-open rDAT homology model. One nanosecond of MD was performed on cocaine bound to DAT, after which cocaine was removed from the binding pocket and LX-10 and LX-11 were independently docked into the outward-open DAT structure. Interactions between BZT, cocaine, LX-10, and LX-11 were evaluated within their respective DAT models after 35 ns of all-atom MD.

The isobaric-isothermal (NPT) ensemble in the NAnoscale Molecular Dynamics (NAMD) program (v2.10b1) was used to perform all MD calculations using a time step of 2 fs, and periodic boundary conditions. Forcefield parameters for the protein, lipids, and water were defined using the CHARMM36 forcefield. *Ab initio* derived forcefield parameters were previously developed and used for assigning parameters for cocaine. CHARMM General Force Field (CGenFF) parameters for BZT and the 2-substituted aryltropanes were obtained from the ParamChem webserver (https://www.paramchem.org/). Langevin dynamics and the damping Langevin piston were utilized to maintain 310 K temperature and 1 atm pressure, respectively. A 20-picosecond (ps) energy minimization was performed to eliminate steric clashes followed by one ns of MD with harmonic restraints were placed on the protein backbone atoms. The atom's restraining potential was scaled from ten to zero kcal/mol/Å. All-atom MD was performed with restraints removed from the backbone atoms for 35 ns. The RMSD of each DAT:inhibitor complex was evaluated after the 35 ns MD with reference to the initial DAT coordinates.

RESULTS

Radioligand Binding Assays. The K_i values determined with the 3ß-aryltropanes with 2ß-substitutions at the DAT ranged from 18.9 for LX-10 to 68.0 nM for LX-19 (Table 3). These

values represent affinities as much as 3-fold higher than those reported previously (Xu et al., 2004) due to the use of a sucrose-phosphate rather than a HEPES buffer (unpublished observations). Additionally, the 2ß-substituted analogs had affinities that were from 10- to 25-fold greater than the respective analogs with α-substitutions. In contrast to the ß-substituted analogs, the α-substituted analogs were generally not selective for DAT over SERT, based on the previously published affinities for the SERT (Xu et al., 2004). None of the compounds had better than mM affinity for either σ_1R or σ_2R binding sites, and those affinities were not appreciably different among the enantiomers (Table 3).

A pair of enantiomers (LX-19 and LX-20) were screened for affinity at 35 mammalian binding sites at a 10 μM concentration of either compound. Compounds were considered inactive because they produced less than 10% displacement at all of the screened sites except those listed in Table 4. Affinities of LX-19 for those sites were uniformly less than that for the DAT, though reasonable affinity measurements were impossible at adrenergic α_{2A}, 5-HT_{2A}, or sodium channel, site 2 because there was greater than 90% displacement of radiolabel by LX-19 at 10 μM (Table 4). The estimated affinity of LX-20 for histamine H_2 receptors was greater than that for the DAT. Additionally at nine other sites the affinity of LX-20 was comparable to or possibly greater than that for the DAT. Finally, the affinity of the compound at dopamine D2 receptors was less than that at the DAT (Table 4).

Locomotor Activity. Cocaine dose-dependently increased ambulatory activity (Fig. 2, squares, repeated in all panels for comparison with the other compounds). The maximum effect of cocaine was approximately 650 counts per min during the first 30 min after injection, and was obtained at 59 μmol/kg. All of the 2ß-substituted compounds dose-dependently stimulated locomotor activity (Fig. 2, filled circles). The dichloro-substituted compound LX-19 (Fig. 2D)

was the most effective and stimulated activity to a maximum similar to that produced by cocaine ($t_{14} = 0.310$, $p = 0.761$). The maximal effects of the other 2ß-substituted compounds were statistically less than the cocaine maximum (all t-values ≥ 2.47, p-values ≤ 0.027).

In contrast to the compounds with 2ß-substituents, those with 2α-substituents generally failed to stimulate locomotor activity (Fig. 2, open symbols). The exception to this was the methyl-substituted LX-22 (Fig. 2E), which increased activity by 163 counts per min. That value was about half, and statistically less than ($t_{14} = 3.14$, $p = 0.007$) the maximum produced by its 2ß-enantiomer, LX-21. The characteristic failure of the 2α-substituted compounds to stimulate locomotor activity occurred despite the assessment across the range of behaviorally active doses from those that had no effect to those that substantially decreased activity. This range typically encompased doses that were at least 10-fold higher than the minimally effective stimulant dose of the corresponding 2ß enantiomers.

Cocaine Discrimination. Cocaine produced a dose-related increase in the percentage of cocaine-appropriate responses (Fig. 3, all top panels, squares). Among the 2ß-substituted aryltropanes, LX-10 (Fig. 3A) was the single compound that fully substituted for cocaine, with 92% of responses on the cocaine-appropriate lever at a dose of 12 µmol/kg (Fig. 3A, top). Several of the other 2ß-substituted analogs (LX-16, LX-19, LX-21; Fig. 3) produced a degree of cocaine-appropriate responding greater than that found following vehicle injections, though none exceeded 60%. In contrast, the remaining 2ß-substituted analogs (LX-13, LX-23) produced maximal substitution for cocaine well below 20% (Fig. 3).

Similarly, none of the 2α-substituted aryltropane analogs produced a level of cocaine-appropriate responding exceeding 20% (Fig. 3). The absence of full substitution for cocaine was

obtained despite the assessment of the entire range of behaviorally active doses the from those having no effects to those that virtually eliminated responding (Fig. 3, bottom panels).

Because it is possible that the time frame for the assessment of discriminative-stimulus effects may have failed to capture the maximal effects of the 2-substituted aryltropanes, the effects of two of the ß-enantiomers, LX-21 and LX-23, were also examined at 30 and 60 min after injection. Increasing the time between treatment and testing did not significantly increase the effectiveness in substituting for cocaine for either compound (Fig. 4, top panels). In addition, at 30 and 60 min after injection, the decreases in response rates produced by the drugs were substantially diminished (Fig. 4, bottom panels). The diminished effects on response rates are indicative of diminished pharmacological effects with time, and that further increases in time between injection and the assessment would not yield greater substitution for cocaine.

The compounds LX-13, LX-21, and LX-23 were selected for studies in combination with cocaine due to their high DAT affinity and their own minimal substitution for cocaine shown in Fig. 3. Each of the 2-substituted aryltropane analogs shifted the dose-effect curve for discriminative-stimulus effects of cocaine to the left, and in a dose-related manner for LX-13 and LX-23 (Fig. 5). The lowest doses studied produced a leftward shift of about 1.5-fold. The higher doses produced shifts of 2.23- and 3.31-fold with LX-13 and LX-23, respectively, which were statistically significant (95% confidence limits of the relative potency values excluded 1.0).

DAT conformation. To compare molecular actions of these compounds with that of cocaine at DAT, the conformation of DAT was probed biochemically using the T316C/C306A construct (substitution of threonine 316 to cysteine and cysteine 306 to alanine). This functional DAT construct has a sole cysteine residue (T316C) accessible from the extracellular side, as measured from alkylation of its sulfhydryl side chain by the cell-impermeant maleimide-PEO_2-

JPET Fast Forward. Published on January 14, 2016 as DOI: 10.1124/jpet.115.230722
This article has not been copyedited and formatted. The final version may differ from this version.

biotin. The reactivity at this position has been shown be altered by inhibitor binding, and used to indicate a DAT conformation open to the extracellular space (Hong and Amara, 2010). Incubation with saturating concentrations (>100-fold K_i values) of the six 2β-enantiomers significantly increased DAT labeling by maleimide-PEO_2-biotin, suggesting a cocaine-like stabilization effect on the outward-open DAT conformation. Additionally, the majority of the 2α-enantiomers (except that LX-15 and LX-20 were inactive) also increased T316C DAT cysteine accessibility, indicating a cocaine-like effect on the DAT conformation.

Molecular Dynamics Simulation of DAT – Ligand Interaction. Initial docking of cocaine, LX-10 or LX-11 to the outward-open DAT conformation, and BZT to the inward-open DAT conformation yielded poses in which each inhibitor was able to occupy subsites A, B, C within the binding site (Wang et al, 2013). Interaction between the positively-charged tropane nitrogen atom of each ligand and the TM1 residue D79 was also observed in each pose. Each inhibitor-DAT complex was then embedded in a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) lipid bilayer and solvated using TIP3P water model with 0.15 M NaCl and 0.15 M KCl.

After 35 ns of MD, the tropane ring of cocaine was still interacting with TM1 residue D79 and TM3 residue Y156 (Fig. 7A). However, the phenyl ring of cocaine was no longer interacting with F325 in a “face-to-edge” interaction. In contrast to cocaine, BZT bound to the inward-open DAT conformation was repositioned by the MD iteration (Fig. 7A). BZT was reoriented towards the intracellular end of the binding pocket, although its tropane ring nitrogen maintained an interaction with D79. Both rings of the diphenyl ether moiety were observed to interact face-to-edge with F76 and F325, allowing the ligand to be stabilized by several hydrophobic interactions within the S1 site.

LX-10 and LX-11 were each, like cocaine, still interacting with TM1 residue D79 and TM3 residue Y156 after 35 ns of MD (Fig. 7A). LX-10 was found to occupy the S1 pocket, with its functional groups overlapping with the placement of cocaine's functional groups (Fig. 7B). The LX-10 tropane and chlorophenyl ether rings were seen to interact with DAT sidechain residues in a similar way as cocaine. In contrast, the diphenyl ether moiety of LX-10 was seen to interact with F325, as it extends further than the 2-methyl ester group of cocaine. Unlike LX-10 and cocaine, the chlorophenyl ring and one of the diphenyl ether rings of LX-11 displayed a BZT-like DAT interaction pattern. Its second diphenyl ether ring was found to form a **π-π** interaction with F325, and a new **π-π** interaction was observed between the chlorophenyl ring of LX-11 and F76, similar to BZT (Fig. 7B). Overall, the positioning of LX-10 in the DAT S1 pocket is more similar to cocaine than is LX-11.

To examine accessibility of the DAT binding pocket from the extracellular side, the distance between two critical residues, R85 on TM1a and D475 on TM10 (Fig. 7C) was measured in the MD simulation. When BZT binds to the inward-open DAT, the average distance was 3.0 ± 0.3Å, substantially smaller than those for DAT binding to cocaine, LX-10 or LX-11 (6.7 ± 0.6Å, 5.1 ± 1.4Å or 5.6 ± 1.0Å, respectively), suggesting BZT is positioned deeper in the DAT binding pocket (Fig. 7D).

DISCUSSION

Previous studies demonstrated that the behavioral potencies of DA uptake inhibitors including cocaine are directly related to their DAT binding affinities (Ritz et al., 1987; Bergman et al, 1989), and the correlation among those effects is greater than that for behavioral effects and affinities at the other monoamine transporters (Ritz et al., 1987), suggesting that behavioral

effects of cocaine-like drugs are due to actions at the DAT (Kuhar et al, 1991). However, an increasing number of DAT inhibitors are being found with effects different from those of cocaine (Reith et al., 2015).

The 2-substituted aryltropane analogs examined in the present study had affinity for the DAT, however, virtually all of the compounds had behavioral effects that were substantially different from those of cocaine. Most of the compounds failed to fully substitute in rats trained to discriminate cocaine from saline injections with the 3-chlorophenyl analog (LX-10) as the only exception. The 2α-substituted compounds failed to stimulate locomotor activity, whereas the 2ß-substituted compounds did so, but with only one compound (LX-19) as effective as cocaine. Thus, although these 2-substituted aryltropanes bind to the DAT, they produce behavioral effects atypical for drugs with DAT affinity.

The absence of cocaine-like behavioral effects of the 2α-substituted aryltropanes in the present study was possibly due to their lower DAT affinity compared to the ß-enantiomers. However, locomotor effects of the α-enantiomers (with the possible exception of LX-15) were assessed at doses sufficient to accommodate their lower affinity. In the drug-discrimination procedure, the dose domain studied was from inactive doses to those that virtually eliminated responding. Because the pairs of enantiomers were typically equipotent in decreasing response rates it is possible that doses of the α-enantiomers sufficient for cocaine-like effects were greater than those that eliminated responding. The similar potencies for effects on response rates of the enantiomeric pairs and their differences in DAT binding affinities suggest that effects on response rates were not DAT-mediated.

The major structural difference between the present aryltropanes and cocaine-like DAT inhibitors (e.g. Carroll et al., 1992) resides in the diphenyl ether substitution on the tropane 2-

position which is a significant substituent of atypical DA uptake inhibitors that are structurally related to BZT (Newman et al., 1995; van der Zee et al., 1980) and the phenylpiperazines (e.g. GBR 12909; van der Zee et al., 1980; Andersen, 1989). Many of the members of those structural groups have various effects atypical of standard DAT inhibitors such as cocaine and methylphenidate. Reith et al. (2015) discussed several mechanisms that may contribute to atypical effects of DAT inhibitors, including off-target actions, slow DAT association, and shifts in DAT conformational equilibrium that decrease its opening to the extracellular space.

The pharmacology of the present series of aryltropanes has some similarities to and differences from that of BZT analogs. Many of the BZT analogs have muscarinic M_1 antagonist effects (Tanda et al., 2009), an off-target action thought by some to interfere with cocaine-like effects (Reith et al., 2015; Tanda et al., 2009). The present compounds have appreciably lower M_1 affinity, and results here are consistent with previous results showing that atypical DAT inhibitors can have effects different from cocaine without having affinity for M_1 receptors (Tanda et al., 2009; Reith et al., 2015).

Previous studies indicate that concurrent DAT and σ_1R inhibition can alter the effects of DAT inhibitors (e.g. Hiranita et al., 2011). However, the relatively low σ_1R binding affinities of the present compounds suggest that actions at that site do not contribute to their atypical effects. The present DAT affinities were obtained in a sucrose-phosphate buffer which yielded higher affinities than those reported previously for binding in a HEPES buffer, and consequently the selectivity of these compounds for the DAT compared to SERT and NET would be greater than that reported previously (Xu et al, 2004). Thus the relatively lower SERT and NET affinities of these aryltropanes are also unlikely to produce effects that interfere with cocaine-like effects.

Further, numerous cocaine analogs that share behavioral effects with cocaine bind to SERT and NET often with affinities as great as that for the DAT (Carroll et al., 1992).

Sites at which the screened compounds were most potent include DA D_3, histamine H_2, muscarinic M_2 and 5-HT_{1A} receptors. Considering these off-target actions with previous findings suggests that none of these is the sole contributor, if there is one at all, to the atypical effects of these compounds. For example, the D_3R affinity of LX-20, which did not stimulate locomotor activity, was greater than that for LX-19, which did stimulate activity, and greater than its affinity for the DAT, suggesting a role for D_3Rs in the atypical effects of LX-20. However, Li et al. (2011) examined a group of BZT analogs that were considered atypical DAT ligands. Within that group, the compound that was most like cocaine, JHW013, had the greatest affinity for D_3Rs. Together the results suggest that DA D_3Rs do not contribute to atypical DAT inhibitor effects.

A previous study indicated that affinity for histamine receptors does not contribute to the atypical effects of BZT analogs (Campbell et al., 2005). Similarly muscarinic M_2 sites seem unlikely as the BZT-derived atypical DAT inhibitors have a wide variety of reported activity at this site. Notably, the N-substituted BZT analog GA-299 had little cocaine-like activity and lacked affinity for the M_2 muscarinic site (Li et al., 2011). Previous studies have also found inconsistencies in affinites at 5-HT_1 binding sites among N-substituted BZT analogs (Katz et al., 2004; Li et al., 2011).

Several studies (Stathis et al., 1995; Desai et al., 2005a; 2005b; Tanda et al., 2009a) suggest that a slow rate of DAT occupancy by various DAT inhibitors can decrease cocaine-like behavioral effects. Slow brain penetration also reduces the reinforcing effectiveness of DA uptake inhibitors (Kimmel et al., 2007; Czoty et al., 2010). It is therefore plausible that slow

DAT occupancy rates with the present aryltropanes renders them ineffective in substituting for cocaine in drug-discrimination paradigms early after injection. To assess this, the time courses of two of the ß-substituted compounds (LX-21, LX-23) that had minimal cocaine-like effects were assessed. Maximal effects were not enhanced at these later times, suggesting that a slow association at the DAT is not responsible for the present atypical effects.

Because of the limited effectiveness of the present aryltropane analogs compared to cocaine, it was of interest to assess whether these compounds might reduce/antagonize the effects of cocaine. It has been previously reported that the discriminative effects of cocaine can be potentiated by DA uptake inhibitors, both "standard" inhibitors that did (Holtzman, 2001; Li et al., 2006), and those that did not (Katz et al., 2004; Tolliver et al., 1999) fully substitute for cocaine. The present compounds enhanced rather than antagonized the discriminative-stimulus of cocaine. Interestingly, compounds that shifted the discriminative effects of cocaine leftward in a previous study (Katz et al., 2004) decreased the self-administration of cocaine (Hiranita et al., 2009), suggesting differences in interactions with cocaine self-administration and discrimination.

Molecular studies have shown that cocaine binding alters DAT residue accessibility to alkylation (Ferrer and Javitch, 1998), and that BZT binding results in a different pattern of DAT residue accessibility (Reith et al, 2001). Recent reports propose that cocaine binding stabilizes an outward-facing DAT conformation (Loland et al., 2008; Hong and Amara, 2010). Further, it was suggested that behavioral effects produced by cocaine-like or atypical DAT inhibitors are related to different conformational states of DAT preferrentially induced by these ligands (Loland et al., 2008). We thus examined whether binding of these 2-substituted aryltropane analogs prefers certain conformational states of DAT. Results from the T316C/C306A DAT

construct are consistent with previous reports (Hiranita et al, 2014; Reith et al., 2015). The cysteine accessibility of T316C was increased by cocaine, but decreased by BZT. Although the present aryltropane analogs did not induce significant locomotor activity or substitute for cocaine in drug discrimination paradigms, all 2ß enantiomers and the 2α enantiomers (except LX-15 and LX-20) significantly increased DAT cysteine accessibility as cocaine did, suggesting that their binding stabilized the outward-facing DAT conformation in a manner similar to cocaine.

Molecular dynamics simulations of DAT–ligand interactions suggest that cocaine binds to DAT in the S1 pocket, preferring an outward-open conformation, whereas BZT appears to have been significantly repositioned, occupying a new novel subsite in the DAT S1 pocket while maintaining several favorable interactions with S1 residues. Combined with the cysteine accessibility data, the results support the notion that BZT binds to DAT in a manner distinct from that of cocaine. Further, both the cysteine accessibility results and the molecular dynamics simulations suggest that LX10 and LX 11, like cocaine prefer an outward-open DAT conformation. Consideration of these data with the behavioral data suggests that the behavioral effects of DAT inhibitors cannot be fully explained by conformational status assumed by the DAT with ligand binding. As with previous reports (Hiranita et al., 2014; Li et al., 2011) the present results suggest that there are likely multiple mechanisms involved in the behavioral effects of atypical DAT inhibitors (Reith et al., 2015).

ACKNOWLEDGMENTS

The authors thank Bettye Campbell and Dawn French for technical support, Lekyla Whitaker for administrative and clerical support, and Dawn French for expert data analysis. We also sincerely thank Drs. Brandon Harvey and Bruce Hope for generously sharing their cell culture facility and biochemical instruments. Portions of this manuscript were presented at Experimental Biology 2015 conference.

Authorship Contributions

Participated in research design: Weimin C. Hong, Theresa A. Kopajtic, Lifen Xu, Stacey A. Lomenzo, Bernandie Jean, Jeffry D. Madura, Christopher K. Surratt, Mark L. Trudell and Jonathan L. Katz

Conducted experiments Weimin C. Hong, Theresa A. Kopajtic, Lifen Xu, Stacey A. Lomenzo, Bernandie Jean, Jeffry D. Madura, Christopher K. Surratt, Mark L Trudell

Contributed new reagents or analytic tools: Weimin C. Hong, Lifen Xu, Stacey A. Lomenzo, Bernandie Jean, Jeffry D. Madura, Christopher K. Surratt, Mark L.Trudell

Performed data analysis: Weimin C. Hong, Theresa A. Kopajtic, Lifen Xu, Stacey A. Lomenzo, Bernandie Jean, Jeffry D. Madura, Christopher K. Surratt, Mark L.Trudell and Jonathan L.Katz

Wrote or contributed to the writing of the manuscript: Weimin C. Hong, Theresa A. Kopajtic, Stacey A. Lomenzo, Bernandie Jean, Jeffry D. Madura, Christopher K. Surratt, Mark L. Trudell and Jonathan L. Katz

REFERENCES

Andersen P H (1989) The dopamine uptake inhibitor GBR 12909: selectivity and molecular mechanism of action. Eur Jour Pharmacology 166: 493-504.

Bergman J, Madras B K, Johnson S E, and Spealman R D (1989) Effects of cocaine and related drugs in nonhuman primates. III. Self-administration by squirrel monkeys. J Pharmacol Exp Ther 251: 150-155.

Carroll F I, Lewin A H, Boja J W, and Kuhar M J (1992) Cocaine receptor: biochemical characterization and structure-activity relationships of cocaine analogs at the dopamine transporter. Journal of Medicinal Chemistry 35: 969-981.

Cheng Y C, and Prusoff W H (1973) Relationship between the inhibition constant (K_i) and the concentration of an inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. Biochem Pharmacol 22: 3099-3108.

Czoty P W, Martelle J L, Carroll F I, and Nader M A (2010) Lower reinforcing strength of the phenyltropane cocaine analogs RTI-336 and RTI-177 compared to cocaine in nonhuman primates. Pharmacol Biochem and Behav 96: 274-278.

Desai R I, Kopajtic T A, Koffarnus M, Newman A H, and Katz J L (2005) Identification of a dopamine transporter ligand that blocks the stimulant effects of cocaine. J Neurosci 25: 1889-1893.

Desai R I, Kopajtic T A, French D, Newman A H, and Katz J L (2005) Relationship between in vivo occupancy at the dopamine transporter and behavioral effects of cocaine. GBR 12909 [1-{2-[bis-(4-fluorophenyl) methoxy] ethyl}-4-(3-phenylpropyl) piperazine], and benztropine analogs. J Pharmacol Exp Ther 315: 397-404.

Ferrer J V, and Javitch J A (1998) Cocaine alters the accessibility of endogenous cysteines in putative extracellular and intracellular loops of the human dopamine transporter. Proc Nat Acad Sci 95: 9238-9243.

Hiranita T, Wilkinson DS, Hong WC, Zou MF, Kopajtic TA, Soto PL, Lupica CR, Newman AH, and Katz JL (2014) 2-Isoxazol-3-phenyltropane derivatives of cocaine: molecular and atypical system effects at the dopamine transporter. J Pharmacol Exp Ther 349: 297-309.

Hiranita T, Soto P L, Newman A H, and Katz J L (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.

Hiranita T, Soto P L, Kohut S J, Kopajtic T, Cao J, Newman A H, Tanda G, and Katz J L (2011) Decreases in cocaine self-administration with dual inhibition of the dopamine transporter and σ receptors. J Pharmacol Exp Ther 339: 662-677.

Hong W C, and Amara S G (2010) Membrane cholesterol modulates the outward facing conformation of the dopamine transporter and alters cocaine binding. Journal of Biological Chemistry 285: 32616-32626.

Holtzman SG (2001) Differential interaction of GBR 12909, a dopamine uptake inhibitor, with cocaine and methamphetamine in rats discriminating cocaine. Psychopharmacology 155:180–186.

Izenwasser S, Newman A H, and Katz J L (1993) Cocaine and several σ receptor ligands inhibit dopamine uptake in rat caudate-putamen. Eur Jour Pharmacol 243: 201-205.

Johanson C, and Fischman M W (1989) The pharmacology of cocaine related to its abuse. Pharmacol Rev 41: 3-52.

Katz J L, Izenwasser S, Kline R H, Allen A C, and Newman A H (1999) Novel 3α-diphenylmethoxytropane analogs: selective dopamine uptake inhibitors with behavioral effects distinct from those of cocaine. J Pharmacol Exp Ther 288: 302-315.

Katz J L, Kopajtic T A, Agoston G E, and Newman A H (2004) Effects of N-substituted analogs of benztropine: diminished cocaine-like effects in dopamine transporter ligands. J Pharmacol Exp Ther 309: 650-660.

Katz J L, Su T P, Hiranita T, Hayashi T, Tanda G, Kopajtic T, and Tsai S Y (2011) A role for sigma receptors in stimulant self administration and addiction. Pharmaceuticals 4: 880-914.

Kimmel H L, O'Connor J A, Carroll F I, and Howell L L (2007) Faster onset and dopamine transporter selectivity predict stimulant and reinforcing effects of cocaine analogs in squirrel monkeys. Pharmacol Biochem Behav 86: 45-54.

Kosterlitz HW, Waterfield AA, and Berthoud V (1973) Assessment of the agonist and antagonist properties of narcotic analgesic drugs, in: *Narcotic Antagonists, Advances in Biochemical Psychopharmacology, Vol 8* (Braude MC, Harris LS , May EL, Smith JP and Villarreal JE eds) pp 319-334, Raven Press, New York.

Krishnamurthy H, and Gouaux E (2012) X-ray structures of LeuT in substrate-free outward-open and apo inward-open states. Nature 481: 469-474.

Kuhar MJ, Ritz MC and Boja, JW (1991) The dopamine hypothesis of the reinforcing properties of cocaine. Trends in Neurosciences 14: 299-302.

Li S M, Kopajtic T A, O'Callaghan M J, Agoston G E, Cao J, Newman A H, and Katz J L (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.

Li S M, Campbell B L, and Katz J L (2006) Interactions of cocaine with dopamine uptake inhibitors or dopamine releasers in rats discriminating cocaine. J Pharmacol Exp Ther 317: 1088-1096.

Loland C J, Desai R I, Zou M F, Cao J, Grundt P, Gerstbrein K, 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.

Madras B K, Fahey M A, Goulet M, Lin Z, Bendor J, Goodrich C, Meltzer PC, Elmaleh DR, Livni E, Bonab AA, and Fischman A J (2006) Dopamine transporter (DAT) inhibitors alleviate specific parkinsonian deficits in monkeys: association with DAT occupancy in vivo. J Pharmacol Exp Ther 319: 570-585.

Newman AH, Kline RH, Allen AC, Izenwasser S, George C and Katz, JL (1995) Novel 4'- and 4',4''-substituted-3α-(diphenylmethoxy)tropane analogs are potent and selective dopamine uptake inhibitors. J Med Chem 38: 3933-3940.

Penmatsa A, Wang K H, and Gouaux E (2013) X-ray structure of dopamine transporter elucidates antidepressant mechanism. Nature 503: 85-90.

Reith M E A, Berfield J L, Wang L C, Ferrer J V, and Javitch J A (2001) The uptake inhibitors cocaine and benztropine differentially alter the conformation of the human dopamine transporter. J Biol Chem 276: 29012-29018.

Reith MEA, Blough BE, Hong WC, Jones KT, Schmitt KC, Baumann MH, Partilla JS, Rothman RB, and Katz JL (2015) Behavioral, biological, and chemical perspectives on atypical agents targeting the dopamine transporter. *Drug Alcohol Depend* **147:** 1-19.

Rice ME, and Cragg SJ (2008) Dopamine spillover after quantal release: rethinking dopamine transmission in the nigrostriatal pathway. Brain Res Rev 58: 303-313.

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.

Schmitt K C, Rothman R B, and Reith M E A (2013) Nonclassical pharmacology of the dopamine transporter: atypical inhibitors, allosteric modulators, and partial substrates. J Pharmacol Exp Ther 346: 2-10.

Stathis M, Scheffel U, Lever S Z, Boja J W, Kuhar M J, and Carroll F I (1995) Rate of binding of various inhibitors at the dopamine transporter in vivo. Psychopharmacology 119: 376-384.

Tanda G, Newman A H, Ebbs A L, Tronci V, Green J L, Tallarida R J, and Katz J L (2009) Combinations of cocaine with other dopamine uptake inhibitors: assessment of additivity. J Pharmacol Exp Ther 330: 802-809.

Tolliver BK, Newman AH, Katz JL, Ho LB, Fox LM, Hsu K, Jr and Berger PS (1999) Behavioral and neurochemical effects of dopamine transporter ligands alone and in combination with cocaine: Characterization of 4-chlorobenztropine in vivo. J Pharmacol Exp Ther: 289: 110-122.

Valchar M, and Hanbauer I (1993) Comparison of [^{3}H] WIN 35,428 binding, a marker for dopamine transporter, in embryonic mesencephalic neuronal cultures with striatal membranes of adult rats. J Neurochem 60: 469-476.

van der Zee P, Koger, H S, Gootjes J, Hespe W (1980) Aryl 1,4-dialk(en)ylpiperazines as selective and very potent inhibitors of dopamine uptake. Eur J Med Chem 15: 363-370.

Wang H, Goehring A, Wang KH, Penmatsa A, Ressler R, Gouaux E (2013) Structural basis for action by diverse antidepressants on biogenic amine transporters. Nature 503: 141–145.

Woolverton WL, Rowlett JK, Wilcox KM, Paul IA, Kline RH, Newman AH, and Katz JL (2000) 3' and 4'-Chloro-substituted analogs of benztropine: intravenous self-administration and in vitro radioligand binding studies in rhesus monkeys. Psychopharmacology 147: 426-435.

Woolverton W L, Hecht G S, Agoston G E, Katz J L, and Newman A H (2001) Further studies of the reinforcing effects of benztropine analogs in rhesus monkeys. Psychopharmacology 154: 375-382.

Xu L, Kulkarni SS, Izenwasser SI, Katz JL, Kopajtic T, Lomenzo SA, Newman AH, and Trudell ML (2004) Synthesis and monoamine transporter binding of 2-(diarylmethoxymethyl)-3ß-aryltropane derivatives. J Med Chem 47:1676-1682.

Yamashita, A Singh S K, Kawate T, Jin Y, and Gouaux E (2005) Crystal structure of a bacterial homologue of Na^+/Cl^--dependent neurotransmitter transporters. Nature 437: 215-223.

FOOTNOTES

The work reported herein was supported in various parts by funds as follows. Work by WCH, TAK, and JLK was supported by the Intramural Research Program of the National Institute on Drug Abuse [ZIA DA000103-26]. WCH was also supported by startup funds from Butler University. The work of JDM and CKS was supported by a grant from the National Institute on Drug Abuse [DA027806]. The efforts by JDM and BJ were funded in part by NIH/NSF grants [R01DA027806 and XSEDE MCB060069 supercomputers]. The work of MLT, LX, and SAL was supported by a grant from the National Institute on Drug Abuse [DA11528].

FIGURE CAPTIONS

Figure 1. Structures of cocaine, benztropine, and 2-substituted aryltropane analogs.

Figure 2. Dose-dependent effects of aryltropane analogs on locomotor activity in mice. Ordinates: horizontal locomotor activity counts after drug administration. Abscissae: dose of drug in μmol/kg, log scale. Each point represents the mean value determined in eight mice with error bars representing SEM. The data are from the 30-min period during the first 60 min after drug administration, in which the greatest stimulant effects were obtained. Note that cocaine is more effective than the other compounds, and that 4',4''-diCl-phenyltropane was the least effective.

Figure 3: Effects of aryltropane analogs in subjects trained to discriminate injections of cocaine from saline. Ordinates for top panels: percentage of responses on the cocaine-appropriate lever. Ordinates for the bottom panels: rates at which responses were emitted (as a percentage of response rate after saline administration). Abscissae: drug dose in μmol/kg (log scale). Each point represents the mean value in from four to six subjects with error bars representing SEM. 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. Note that only LX-10 fully (>80% cocaine-appropriate responding) substituted for cocaine, however, the 3'-substituted compounds produced a partial substitution that was greater than levels after 4'-Cl-phenyltropane and vehicle.

Figure 4. Time course of effects of aryltropane analogs in rats trained to discriminate injections of cocaine from saline at various times after injection. Top ordinates: percentage of responses on the cocaine-appropriate key. Bottom ordinates: rates at which responses were emitted (as a percentage of response rate after saline administration). Abscissae: drug dose in mg/kg (log scale). Each point represents the effect in six rats. Note that with pretreatment times of up to one hour the effects of the drugs in substituting for cocaine were not significantly increased, and the effects on response rates decreased

Figure 5: Changes in the cocaine dose-effect curve for discriminative stimulus effects produced by pretreatments with the aryltropane analogs. Ordinates: percentage of responses on the cocaine-appropriate lever. Abscissae: cocaine dose in mg/kg (log scale). Each point represents the mean value in from four to six subjects with error bars representing SEM. 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. Note that each of the aryltropane analogs shifted the cocaine dose-effect curve to the left.

Figure 6: Modulation of conformation of DAT by 2-substituted aryltropane analogs. Effects on the cysteine accessibility of DAT T316C/C306A by cocaine, BZT, 2β-substituents (panel A) or 2α-substituents (panel B) were probed with maleimide-PEO_2-biotin and quantified by immunoblotting (see Methods for details). Summarized results (average ± SEM) in A: from n = 4–7 experiments or B: n = 4–5 experiments, with representative blots. Quantified DAT band densities were analyzed by one-way ANOVA with post-hoc Dunnett's test, * $P<0.05$, # $P<0.01$ compared with vehicle.

Figure 7: Molecular model of DAT inhibitors within the binding pocket. DAT inhibitors were independently docked into DAT, then subjected to 35 ns MD to generate poses for each DAT-inhibitor combination. A) BZT (yellow) in inward-open ("IO") DAT is superimposed with cocaine (red) in outward-open ("OO") DAT. B) LX-10 (blue) and LX-11 (green) are both in outward-open DAT and superimposed for comparison. DAT side chains F76 (TM1a), D79 (TM1a), Y156 (TM 3), F319 (TM 6) and F325 (TM 6) are color-coded to match the pose of their respective ligands. C) Final poses of benztropine (yellow stick model) and cocaine (red stick) in DAT binding pocket after 35 ns of MD. Positions of R85 and D475 are shown as space-filling atoms in DAT bound with BZT (yellow) or cocaine (red), respectively. In the inward-open DAT conformation R85 (TM1) and D475 (TM10), shown as yellow space-filling atoms, are closer in distance allowing the residues to form ionic interactions. D) The average distance between R85 (TM1a) and D475 (TM10) was also measured for LX10 (blue) and LX11 (green) and compared to cocaine (red) and BZT (yellow) in their respective DAT conformations. * $P<0.01$ one-way ANOVA; error bars represent the standard deviation.

JPET Fast Forward. Published on January 14, 2016 as DOI: 10.1124/jpet.115.230722
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1: Specific conditions used for studies of displacement of radioligands by 3ß-aryltropanes.

Assay	**Radiolabel**	**Tissue**	**Incubation Buffer**	**Incubation**	**Nonspecific Binding**
DAT	0.50 nM [^{3}H]WIN 35,428 (Perkin-Elmer, Boston, MA)	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_2HPO_4, 2.26 mM NaH_2PO_4, pH adjusted to 7.4)	120 min on ice	100 µM cocaine HCl
σ_1R	3.0 nM [^{3}H](+)-pentazocine (Perkin-Elmer)	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 µM haloperidol
σ_2R	3.0 nM [^{3}H]1,3-di-*o*-tolylguanidine (Perkin-Elmer) 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 µM haloperidol

Table 2: Assay Conditions for Activity at Various Receptor Sites by Examining Competition with the Appropriate Radioligands (ProfilingScreen®, MDS Panlabs Pharmacology Services, Bothell, Washington). σ_1R and σ_2R binding was assessed, however these results were not used as activity at those sites wereassessed more closely in our own laboratory.

Assay Target Site	Ligand	Non-Specific Binding	Tissue	Incubation
Adenosine A_1	1 nM [^{3}H]DPCPX	100 µM R(-)-PIA	Human recombinant CHO cells	90 min @ 25°C
Adenosine A_{2A}	0.05 µM [^{3}H]CGS-21680	50 µM NECA	Human recombinant HEK-293 cells	90 mm. @ 25°C
Adrenergic α_{1A}	0.25 nM [^{3}H]prazosin	10 µM phentolamine	Rat submaxillary gland	60 min @ 25°C
Adrenergic α_{2A}	1.0 nM [^{3}H]MK-912	10 µM WB-4101	Human recombinant insect Sf9 cells	60 min @ 25°C
Adrenergic β_1	0.03 nM [^{125}I]-cyanopindolol	100 µM S(-)-propranolol	Human recombinant Rex 16 cells	120 min @ 25°C
Adrenergic β_2	0.2 nM [^{3}H]CGP-12177	10 µM ICI-118551	Human recombinant CHO-NBR1 cells	60 min @ 25°C
Ca^{++} Channel-L, dihydropyridine	0.1 nM [^{3}H]nitrendipine	1 µM nifedipine	Rat cortex	90 min @ 25°C
Dopamine D_1	1.4 nM [^{3}H]SCH 23390	10 µM (+)-butaclamol	Human recombinant CHO cells	120 min @ 37°C
Dopamine D_{2L}	0.16 nM [^{3}H]spiperone	10 µM haloperidol	Human recombinant CHO cells	120 min @ 25°C
Dopamine D_3	0.7 nM [^{3}H]spiperone	25 µM S(-)-sulpiride	Human recombinant CHO cells	120 min @ 37°C
Dopamine $D_{4.2}$	0.5 nM [^{3}H]spiperone	10 µM haloperidol	Human recombinant CHO cells	120 min @ 25°C
GABA Transporter	6 nM [^{3}H]GABA	10 µM NO-711	Rat cortex	20 min @ 25°C
$GABA_A$, Agonist	1 nM [^{3}H]muscimol	100 nM muscimol	Rat brain (excluding cerebellum)	10 min @ 4°C
$GABA_A$, Central Benzodiazepine	1 nM [^{3}H]flunitrazepam	10 µM diazepam	Rat brain (excluding cerebellum)	60 min @ 25°C
$GABA_B$	0.6 nM [^{3}H]CGP54626	100 µM CGP54626	Rat brain	20 min @ 25°C
Glutamate, Kainate	5 nM [^{3}H]kainite	1 mM L-glutamate	Rat brain (excluding cerebellum)	60 min @ 4°C
Glutamate, NMDA, phencyclidine	4 nM [^{3}H]TCP	1 µM MK-801	Rat cortex	45 min @ 25°C
Histamine H_1, Central	3 nM [^{3}H]pyrilamine	1 µM pyrilamine	Guinea pig brain	60 min @ 25°C
Histamine H_2	10 pM [^{125}I]iodoaminopotentidine	100 µM tiotidine	Guinea pig striatum	120 min @ 25°C
Histamine H_3	1 nM [^{3}H]N-α-methylhistimine	1 µM N-α-methylhistimine	Rat brain	30 min @ 25°C
Imidazoline I_2, Central	2 nM [^{3}H]idazoxan	1 µM idazoxan	Rat cortex	30 min @ 25°C
Muscarinic M_2	0.29 nM [^{3}H]N-methylscopolamine	1 µM atropine	Human recombinant insect sf9 cells	60 min @ 25°C
Nicotinic Acetylcholine, Central	2 nM [^{3}H]cytisine	100 µM nicotine	Rat brain	75 min @ 4°C
Norepinephrine Transporter	0.2 nM [^{125}I]RTI-55	10 µM desipramine	Human recombinant MDCK cells	180 min @ 4°C
Opiate-δ	0.9 nM [^{3}H]naltrindole	10 µM naloxone	Human recombinant CHO-K1 cells	60 min @ 25°C
Opiate-κ	0.6 nM [^{3}H]diprenorphine	10 µM naloxone	Human recombinant HEK-293 cells	60 min @ 25°C
Opiate-µ	0.6 nM [^{3}H]diprenorphine	10 µM naloxone	Human recombinant CHO-K1 cells	60 min @ 25°C
Phorbol ester	3 nM [^{3}H]PDBu	1 µM PDBu	Mouse brain	60 min @ 25°C
Potassium Channel [K_{ATP}]	5 nM [^{3}H]glyburide	1 µM glyburide	Syrian hamster pancreatic β cells HIT-T15	120 min @ 25°C
Serotonin, 5-HT_{1A}	1.5 nM [^{3}H]8-OH-DPAT	10 µM metergoline	Human recombinant CHO cells	60 min @ 25°C
Serotonin, 5-HT_{2A}	0.5 nM [^{3}H]ketanserin	1 µM mianserin	Human recombinant CHO-K1 cells	60 min @ 25°C
Serotonin, 5-HT_3	0.69 nM [^{3}H]GR-65630	10 µM MDL-72222	Human recombinant HEK-293 cells	60 min @ 25°C
Sigma, σ_1	8 nM [^{3}H]haloperidol	10 µM haloperidol	Human Jurkat cells TIB-152	240 min @ 25°C
Sigma, σ_2	3 nM [^{3}H]ifenprodil	10 µM ifenprodil	Rat brain	60 min @ 37°C
Sodium Channel, site 2	1.5 nM [^{3}H]batrachotoxin A 20-α-benzoate	100 µM veratridine	Rat brain	30 min @ 37°C

JPET Fast Forward. Published on January 14, 2016 as DOI: 10.1124/jpet.115.230722
This article has not been copyedited and formatted. The final version may differ from this version.

Table 3: Affinities (K_i values in nM) of the 3ß-aryltropane cocaine analogs obtained for displacement of the designated radioligands for DAT and σ_1R and σ_2R proteins. Numbers are K_i values in nM with S.E.M.

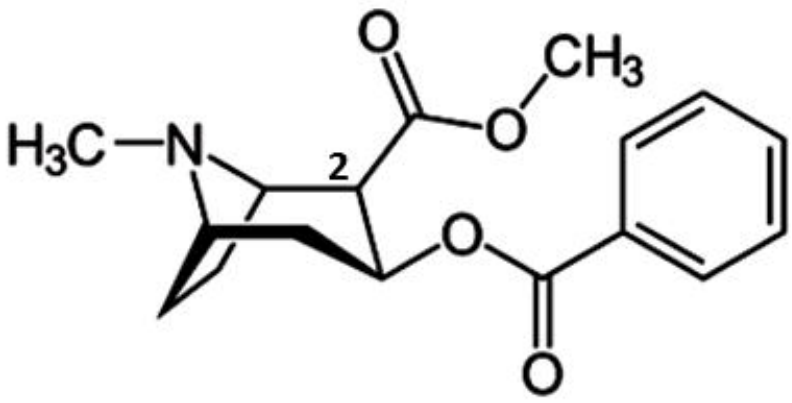
Compound (and 2-position conformation)	***DAT K_i Value [^{3}H]WIN 35,428***	***σ_1R K_i Value [^{3}H](+)-Pentazocine***	***σ_2R K_i Value [^{3}H]DTG***
Cocaine	98.1 ± 6.58	9,040 ± 1,150	15,000 ± 1,613
LX-10 (ß)	18.9 ± 0.19	5,090 ± 528	1,890 ± 63.5
LX-11 (α)	352 ± 44.1	5,940 ± 419	1,490 ± 179
LX-13 (ß)	22.8 ± 2.79	5,370 ± 596	2,490 ± 283
LX-12 (α)	353 ± 41.5	5,500 ± 750	1,700 ± 25.9
LX-16 (ß)	34.2 ± 1.29	2,060 ± 317	2,720 ± 267
LX-15 (α)	860 ± 62.6	2,270 ± 232	3,080 ± 53.7
LX-19 (ß)	68.0 ± 10.2	8,450 ± 349	3,000 ± 310
LX-20 (α)	703 ± 69.5	3,410 ± 333	2,910 ± 247
LX-21 (ß)	20.6 ± 1.79	943 ± 81.1	1,340 ± 311
LX-22 (α)	401 ± 13.0	3,530 ± 493	1,980 ± 85.1
LX-23 (ß)	40.8 ± 3.32	4,620 ± 292	1,840 ± 217
LX-24 (α)	651 ± 60.4	2,910 ± 233	1,760 ± 177

Table 4: Activities derived from the receptor screen at various sites expressed as % displacement at 10 μM and estimated K_i values relative to DAT affinity. Sites at which at least one of the compounds showed greater than 50% inhibition of radioligand binding at 10 μM are shown.

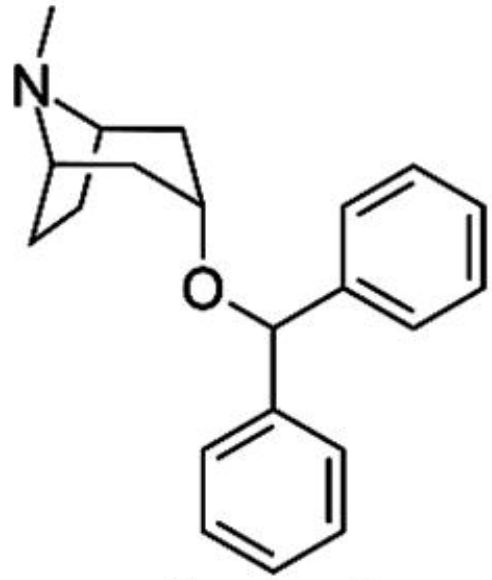
Target	% Inhibition		Relative to DAT[1]	
	LX19 (ß)	LX20 (α)	LX19 (ß)	LX20 (α)
Adenosine A_{2A}	57	Inactive	60	Inactive
Adrenergic α_{2A}	94	99	<7.3	<0.56
DA D_1R	76	80	22	1.8
DA D_2LR	Inactive	63	Inactive	2.6
DA D_3R	60	97	32	<0.56
Histamine H_2	Inactive	69	Inactive	0.16
Muscarinic M_2	85	99	10	<0.53
Opioid δ	58	76	36	1.5
Opioid κ	84	77	12	1.6
Opioid μ	Inactive	52	Inactive	5.3
5-HT_{1A}	65	102	42	<0.74
5-HT_{2A}	96	98	<5.1	<0.45
Na Channel, Site 2	94	98	<17	<1.4

[1]See Methods section for details regarding derivation of affinities relative to DAT.

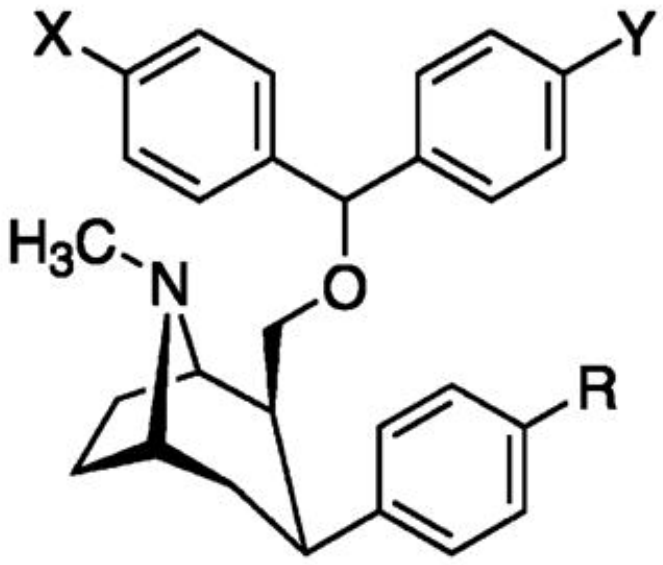
Briefly, for sites at which compounds produced between 10 and 90% displacement, an IC_{50} value was derived by linear interpolation assuming a displacement curve spanning a 100-fold domain of concentrations. For sites at which the displacement was >90%, IC_{50} values were estimated to be <1 μM. The compound was considered inactive at sites for which the displacement by 10 μM was <10%. K_i values were derived from estimated IC_{50} values and the Cheng-Prusoff equation using K_d values of radioligands provided by the vendor. This method follows a practice used by Kosterlitz et al. (1973) to estimate potencies of opioids for inhibition of guinea pig ileum contractions at single concentrations. The K_i values are presented in the table as a ratio K_i values (LX compound/DAT), with values for the DAT determined in the present study (Table 3).

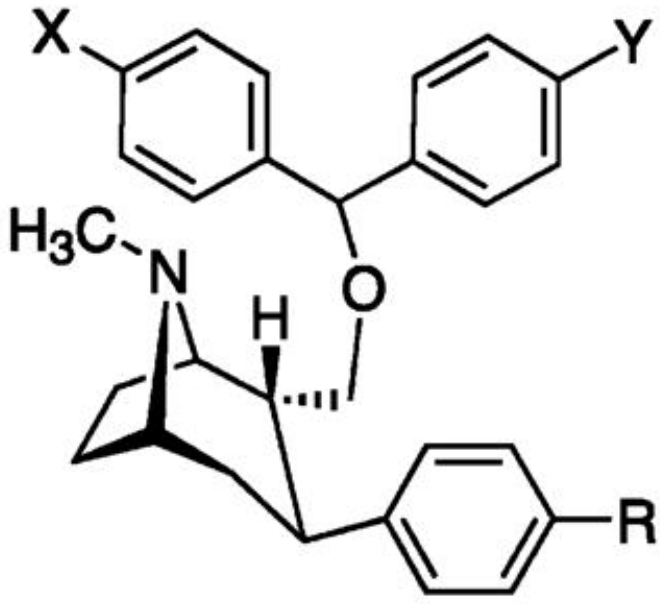


Cocaine



Benztropine





2-substituted aryltropanes

Figure 1

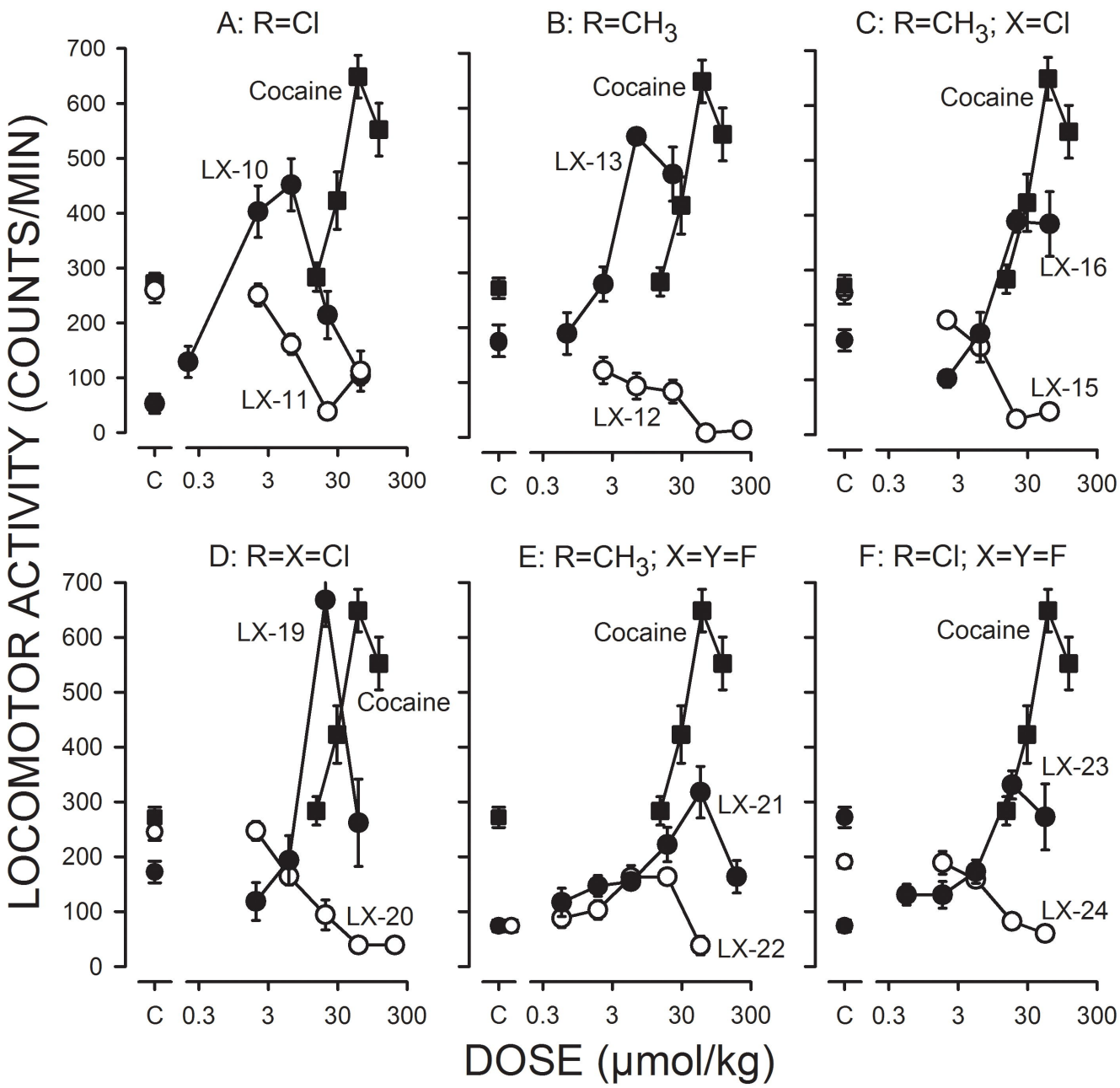

A: R=Cl
B: R=CH3
C: R=CH3; X=Cl
D: R=X=Cl
E: R=CH3; X=Y=F
F: R=Cl; X=Y=F
Cocaine
LX-10
LX-11
LX-13
LX-12
LX-16
LX-15
LX-19
LX-20
LX-21
LX-22
LX-23
LX-24
LOCOMOTOR ACTIVITY (COUNTS/MIN)
DOSE (µmol/kg)
0
100
200
300
400
500
600
700
C
0.3
3
30
300

Figure 2

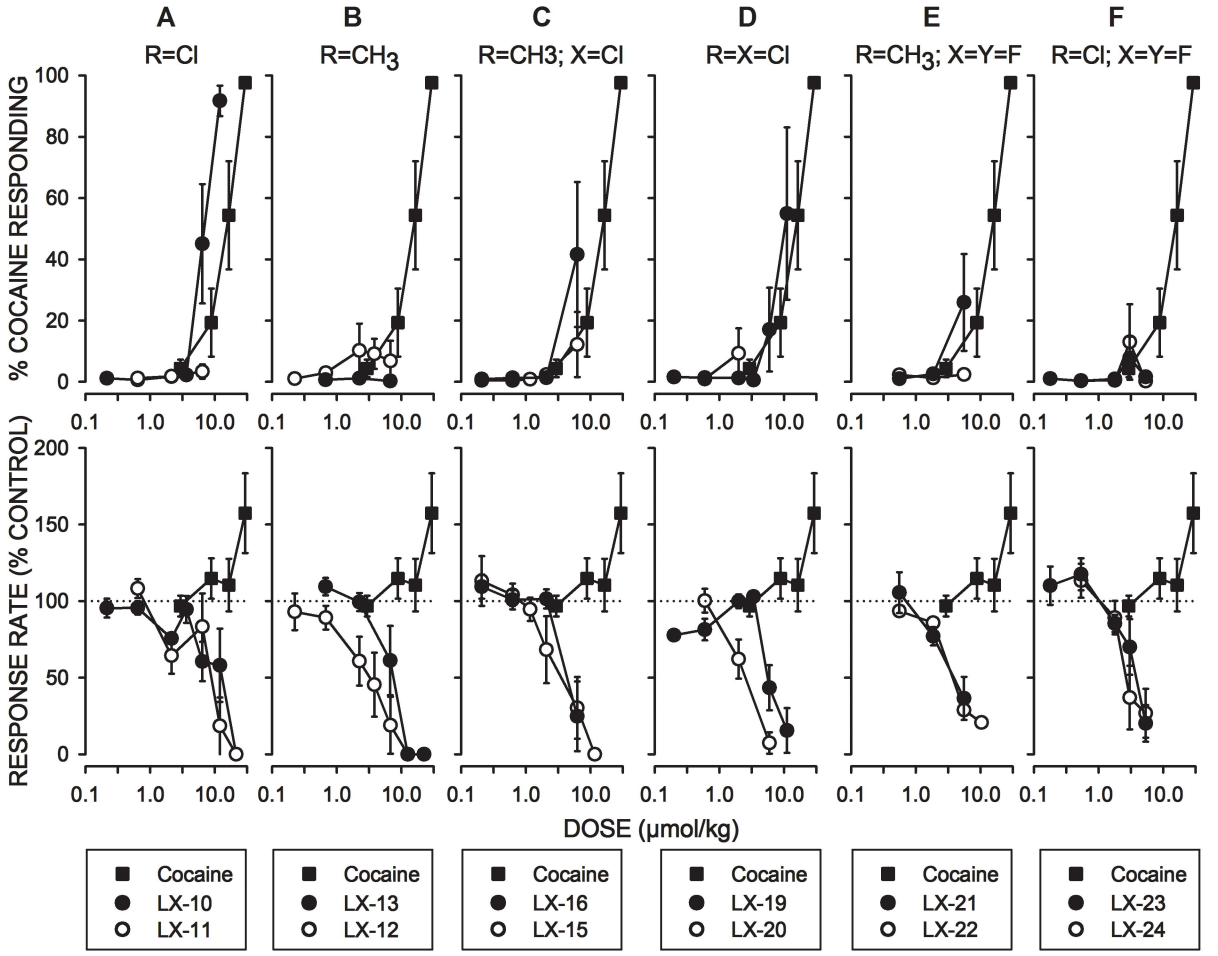

A
R=Cl
B
R=CH3
C
R=CH3; X=Cl
D
R=X=Cl
E
R=CH3; X=Y=F
F
R=Cl; X=Y=F
% COCAINE RESPONDING
RESPONSE RATE (% CONTROL)
DOSE (µmol/kg)
Cocaine
LX-10
LX-11
Cocaine
LX-13
LX-12
Cocaine
LX-16
LX-15
Cocaine
LX-19
LX-20
Cocaine
LX-21
LX-22
Cocaine
LX-23
LX-24

Figure 3

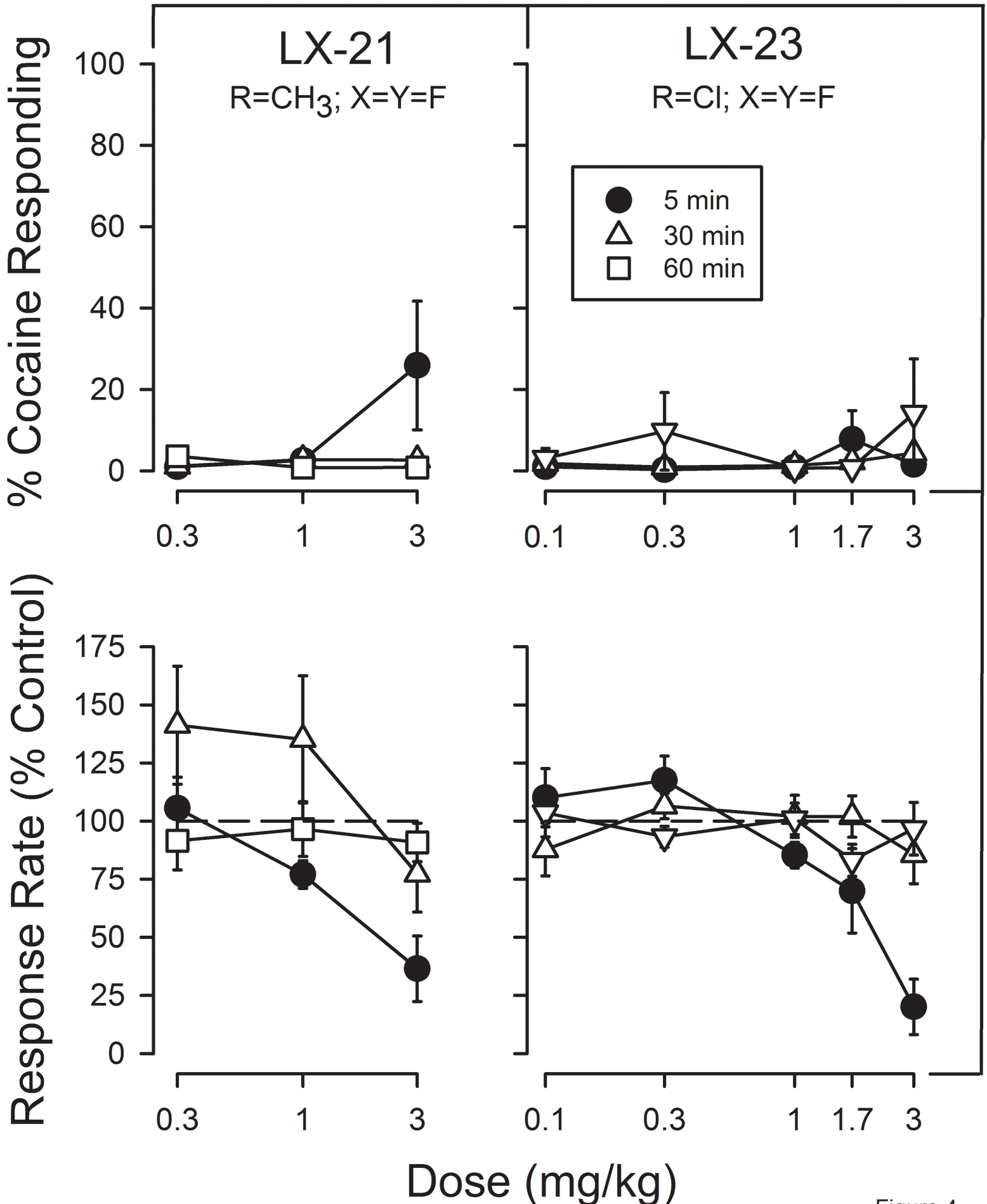

LX-21
R=CH3; X=Y=F
LX-23
R=Cl; X=Y=F
5 min
30 min
60 min
% Cocaine Responding
Response Rate (% Control)
Dose (mg/kg)
0
20
40
60
80
100
0
25
50
75
100
125
150
175
0.3
1
3
0.1
0.3
1
1.7
3

Figure 4

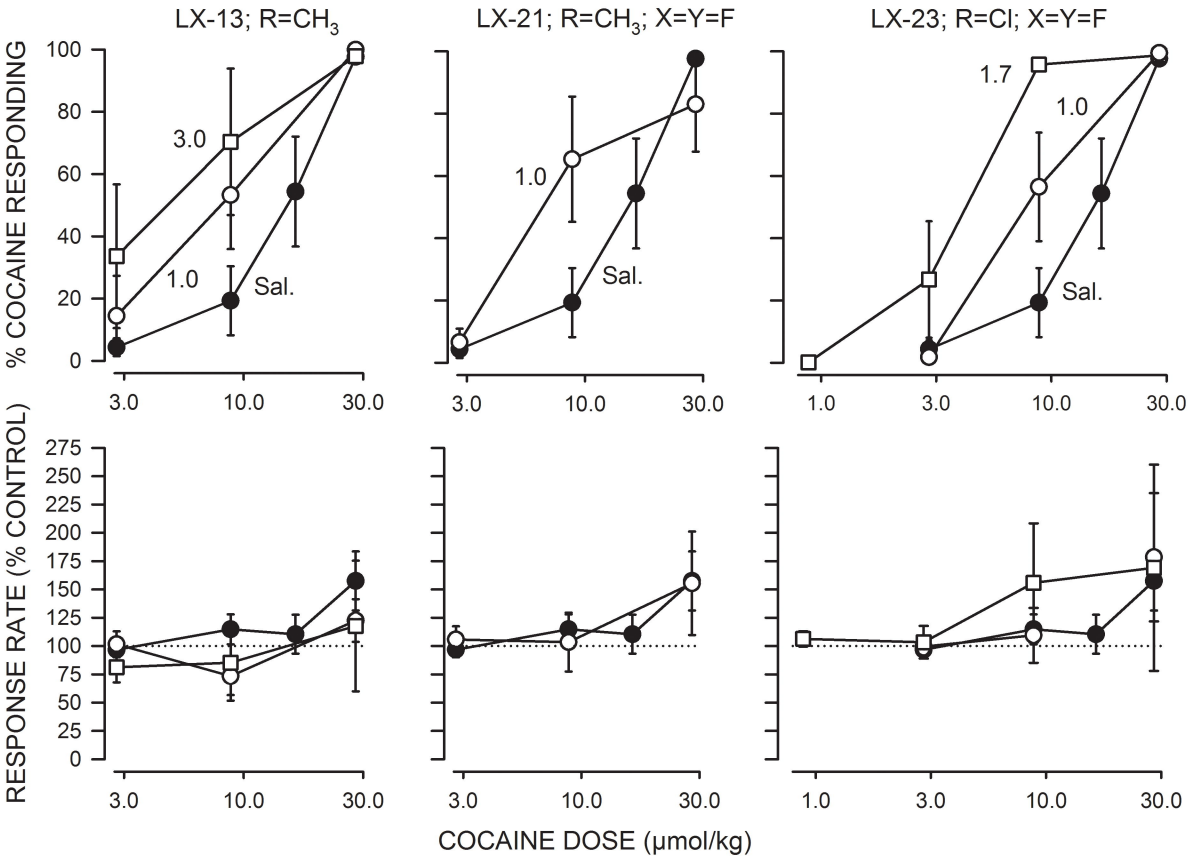
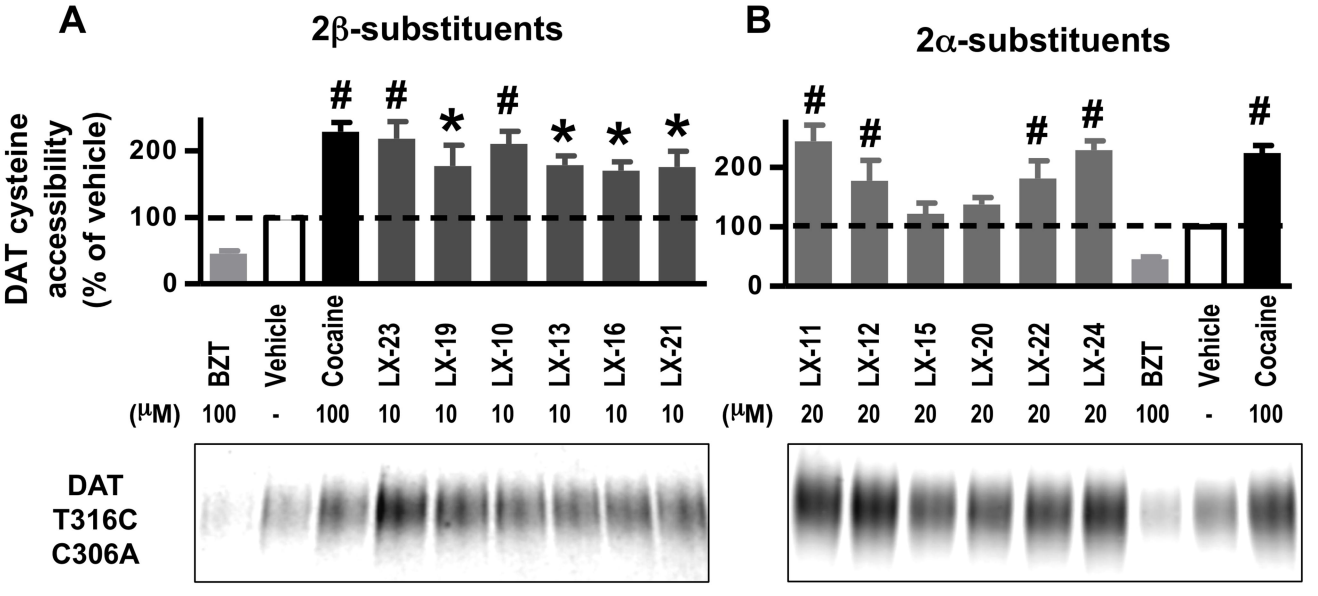
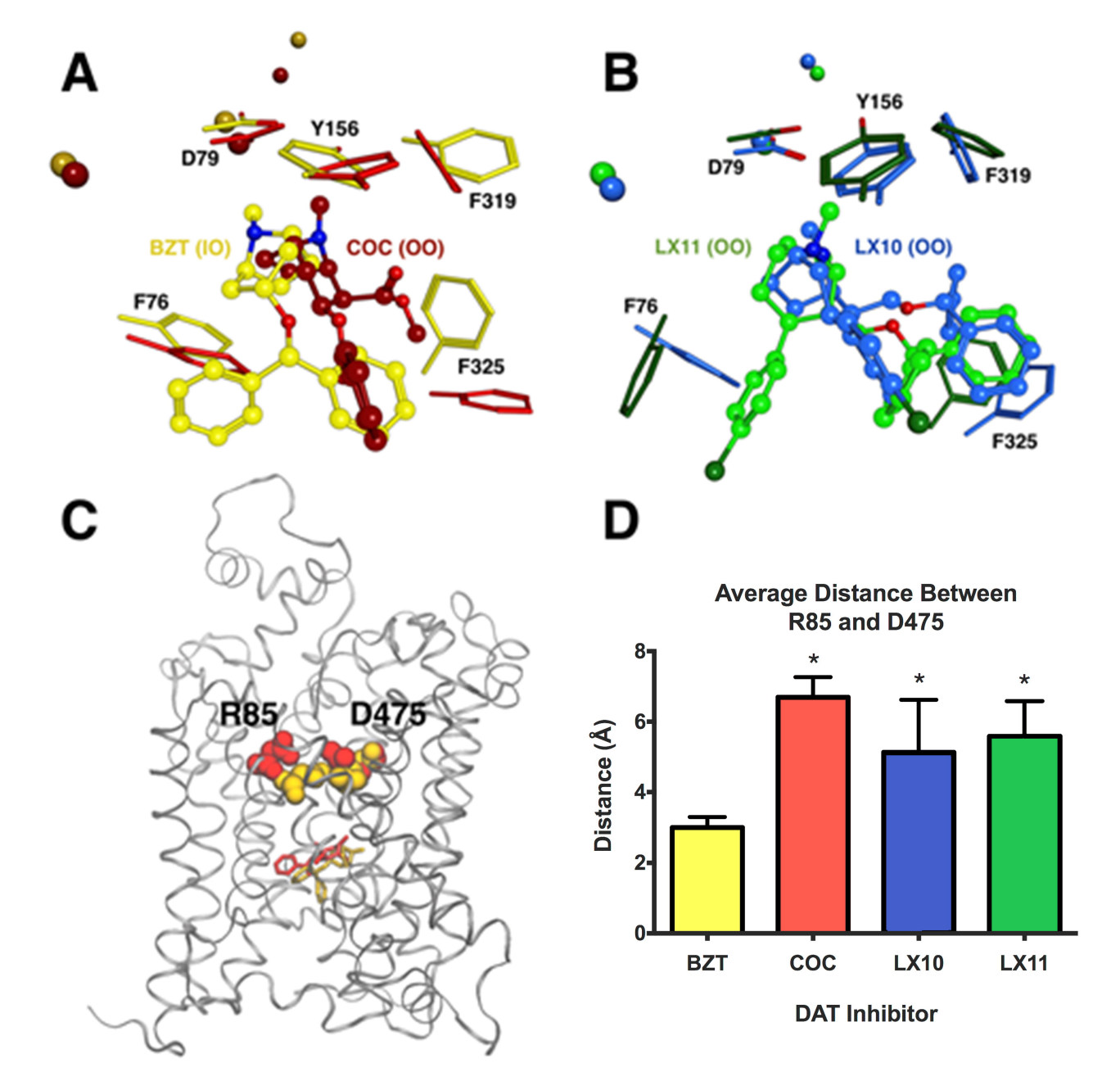

LX-13; R=CH3
LX-21; R=CH3; X=Y=F
LX-23; R=Cl; X=Y=F
% COCAINE RESPONDING
RESPONSE RATE (% CONTROL)
COCAINE DOSE (µmol/kg)
3.0
1.0
Sal.
1.7
100
80
60
40
20
0
275
250
225
200
175
150
125
100
75
50
25
0
1.0
3.0
10.0
30.0

Figure 5


A
2β-substituents
B
2α-substituents
DAT cysteine accessibility (% of vehicle)
200
100
0
#
*
BZT
Vehicle
Cocaine
LX-23
LX-19
LX-10
LX-13
LX-16
LX-21
LX-11
LX-12
LX-15
LX-20
LX-22
LX-24
(μM)
100
-
10
20
DAT
T316C
C306A


A
Y156
D79
F319
BZT (IO)
COC (OO)
F76
F325
B
Y156
D79
F319
LX11 (OO)
LX10 (OO)
F76
F325
C
R85
D475
D
Average Distance Between
R85 and D475
Distance (Å)
8
6
4
2
0
*
*
*
BZT
COC
LX10
LX11
DAT Inhibitor