1521-0103/366/3/527–540$35.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

https://doi.org/10.1124/jpet.118.250498
J Pharmacol Exp Ther 366:527–540, September 2018

The Journal of PHARMACOLOGY And Experimental Therapeutics

Dopamine Transporter Dynamics of *N*-Substituted Benztropine Analogs with Atypical Behavioral Effects[S]

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Received May 8, 2018; accepted June 22, 2018

ABSTRACT

Atypical dopamine transporter (DAT) inhibitors, despite high DAT affinity, do not produce the psychomotor stimulant and abuse profile of standard DAT inhibitors such as cocaine. Proposed contributing features for those differences include off-target actions, slow onsets of action, and ligand bias regarding DAT conformation. Several 3α-(4′,4′′-difluoro-diphenylmethoxy)tropanes were examined, including those with the following substitutions: *N*-(indole-3′′-ethyl)- (GA1-69), *N*-(*R*)-2′′-amino-3′′-methyl-*n*-butyl- (GA2-50), *N*-2′′aminoethyl- (GA2-99), and *N*-(cyclopropylmethyl)- (JHW013). These compounds were previously reported to have rapid onset of behavioral effects and were presently evaluated pharmacologically alone or in combination with cocaine. DAT conformational mode was assessed by substituted-cysteine accessibility and molecular dynamics (MD) simulations. As determined by substituted-cysteine alkylation, all BZT analogs except GA2-99 showed bias for a cytoplasmic-facing DAT conformation, whereas cocaine stabilized the extracellular-facing conformation. MD simulations suggested that several analog-DAT complexes formed stable R85-D476 "outer gate" bonds that close the DAT to extracellular space. GA2-99 diverged from this pattern, yet had effects similar to those of other atypical DAT inhibitors. Apparent DAT association rates of the BZT analogs in vivo were slower than that for cocaine. None of the compounds was self-administered or stimulated locomotion, and each blocked those effects of cocaine. The present findings provide more detail on ligand-induced DAT conformations and indicate that aspects of DAT conformation other than "open" versus "closed" may facilitate predictions of the actions of DAT inhibitors and may promote rational design of potential treatments for psychomotor-stimulant abuse.

Introduction

Discovery research for compounds with efficacy in treating stimulant abuse has focused on several classes of compounds with dopamine transporter (DAT) inhibitory action similar to that of cocaine, but without the effects of standard DAT inhibitors (Reith et al., 2015). These "atypical" DAT inhibitors are of basic interest as they run counter to the hypothesis that the effects of cocaine related to its abuse result from DAT binding (Kuhar et al., 1991). Much of the evidence for that hypothesis comes from studies of various compounds that inhibit the DAT and produce effects in laboratory animals similar to those of cocaine, with in vivo potencies that correspond to their DAT-binding affinities (Ritz et al., 1987; Bergman et al., 1989). The atypical DAT inhibitors are also of practical interest as potential leads for the development of treatments for stimulant abuse.

This work was supported by the National Institute on Drug Abuse, Intramural Research Program, [1ZIA DA000103-27], Extreme Science and Engineering Discovery Environment [XSEDE computational Grant MCB060069], and Butler University Faculty startup funds.

Parts of this study were presented previously: Hiranita T, Li LB, Hayashi S, Cao JJ, Newman AH, and Katz JL (2012) The stereotypy-inducing effects of atypical dopamine uptake inhibitors do not account for their blockade of cocaine self administration. *Annual Meeting at Experimental Biology*; 2012 Apr 21–25; San Diego, CA.

Wasko MJ, Madura JD, Hong WC, Katz JL, and Surratt CK (2017) In silico molecular dynamics analysis of dopamine transporter conformational preference as a function of occupancy with inhibitors of low abuse potential. *Annual Meeting at Experimental Biology*; 2017 Apr 22–26; Chicago, IL.

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https://doi.org/10.1124/jpet.118.250498.

[S] This article has supplemental material available at jpet.aspetjournals.org.

ABBREVIATIONS: ANOVA, analysis of variance; BZT, benztropine; CSF, cerebrospinal fluid; DAT, dopamine transporter; EXT, extinction; GA1-69, *N*-(indole-3′′-ethyl)-3α-(4′,4′′-difluoro-diphenylmethoxy)tropane HCl; GA2-50, *N*-(*R*)-2′′-amino-3′′-methyl-*n*-butyl-3α-(4′,4′′-difluoro-diphenylmethoxy) tropane HBr; FR, fixed ratio; GA2-99, *N*-2′′aminoethyl-3α-(4′,4′′-difluoro-diphenylmethoxy)tropane HBr; JHW013, *N*-(cyclopropylmethyl)-3α-(4′,4′′-difluoro-diphenylmethoxy)tropane HCl; MD, molecular dynamics; LED, light-emitting diode; PBS, phosphate-buffered saline; PBSCM, PBS with $CaCl_2$ and $MgCl_2$; PEO_2, polyethylene oxide; TM, transmembrane domain; WIN35,428, (−)-3β-(4-fluorophenyl)-tropan-2-β-carboxylic acid methyl ester tartrate; WT, wild type.

Among the atypical DAT inhibitors are analogs of benztropine (BZT) that bind the DAT with high affinity, but produce behavioral effects that substantially depart from those of cocaine (reviewed by Tanda et al., 2009a). For example, BZT analogs were less effective than cocaine in producing archetypical cocaine-like effects, such as stimulating nucleus accumbens dopamine (DA) levels (Tanda et al., 2005, 2009b), and were substantially less effective than cocaine in stimulating locomotor activity (Katz et al., 1999; Velázquez-Sánchez et al., 2009), substituting in rats trained to discriminate cocaine from saline injections (Katz et al., 1999, 2004), producing place conditioning (Li et al., 2005; Velázquez-Sánchez et al., 2009) or maintaining self-administration (Woolverton et al., 2000; Ferragud et al., 2009; Hiranita et al., 2009). BZT analogs block many of the behavioral effects of cocaine related to its abuse, including locomotor stimulation (e.g., Desai et al., 2005; Ferragud et al., 2009) and sensitization (Velázquez-Sánchez et al., 2013), place conditioning (Velázquez-Sánchez et al., 2009), and, importantly, cocaine or amphetamine or methamphetamine self-administration (Hiranita et al., 2009, 2014b; Velázquez-Sánchez et al., 2013).

Beuming et al. (2008) used the crystal structure of the bacterial leucine transporter (Yamashita et al., 2005) to model ligand binding to the DAT primary substrate pocket, S1. BZT analogs, as opposed to cocaine analogs, preserved a distance between Tyr156 and Asp79 that allowed hydrogen bonding between the two residues. That bond was suggested to close the S1 binding pocket in the presence of a substrate molecule, shielding the substrate from the extracellular space (Beuming et al., 2008). *Drosphila* DAT, crystallized in the presence of cocaine, showed a predominant outward-facing conformation (Wang et al., 2015). Studies of the binding of the radiolabeled BZT analog *N*-(*n*-butyl)-3α-[bis-(4′-fluorophenyl)methoxy]-tropane (JHW007) indicate that, as opposed to standard DAT inhibitors such as cocaine, its binding is insensitive to Na^+ (Kopajtic et al., 2010), suggesting again that DAT binding of BZT analogs differs from that of standard DAT inhibitors.

Pharmacological studies have suggested conformational changes in the DAT that are induced or stabilized by uptake inhibitors, consistent with the modeling studies. Loland et al. (2008) found that cocaine analogs were less potent as DA-uptake inhibitors in cells transfected with a Y335A human DAT (hDAT) mutant that assumes an inward (cytoplasmic)-facing conformation than in cells transfected with the WT hDAT. The potencies of BZT analogs in Y335A hDAT cells were relatively unchanged. A categorical relationship between the DAT conformation and the behavioral effects of the compounds was suggested in the Loland et al. (2008) study. However, a study of 3β-aryltropane DAT inhibitors with 2β- or 2α-diarylmethoxy substitutions found atypical behavioral effects despite cocaine-like changes in cysteine accessibility in a T316C/C306A hDAT mutant, indicative of outward-facing DAT conformations (Hong et al., 2016).

Li et al. (2011) reported several *N*-substituted BZT analogs (Fig. 1) that, despite DAT selectivity and nanomolar affinity, were substantially less effective than cocaine in producing cocaine-like behavioral effects. One hypothesis for atypical effects of DAT inhibitors is a slow onset of action, allowing compensatory mechanisms to blunt standard DAT-inhibitor effects. Another hypothesis suggests off-target effects of DAT inhibitors interfere with standard effects, with recent studies indicating that off-target sigma-receptor activity produced atypical DAT-inhibitor

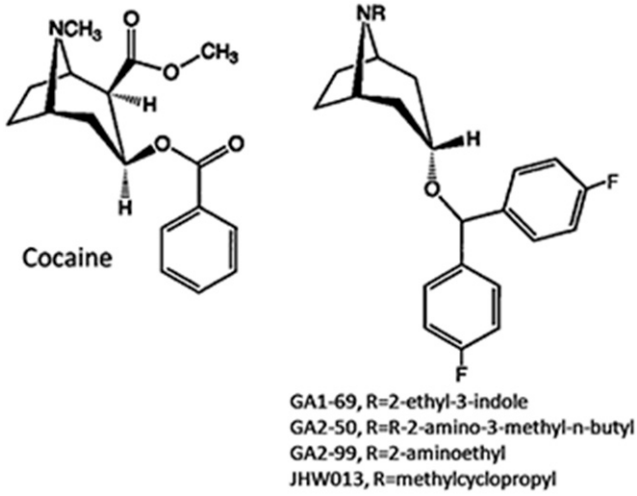

Fig. 1. Chemical structures of cocaine and the presently studied BZT analogs.

effects (Katz et al., 2017). However, the atypical *N*-substituted BZT analogs reported by Li et al. (2011) have rapid onsets of action, and two of the compounds have low sigma-receptor compared with DAT affinity. Therefore, the present study further examined effects of these compounds in combination with cocaine and assessed their molecular interactions with the DAT. Induced or stabilized DAT conformations were addressed using a cysteine-accessibility assay as well as molecular dynamics (MD) of in silico inhibitor-DAT complexes, suggesting how these compounds may yield their atypical effects.

Methods

All animals used in these studies were housed in a temperature- and humidity-controlled animal facility with a 12-hour light/dark cycle (lights on at 7:00 AM). Experiments were conducted during the light phase, and subjects were allowed to habituate to the animal facility for at least 1 week before experiments. Food and water were available at all times, except during experimental sessions and as indicated below. Experiments were conducted in accordance with National Institutes of Health Guidelines under protocols approved by the institutional Animal Care and Use Committee within a facility fully accredited by AAALAC International.

Ligand-Induced DAT Cysteine Accessibility. As previously described (Hong and Amara, 2010; Hiranita et al., 2014a; Hong et al., 2016), human embryonic kidney 293 cells were stably transfected with T316C/C306A hDAT. Confluent cells in 12-well plates 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 15 minutes at 4°C. Cells were then further incubated with 0.5 mg/ml maleimide-PEO_2-biotin (Pierce Biotechnology, Rockford, IL) in the presence of DAT inhibitors for 45 minutes at 4°C, followed by quenching with 100 mM cysteine in PBSCM for 15 minutes at 4°C. Cells were then washed, harvested, and lysed with lysis buffer (1% Triton X-100, NaCl 150 mM, EDTA 1 mM, Tris 10 mM, pH 7.5 and protease inhibitors). Biotinylated protein in the lysates was concentrated with 30 μl NeutrAvidin agarose beads (Pierce Biotechnology) overnight at 4°C. Following extensive washes of beads, biotinylated proteins were eluted with sodium dodecyl sulfate sample buffer, separated by polyacrylamide gel electrophoresis, and transferred to membranes for immunoblot with DAT antibody (MAB369; Millipore, Burlington, MA). Optical densities of chemiluminescent DAT bands were quantified using the National Institutes of Health ImageJ software (Bethesda, MD) and normalized to percent of vehicle

values. Because maleimide-PEO_2-biotin is membrane impermeable and thiol selective, it probes cysteine accessibility of mature, glycosylated DAT (~75 kDa) on the cell surface.

MD Simulation of DAT-Ligand Interactions. An hDAT homology model in the outward-facing conformation was constructed with Molecular Operating Environment (MOE) software (Chemical Computing Group, Montreal, Canada) using the *Drosophila* DAT (dDAT) crystal structure (4M48) (Penmatsa et al., 2013) as a template. The chemical structures of cocaine, BZT, GA1-69, and GA2-99 were initially created with ChemDraw (Perkin-Elmer, Bridgeville, PA) and then reconstructed as a 3D rendering and docked with MOE into the hDAT S1 binding pocket. The protein-ligand complex was embedded in a 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) lipid membrane and solvated with 0.15 M NaCl using High Throughput Molecular Dynamics (HTMD) software (Acellera Corp., Stanmore, UK). The Orientations of Proteins in Membranes (OPM) server (http://opm.phar.umich.edu) was used to define the angle of DAT within the POPC membrane. MD simulations were calculated using ACEMD software (Acellera), with the CHARM36 force field set at 310 Kelvin, with periodic boundary conditions using 4-fs time steps. Ligand parameters were defined using the CHARMM General Force Field (CGenFF) program through the ParamChem server (https://cgenff.paramchem.org). Each system was minimized for 2 ps and equilibrated for 40 ns, followed by 400 ns of MD simulation. Data analysis was completed using Visual Molecular Dynamics (VMD) software (University of Illinois, Urbana-Champaign, IL). MD traces measured the distance in Angstroms between the TM1-TM10 salt bridge residues (R85-D476) that contribute to the DAT outer gate between the S1 pocket and the extracellular space (Pedersen et al., 2014). Choosing the most distal nitrogen and oxygen atoms for each side chain, an average R85-D476 distance was calculated from the four distances. This minimized misleading distance changes due to side chain rotation. The average R85-D476 distance was plotted over the course of the simulation (time, nanosecond).

In Vivo Displacement of [^{125}I]RTI-121. Swiss-Webster male mice (Taconic Farms, Germantown, NY) weighing 20–25 g were injected (i.v.) with 2 μCi of [^{125}I]RTI-121 followed 2 hours later, at the time of its maximal DAT occupancy (Scheffel et al., 1989; Lever et al., 1996), by cervical dislocation and tissue harvest. Whole brains were rapidly removed, and striatum and cerebellum were dissected on ice. After dissection, each brain region was placed into separate plastic 55 × 12 mm vials (Rohren Tubes; Sarstedt AG, Nümbrecht, Germany) and weighed, and tissue radioactivity was measured using an automated gamma counter (Micromedic Systems, 10/600 PLUS; ICN Biomedicals Inc., Santa Ana, CA). Test compounds (GA1-69, GA2-50, GA2-99, JHW013) were assessed as displacers of [^{125}I]RTI-121 after injection at various doses (based on Li et al., 2011) and times relative to tissue harvest. Each data point was determined typically in sets of three to seven mice.

For the analysis of in vivo binding data, regional radioactivity levels (counts per min, cpm) were divided by gram weight of the tissue (cpm/tissue weight). Specific binding was calculated as counts per minute/tissue weight in striatum divided by that in cerebellum minus 1 (S/C–1), which is based on the observation that DAT molecules are highly concentrated in the striatum and relatively absent in the cerebellum (Scheffel et al., 1989). These values were expressed as a percentage of specific binding after vehicle injection. Data were analyzed using two-way analysis of variance (ANOVA) and post hoc Tukey's tests to determine significance of effects for individual doses and time periods. The data for the effects of cocaine were collected contemporaneously with the present data, but have been reported previously (Hiranita et al., 2014a) and are included in this report for comparison.

Locomotor Activity and Stereotypy. Experimentally naive Swiss-Webster male mice (Charles-River Laboratories, Raleigh, NC) or Sprague-Dawley rats (Taconic Farms) were placed singly in clear acrylic chambers (40 cm^3) contained within monitors (Accuscan Instruments, Inc., Columbus, OH) that were equipped with light-sensitive detectors. The detectors were spaced 2.5 cm apart along two perpendicular walls with infrared light sources mounted on the opposing walls and directed at the detectors. Activity counts were registered for each interruption of a light beam. Mice were injected twice (intraperitoneally, i.p., in volumes of 1 ml/100 g) and immediately placed in the chambers for 60 minutes. Rats were placed in the chambers for 60 minutes before injection to allow habituation to the chamber. The data collection started 5 minutes after injection of the rats, with activity counts cumulated for each 10-minute period. As the effects of cocaine, WIN35,428, and each of the BZT analogs (GA1-69, GA2-50, GA2-99, JHW013) have their most pronounced effects immediately after injection (Li et al., 2011), only the first 30 minutes are reported; however, there were no substantial differences in conclusions based on the first 30 minutes or the entire 60-minute period. Each dose or dose combination was studied in six subjects. WIN35,428 was not studied in mice. Activity counts are presented as group means (± S.E.M.) and statistical significance of results was assessed using two-way ANOVA for repeated measures with cocaine and test compound doses as factors. Significant results were followed by post hoc Tukey's tests, and changes were considered significant when $P < 0.05$.

Visual observations of subjects were conducted during automatic recording of locomotor activity in rats. Behavior of each subject was observed for 1 minute, every 10 minutes over the course of 60 minutes. Scoring of the observations was conducted by observers blind to treatment conditions according to a modification of a published scale to identify stereotypy (Kalivas et al., 1988). The scoring was as follows: 1) asleep or still; 2) grooming (any kind of grooming, scratching, or licking for more than 3 consecutive seconds); 3) locomotion (horizontal movement of greater than half of body length during 10 seconds), rearing (both forepaws raised from the cage floor), or sniffing (more than 3 consecutive seconds); 4) any combination of two: locomotion, rearing, or sniffing; and 7.5) head bobbing. Modification of the previous scale was made based on the current frequencies and dose-dependencies of the observed behavioral categories and designed to correspond closely by definition and outcome to the original published scale. The observers had previous extensive training with regard to recognition of the behavioral categories but were blind to treatments. Stereotypy scores were presented as group means (± S.E.M.), because this treatment, despite the nominal nature of the data, has proven robust under these mathematical operations.

Cocaine Discrimination. Experimentally naive male Sprague-Dawley rats (Charles River Laboratories) were individually housed and maintained at 325–350 g by controlled daily feedings that occurred at least 1 hour after sessions. Sessions were conducted at the same time daily, with subjects placed in 29.2 × 24.2 × 21 cm operant-conditioning chambers (modified ENV-001; Med Associates, Fairfax, VT) containing two response keys (levers requiring a downward force of 0.4 N) with pairs of green and yellow light-emitting diodes above each. A dispenser delivered 45-mg food pellets (BioServ, Flemington, NJ) to a tray located between the response keys. A light mounted near the ceiling provided overall illumination. The chamber was contained within a sound-attenuating, ventilated enclosure that was supplied with white noise to mask extraneous noise.

Rats were initially trained with food reinforcement to press both levers and were subsequently trained to press one lever after cocaine (10 mg/kg, i.p.) and the other after saline (i.p.) injection. Responses always produced an audible click. The ratio of responses to food pellets (fixed ratio or FR) was gradually increased until, under the final conditions, the completion of 20 consecutive responses on the cocaine- or saline-appropriate lever produced food. The right versus left assignments of cocaine and saline keys were counterbalanced among subjects. Subjects were injected and placed in chambers with the session proper starting after a 5-minute time-out period during which lights were off and responses had no scheduled consequences. Following the time-out, the house light was turned on until completion of the FR 20-response requirement and the presentation of food. Sessions ended after 20 food presentations or 15 minutes, whichever occurred first, and were conducted 5 days/week, with cocaine or saline sessions scheduled in a double-alternation sequence.

Testing of GA1-69, GA2-50, GA2-99, and JHW013 was initiated after subjects met the criteria on four consecutive sessions of at least 85% cocaine- or saline-appropriate responding (two sessions of each) over the entire session and the first FR. Test sessions were conducted with the pre-session administration of different doses of cocaine, or the *N*-substituted BZT analogs. Test sessions were identical to training sessions with the exception that 20-consecutive responses on either lever were reinforced. Mean values for overall rates of responding on both keys and the percentage of responses emitted on the cocaine-appropriate key were calculated.

Drug Self-Administration. Male Sprague-Dawley rats (Taconic Farms) surgically prepared with venous catheters were maintained at approximately 320 g ($N = 12$) throughout the study. Food (Scored Bacon Lover Treats; Bioserv, Flemington, NJ) and tap water were available in their home cages with daily food rations adjusted to maintain individual body weights at 320 g. Subjects were placed in 25.5 × 32.1 × 25.0 cm operant-conditioning chambers (modified ENV-203; Med Associates, Fairfax, VT) which were enclosed within sound-attenuating cubicles equipped with a fan for ventilation and white noise to mask extraneous sounds. A syringe pump (Model 22; Harvard Apparatus, Holliston, MA) was placed above each sound-attenuating enclosure for delivery of injections from a 10-ml syringe. The syringe was connected to the subject's catheter by Tygon tubing through a single-channel fluid swivel (375 Series Single Channel Swivels, Instech Laboratories, Plymouth Meeting, PA) balanced above the chamber. The tubing from the swivel to the subject's catheter was protected by a surrounding metal spring.

Sessions were conducted daily, during which subjects were initially trained to press the right lever with food reinforcement (45-mg food pellets; Bioserv) under an FR five-response schedule of reinforcement (each fifth response produced a food pellet). Once pressing was reliable, catheters were surgically implanted into the jugular veins and subjects were allowed 7 days of recovery before cocaine self-administration studies were initiated. One group of subjects continued with food reinforcement and without surgery.

Cocaine self-administration or food reinforcement sessions were conducted in 2-hour daily sessions, during which three light-emitting diodes (LEDs) above the right lever were illuminated when food or cocaine injections were available. Each fifth response turned off the LEDs and activated the infusion pump (1.0 mg/kg) or the food-pellet dispenser with a following 20-second time-out period, during which LEDs were off and responding had no scheduled consequences. After the time out, the LEDs were illuminated and responding again had scheduled consequences. The experimental sessions were divided into five 20-minute components, each preceded by a 2-minute time-out period, in which different doses or amounts of food were available. The doses and order were as follows: no injection (also referred to as extinction, or EXT, as responses had no scheduled consequences), 0.03, 0.10, 0.32, and 1.0 (mg/kg)/injection. The food amounts were as follows: EXT (no food), 1, 2, 3, and 4 pellets (45 mg each). A sample injection of cocaine or food delivery at the corresponding dose or amount occurred independently of responding at the start of each component. Subjects studied with food reinforcement were given their daily (~15 g) ration of food 60 minutes before sessions to ensure that response rates approached those maintained by cocaine.

Training continued until performances were stable from one session to the next. The effects of substitution of a progression of doses of WIN35,428, GA1-69, GA2-50, GA2-99, and JHW013 for cocaine or pre-session treatments with those compounds were separated by at least 72 hours and were conducted only if performances were stable. Tests were conducted with a mixed order of drugs and doses, which were administered 5 minutes before sessions.

The response rates were calculated by dividing the total responses by the elapsed time during components (excluding timeout periods). The statistical significance of effects on response rates was assessed by one- or two-way (as appropriate) repeated-measures ANOVA, with post hoc Tukey's tests.

Compounds. The compounds studied were cocaine HCl (Sigma-Aldrich, St. Louis, MO), WIN35,428 (National Institute on Drug Abuse, Drug Supply Program), BZT mesylate, and several *N*-substituted analogs of BZT (Fig. 1): GA1-69: *N*-(indole-3′′-ethyl)-3α-(4′,4′′-difluoro-diphenylmethoxy)tropane HCl; GA2-50: (R)-2′′-amino-3′′-methyl-n-butyl-3α-(4′,4′′-difluoro-diphenylmethoxy)tropane HBr; GA2-99: *N*-2′′aminoethyl-3α-(4′,4′′-difluoro-diphenylmethoxy)tropane HBr; and JHW013: *N*-(cyclopropylmethyl)-3α-(4′,4′′-difluoro-diphenylmethoxy)-tropane HCl. The synthesis of these analogs was conducted in the Medicinal Chemistry Section of the National Institute on Drug Abuse Intramural Research Program and has been described previously (Agoston et al., 1997; Robarge et al., 2000). All compounds were dissolved in distilled water with heat and sonication as necessary, except cocaine, which was dissolved in saline. All solutions were administered i.p. at 1 ml/kg, except GA2-50, which was given at 2 and 3.4 ml/kg at the higher doses due to solubility limitations. GA1-69 was initially dissolved with 5% DMSO/5% Tween 80 (or for studies involving intravenous injection dissolved with lactic acid) and diluted with distilled water.

Results

Ligand-Induced DAT Cysteine Accessibility. We showed previously that binding of cocaine and BZT had opposite effects on the sulfhydryl side chain accessibility of the T316C/C306A hDAT construct, a DAT conformation sensor that retains DA uptake and inhibitor binding (Hiranita et al., 2014a). Here, effects of saturating concentrations (>100-fold higher K_i values) of the four *N*-substituted BZT analogs were compared with those of vehicle, BZT, and cocaine (Fig. 2A). Maleimide-PEO_2-biotin (membrane impermeant, thiol selective) was used to probe cysteine accessibility of mature, glycosylated DAT (~75 kDa) on the cell surface. Because labeling was done at 4°C to prevent membrane trafficking of DAT, changes in accessibility reflected the degree of exposure of T316C to the extracellular milieu and not changes in expression levels of DAT on cell surface. Cocaine significantly increased DAT labeling compared with vehicle, suggesting a stabilization of the DAT in the outward-facing conformation. Furthermore, evidence of outward-facing conformation was also obtained with the standard DAT inhibitor, WIN35,428 (Supplemental Fig. 1). In contrast, BZT, GA1-69, GA2-50, and JHW013 significantly decreased T316C accessibility, suggesting a stabilization of inward-facing DAT. At the concentrations studied, GA2-99 did not significantly register a DAT conformation preference.

Known structures of DAT homologs further support the interpretation of these biochemical data. Although the precise molecular architecture of hDAT when assuming inward- or outward-facing conformations remains unknown, atomic structures of ligand-free inward-facing $LeuT_{Aa}$ (a bacterial homolog of hDAT) and cocaine-bound outward-facing dDAT have been elucidated (Krishnamurthy and Gouaux, 2012; Wang et al., 2015, PDB codes 3TT3 and 4XP4, respectively). The two structures exhibit substantial conformational rearrangement on several key domains, including transmembrane domain 6a (TM6a), which contains T316. Based on sequence alignment, G249 in $LeuT_{Aa}$ corresponds to T316 in hDAT, and it is located inside the protein and not accessible from the extracellular side in the inward-facing structure (Fig. 2B). In contrast, the corresponding residue T315 in outward-facing dDAT is exposed on the surface of a vestibule accessible from the extracellular side (Fig. 2C). Crystal structure of WIN35,428-bound dDAT (Wang et al., 2015, PDB code 4XPG) also shows an outward-facing conformation. In the ligand

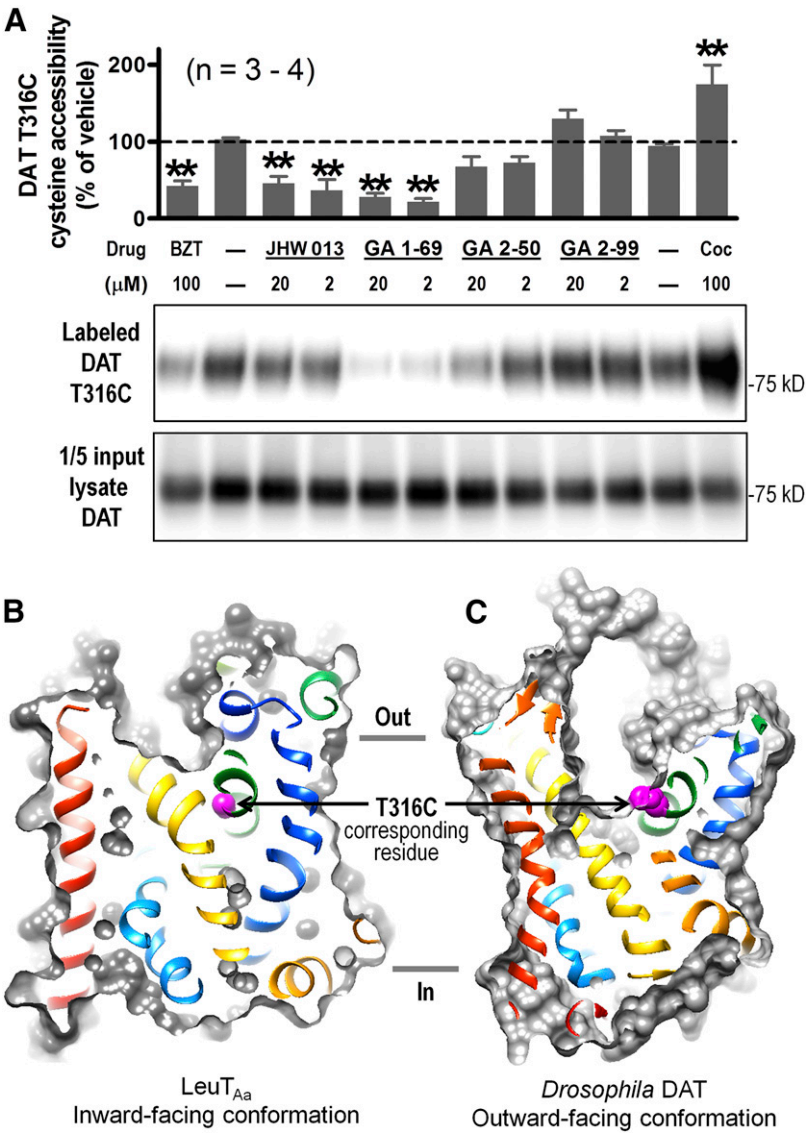

Fig. 2. Modulation of hDAT T316C/C306A cysteine accessibility by BZT analogs. (A) Human embryonic kidney 293 cells expressing T316C/C306A hDAT were incubated with drugs at 4°C for 15 minutes, followed by incubation with drugs at 4°C for 45 minutes in the presence of maleimide-PEO_2-biotin, a membrane-impermeant, thiol-selective probe that reacts with accessible cysteine residues in cell surface proteins. After cell lysis, labeled DAT proteins were enriched with NeutrAvidin beads, separated by SDS-PAGE, and detected by immunoblotting (see *Methods* for details). Summarized results (average ± S.E.M., top panel) are from $n = 3$ to 4 experiments, with a representative immunoblot showing labeled DAT (~75 kDa), which is glycosylated on the cell surface (middle panel). Quantified DAT band densities were normalized to vehicle treatment. $^{**}P < 0.01$, compared with vehicle, one-way ANOVA with post hoc Dunnett's test. DAT proteins in cell lysates were of approximately the same amount (bottom panel). "Coc" represents cocaine. (B and C) Structure cartoons of inward-facing $LeuT_{Aa}$ and outward-facing dDAT (PDB codes 3TT3 and 4XP4, respectively), generated using UCSF Chimera 1.12 software. Both show cross-section surface representations with the residue corresponding to T316C in hDAT (G249 in $LeuT_{Aa}$; T315 in dDAT) in focus (highlighted in magenta). In the outward-facing conformation, the residue is exposed on the surface of a vestibule accessible from the extracellular side; it is buried inside the protein in the inward-facing conformation. TMs are colored in a rainbow pattern from blue to red, with TM12 (red) aligned to the left.

binding pocket, the two standard DAT inhibitors, cocaine and WIN35,428, adopt very similar poses (Supplemental Fig. 2). Previously we showed maleimide-PEO_2-biotin does not react with C90 in hDAT (Hong and Amara, 2010). The residue corresponding to C90 also does not appear to be exposed on the protein surface in either of these structures. Therefore, varying degrees of labeling of DAT at T316C suggested that cocaine or BZT analogs displayed differential propensities to stabilize outward- or inward-facing conformations of DAT.

Molecular Dynamics Simulation of Inhibitor-DAT Complexes. Cocaine, BZT, GA1-69, or GA2-99 was docked in the S1 binding pocket (Fig. 3) of an hDAT homology model in its outward-facing conformation. A 400-ns MD simulation was performed to identify ligand positioning and possible DAT conformational changes due to ligand binding. Whether the outer gate was closed was assessed by measuring the distance between distal nitrogen and oxygen atoms of outer gate salt bridge side chains R85 (TM1) and D476 (TM10) (Pedersen et al., 2014). An intact bridge (a direct ionic bond between the side chains) and therefore a closed gate would be reflected by a distance of 3 to 4 Å on the MD trace. Greater distances between the side chains should indicate an open outer gate and, presumably, exposure of the TM6 alkylation target T316C to the extracellular side of the bilayer, which was observed for MD simulations with cocaine and GA2-99 (Fig. 3, top row). The R85-D476 distance fluctuated throughout the cocaine-DAT simulation, whereas this distance was more consistent and averaged approximately 6 to 7 Å during GA2-99-DAT simulations. Distances consistent with an intact salt bridge (closed gate) were observed in the BZT and GA1-69 simulations beyond the 200-ns mark (Fig. 3, top row).

In Vivo Displacement of [^{125}I]RTI-121. Cocaine produced a dose- and time-dependent displacement of [^{125}I]RTI-121 in striatum (Fig. 4; top left). Maximal displacement of approximately 80% of specific [^{125}I]RTI-121 binding in striatum was obtained at 45–60 minutes after injection of the highest dose studied, 40 mg/kg. ANOVA showed a significant effect of dose ($F_{3,35} = 107$; $P < 0.0001$) and time ($F_{3,35} = 25.1$; $P < 0.0001$), and a significant interaction of the two ($F_{9,35} = 3.15$; $P = 0.007$). At the highest dose, cocaine displaced at a rate of 1.96 (±0.168, S.E.M.) %/min of [^{125}I]RTI-121 specific binding.

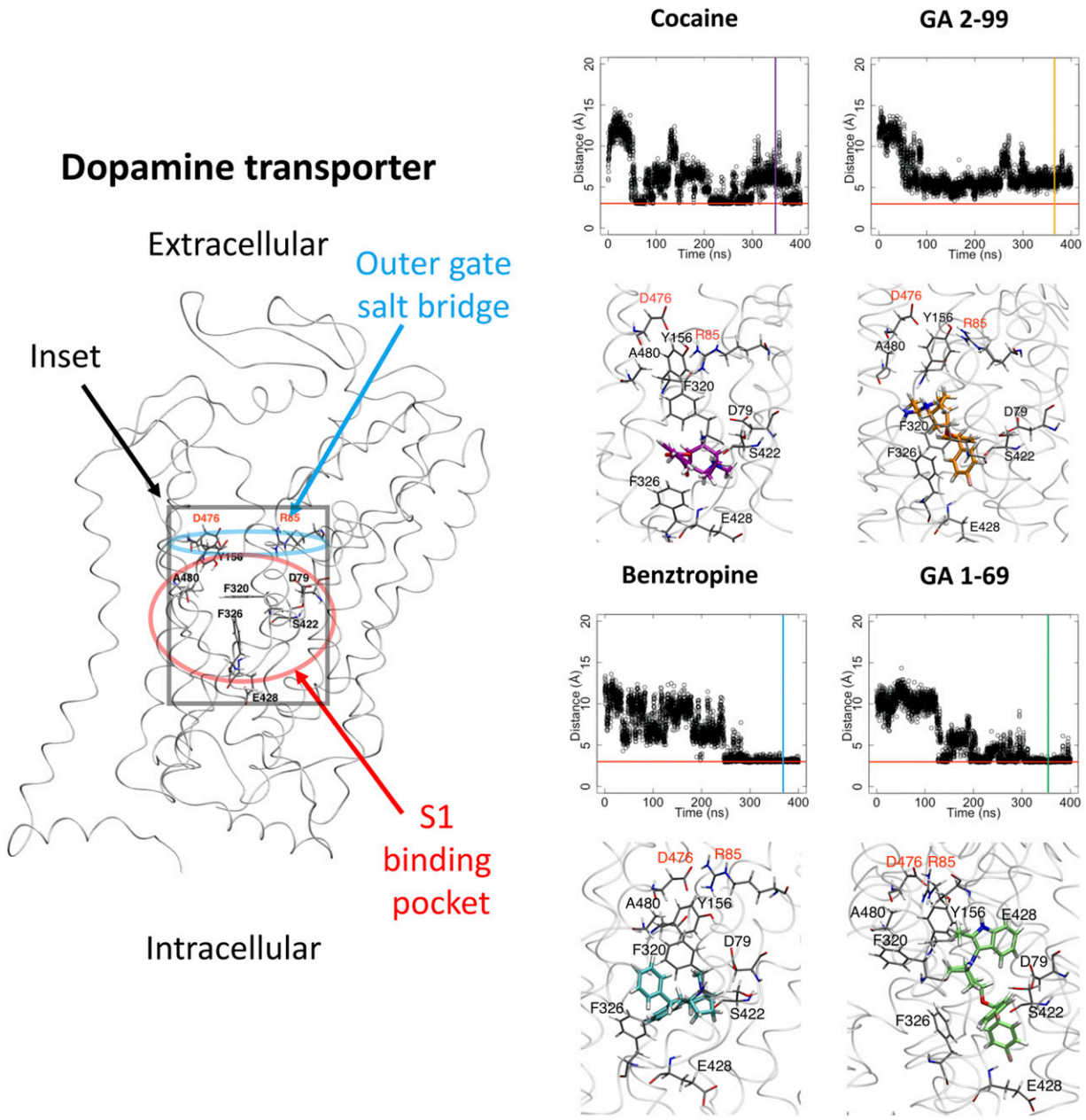

Fig. 3. Molecular dynamics (MD) of inhibitor-DAT complexes. Leftmost column: Side view of hDAT computational model featuring the approximate S1 ligand binding pocket (inscribed by orange oval) and R85-D476 salt bridge strut of the outer gate. Remaining columns: Top row: MD traces (n = 2–4) of representative simulations of each of four inhibitors docked to the DAT model. Ordinate indicates average distance between the outer gate R85 terminal nitrogen atoms and D476 carboxylate oxygen atoms as a function of simulation time point. Average distance for an intact salt bridge (~3 Å) is represented by the red horizontal bar. A vertical line after 300 ns and color-coded by inhibitor ligand indicates the time point at which "snapshot" poses were taken. Bottom row: Side view snapshot poses of DAT MD simulations involving cocaine (purple), GA2-99 (gold), BZT (cyan), or GA1-69 (green). For each pose, nearby surrounding S1 pocket side chains are shown (atomtype color), with the outer gate R85 and D476 residues annotated in red.

The BZT analog, GA1-69, also displaced [^{125}I]RTI-121 in striatum in a dose- and time-dependent manner (Fig. 4; top right). Maximal displacement within the range of doses and times studied was less than that for cocaine, ~26% of specific [^{125}I]RTI-121 binding in striatum at 30 minutes after injection of the 10 and 17 mg/kg doses. Whereas the effect of dose was statistically significant ($F_{3,25} = 7.83$; $P < 0.001$), the effect of time was not ($F_{2,25} = 0.77$; $P = 0.474$). At the two highest doses, GA1-69 displaced at a rate of 1.00 (±0.163) %/min and 0.886 (±0.202) %/min of [^{125}I]RTI-121 specific binding.

Displacement of [^{125}I]RTI-121 in vivo by GA2-50 depended on dose and time, with approximately 40% of specific [^{125}I]RTI-121 binding in striatum obtained at 30 minutes after injection of 56 mg/kg. A higher dose produced greater displacement of [^{125}I]RTI-121 at 5 and 10 minutes after injection but also produced lethality between 10 and 30 minutes after injection and, consequently, was not studied further. The ANOVA showed significant effects of dose ($F_{4,63} = 11.9$; $P < 0.0001$) and time ($F_{4,63} = 10.4$; $P < 0.0001$), although no significant interaction of the two ($F_{16,63} = 1.25$; $P = 0.256$). At the 56 mg/kg dose, GA2-50 displaced at a rate of 1.50 (±0.189) %/min of [^{125}I]RTI-121

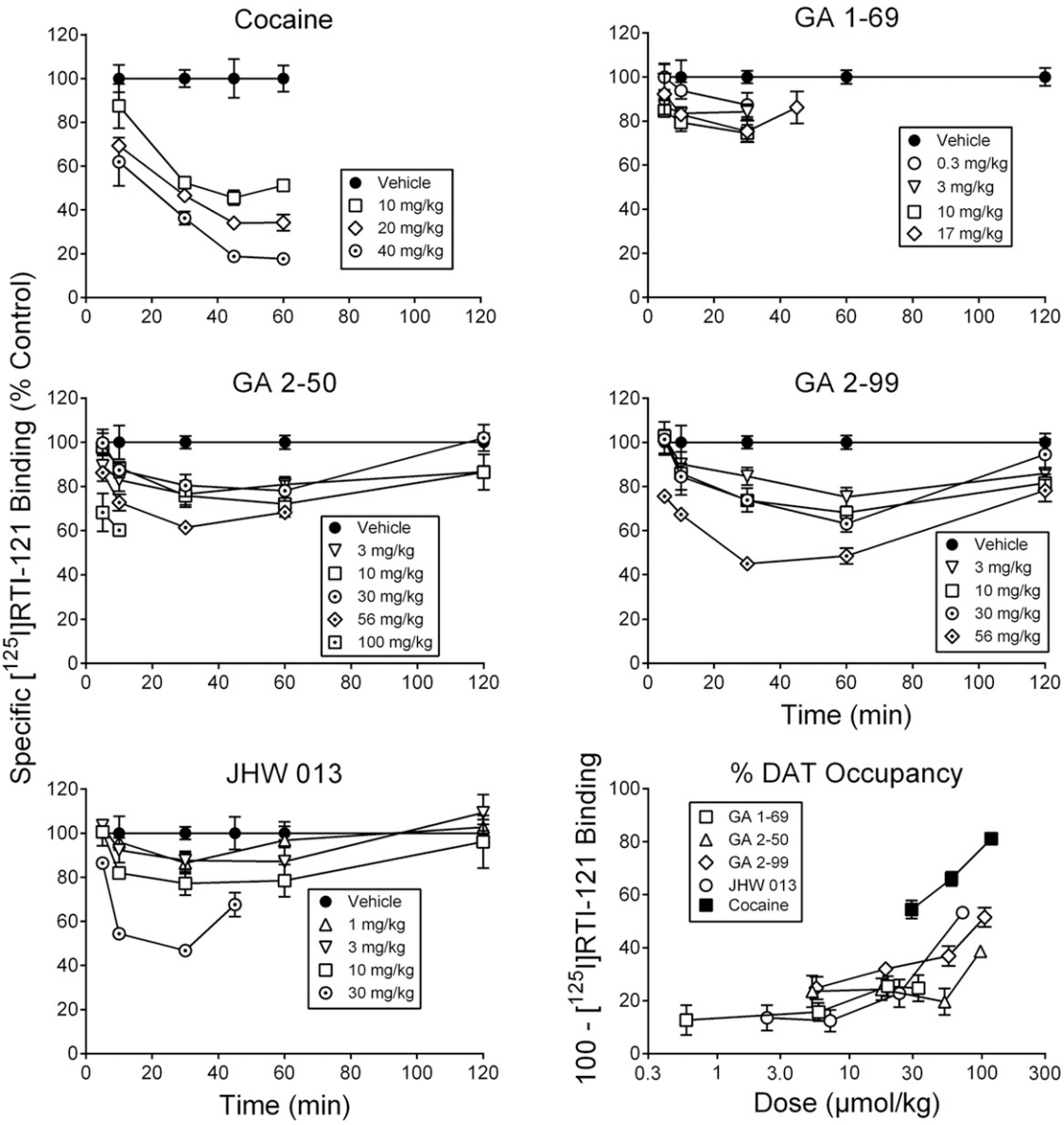

Fig. 4. In vivo displacement of specific [^{125}I]RTI-121 accumulation in mouse striatum at various times following intraperitoneal injection of cocaine, GA1-69, GA2-59, GA2-99, and JHW013. Ordinates: specific [^{125}I]RTI-121 binding as a percentage of that obtained after vehicle injection. Abscissae: time. For each point, the number of replicates was typically from three to seven. The bottom right panel shows maximal occupancy of [^{125}I]RTI-121 binding sites as a function of dose. Ordinates, %DAT occupancy. Abscissae, drug dose in micromoles per kilogram. Note that maximal displacement of [^{125}I]RTI-121 was obtained at different time points after injection. Those times were for cocaine, 45 minutes at 40 mg/kg; GA1-69, 30 minutes at 17 mg/kg; GA2-50, 10 minutes at 100 mg/kg; GA2-99, 30 minutes at 56 mg/kg; and JHW013, 30 minutes at 30 mg/kg.

specific binding. At the 100 mg/kg dose, GA2-50 displaced [^{125}I] RTI-121 specific binding at a higher rate, although only two points provided this value.

As with the other compounds, GA2-99 and JHW013 both displaced [^{125}I]RTI-121 binding in a manner related to dose and time. Maximal displacement of [^{125}I]RTI-121 binding produced by each of these two compounds was 55% (GA2-99) or 53% (JHW013), values greater than those for the other two *N*-substituted BZT analogs. The statistical analyses for the two drugs indicated significant effects of dose ($F_{4,61}$ = 32.7, $F_{3,36}$ = 6.82, respectively; P values < 0.001) and time ($F_{4,61}$ = 18.5, $F_{3,36}$ = 4.09, respectively; P values < 0.05). At the highest dose, GA2-99 displaced at a rate of 1.12 (±0.176) %/min of [^{125}I]RTI-121 specific binding. In contrast, JHW013 displaced at a rate of 2.07 (±0.301) %/min of [^{125}I]RTI-121 specific binding.

The bottom right panel of Fig. 4 shows DAT occupancy produced by the four compounds derived from the times at which the greatest behavioral effects were obtained. Each of the compounds had dose-related effects, with those of cocaine greater than the other compounds across the range of doses studied. A plateau in DAT occupancy was obtained with GA1-69 at approximately 25%, an amount that is the lowest among the compounds. Both GA2-99 and JHW013, at their highest doses, reached levels of occupancy comparable to that produced by 10 mg/kg cocaine, whereas GA2-50 at the highest dose tested produced less DAT occupancy.

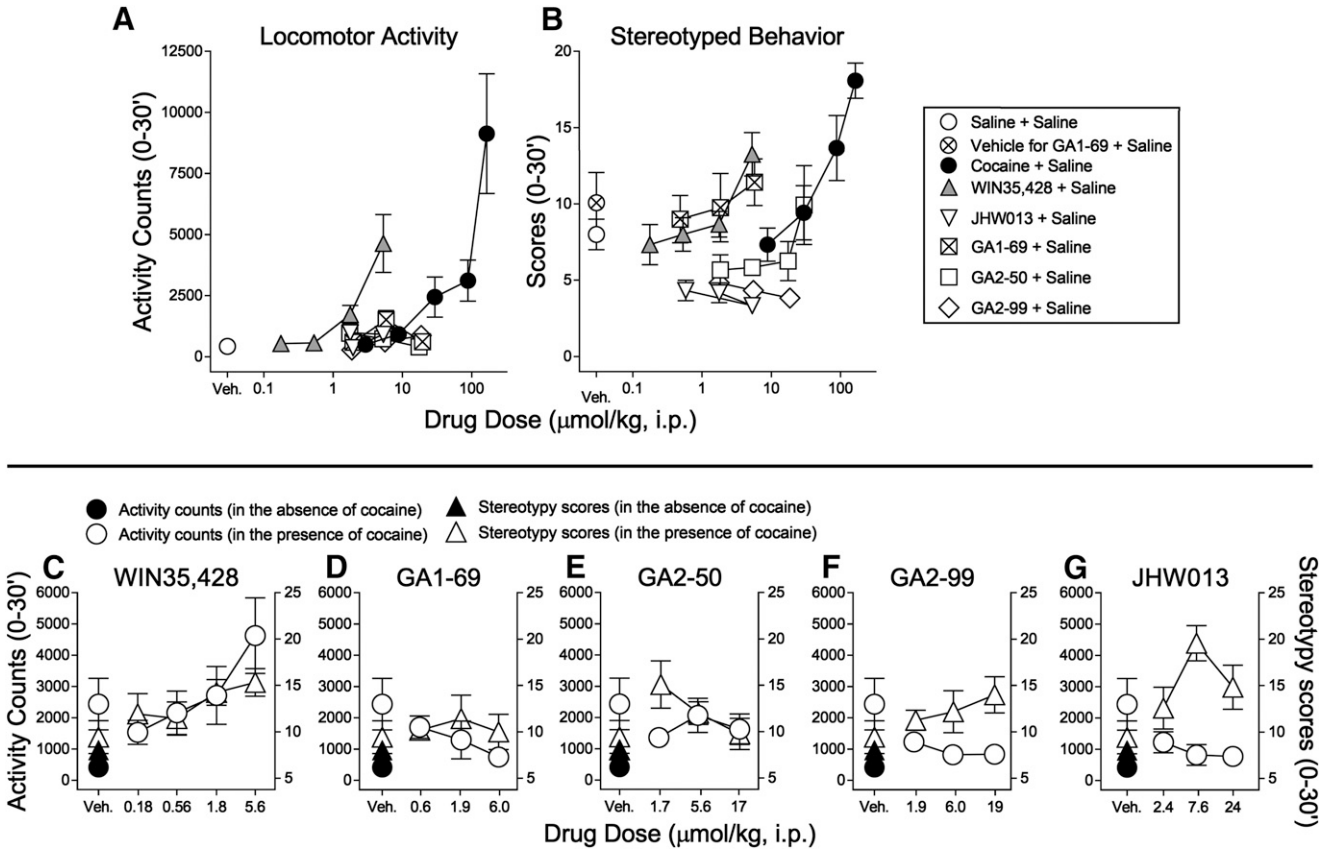

Fig. 5. Effects of *N*-substituted BZT analogs on locomotor activity and stereotyped behavior in rats. (A) Effects on horizontal locomotor activity. Abscissae: drug dose in micromoles per kilogram, log scale; Ordinates: horizontal locomotor activity counts per minute over a 30-minute period after drug administration. Each point represents the average effect determined in six subjects. (B) Effects on stereotypy determined as described in the *Methods*. Abscissae: drug dose in micromoles per kilogram, log scale; Ordinates: stereotypy score determined over a 30-minute period after drug administration. Each point represents the average effect determined in six subjects. (C–G) Effects of combinations of BZT analogs or WIN35,428 with cocaine. Abscissae: dose of BZT analog or WIN35,428 (log scale) alone (filled symbols) or in combination with a 29.4 μmol/kg (10 mg/kg) dose of cocaine (open symbols) that produced intermediate changes in locomotor activity or stereotypy. Ordinates: horizontal locomotor activity counts per minute (circles) or stereotypy score (triangles) determined over a 30-minute period after drug administration. Vertical bars represent the S.E.M. The data are from the 30-minute period immediately after drug administration. Note that none of the BZT analogs produced a stimulation of activity or stereotypy that was equivalent to that of cocaine and that all but GA2-50 blocked the stimulation of activity produced by cocaine, whereas none of the compounds blocked cocaine-induced stereotypy.

Locomotor and Stereotyped Behavior. Cocaine produced dose-related increases in locomotor activity in rats during the first 30 minute after injection (Fig. 5A, filled circles). The maximal effects of cocaine were obtained at 165 μmol/kg (56 mg/kg); higher doses were not tested. Similar effects were obtained with the 10-fold more potent cocaine analog, WIN35,428, though at its highest tested dose the maximal effects were less than those obtained with cocaine (Fig. 5A, filled triangles). The effects of dose on locomotor activity levels produced by both drugs were significant ($F_{5,30}$ = 8.79, $F_{4,25}$ = 9.96, respectively, P values < 0.001). In contrast, none of the N-substituted BZT analogs over the range of doses studied comparably increased activity (Fig. 5A). However, the small increases with doses of GA1-69 were statistically significant ($F_{3,20}$ = 5.59, $P < 0.05$). There were no significant increases in activity produced by these compounds in the second 30 minutes after injection (data not shown).

Cocaine also increased the incidence of stereotypy signs, producing a dose-related increase in stereotypy scores (Fig. 5B, filled circles). The maximal effects of cocaine were obtained at 165 μmol/kg (56 mg/kg), the highest dose tested. Similar effects were obtained with the 10-fold more potent cocaine analog, WIN35,428 (Fig. 5B, filled triangles), although as with locomotor activity, the highest dose of WIN35,428 produced maximal effects that were less than those obtained with cocaine. In contrast to effects obtained with cocaine and WIN35,428, none of the *N*-substituted BZT analogs over the range of doses and times studied increased stereotypy scores (Fig. 5B) above values obtained with the corresponding vehicle control.

The effects of increasing doses of each compound in combination with 29.4 μmol/kg (10 mg/kg) cocaine are shown in the bottom portion of Fig. 5. That dose of cocaine was selected for studies of drug combinations, as it produced only a moderate enhancement of both locomotion and stereotypy (compare filled, without cocaine, and open, with cocaine, symbols above "Veh.") and would therefore be suitable for demonstrations of diminished or enhanced effects of cocaine. WIN35,428 produced a dose-related enhancement of locomotor stimulation ($F_{4,50}$ =6.79. $P < 0.001$) and stereotypy induced by cocaine (Fig. 5C). In contrast, all BZT analogs (Fig. 5, D, F, and G) except GA2-50 (Fig. 5E) dose dependently decreased the locomotor-stimulant effects of cocaine. Over the same range of doses, all BZT analogs except GA1-69 at some doses enhanced the effects of cocaine on stereotypy scores,

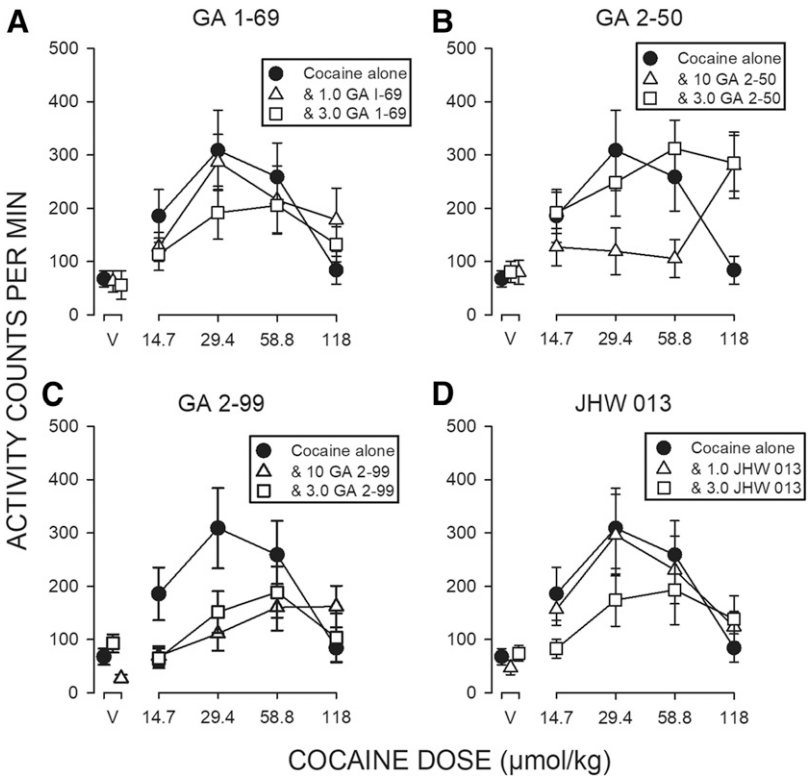

Fig. 6. Dose-dependent interactions of *N*-substituted BZT analogs with cocaine on locomotor activity in mice. Ordinates: horizontal locomotor activity counts after drug administration. Abscissae: dose of cocaine in milligrams per kilogram, log scale. Each point represents the average effect determined in six mice. Vertical bars represent the S.E.M. Filled points represent the effects of cocaine administered with saline. Disconnected points on the left above V represent the effects of the BZT analogs administered with vehicle. The data are from the 30-minute period immediately after drug administration. Note that each of the BZT analogs [GA1-69 (A), GA2-50 (B), GA2-99 (C), JHW013 (D)] at some doses blocked the stimulation of activity produced by cocaine.

although with the exception of JHW013, the maximal increases were less than those obtained with WIN35,428.

Cocaine also produced dose-related increases in locomotor activity in mice ($F_{4,23} = 4.66$, $P = 0.007$) during the first 30 minutes after injection, with 10 mg/kg producing the maximal effect (Fig. 6, filled symbols). The increase in activity counts at a dose of 10 mg/kg was significant compared with control injections based on post hoc comparisons ($q = 4.82$; $P = 0.019$). None of the *N*-substituted BZT analogs over the range of doses studied significantly altered activity counts (Fig. 6, open points above V), with the exception of GA2-99 ($F_{2,14} = 18.6$; $P < 0.001$), which decreased activity at the highest dose (10 mg/kg) examined ($q = 5.05$; $P < 0.05$).

Pretreatment with GA1-69 (1.0 and 3.0 mg/kg) produced a trend toward dose-dependent antagonism of the locomotor-stimulant effects of cocaine (Fig. 6A, compare filled and open symbols), with a decrease in maximal effects of cocaine. A two-way ANOVA revealed a significant main effect of cocaine dose ($F_{4,73} = 9.79$, $P < 0.001$), but no effect for GA1-69 dose ($F_{2,73} = 1.25$, $P = 0.293$) or the interaction of cocaine and GA1-69 doses ($F_{8,73} = 0.777$, $P = 0.624$).

Both GA2-50 and GA2-99 (each at 3.0 and 10.0 mg/kg) produced dose-dependent antagonism of the locomotor-stimulant effects of cocaine (Fig. 6, B and C; compare filled and open symbols), with rightward shifts in the cocaine dose-effect curve at active doses. Two-way ANOVAs for GA2-50 and GA2-99 indicated significant main effects of cocaine dose ($F_{4,73} = 6.13$ and 6.95, respectively, P values<0.001), BZT analog dose ($F_{2,73} = 4.36$ and 5.14, respectively, P values < 0.016) and the interactions of the two factors ($F_{8,73} = 3.55$ and 2.16, respectively, P values < 0.040).

Pretreatment with JHW013 (1.0 and 3.0 mg/kg) produced a trend toward dose-dependent antagonism of the locomotor-stimulant effects of cocaine (Fig. 6D, compare filled and open symbols), with a decrease in maximal effects of cocaine. A two-way ANOVA revealed a significant main effect of cocaine dose ($F_{4,73} = 8.43$, $P < 0.001$) with neither an effect of JHW013 dose ($F_{2,73} = 1.41$, $P = 0.252$) nor the interaction of the two factors ($F_{8,73} = 0.768$, $P = 0.632$).

Cocaine Discrimination. As shown previously, cocaine produced a dose-related increase in the percentage of drug-appropriate responses in subjects trained to discriminate cocaine (10 mg/kg) from saline injections (Fig. 7, top row, filled symbols). ANOVA indicated a significant effect of cocaine dose ($F_{3,15} = 15.1$, $P < 0.001$) and an ED_{50} value of 4.04 (95% confidence limits: 3.01–5.58) mg/kg. The cocaine analog, WIN35,428 fully substituted for cocaine (ED_{50} value of 0.347 (95% confidence limits: 0.122–0.689 mg/kg, data not shown). In contrast to the effects of cocaine and WIN35,428, none of the *N*-substituted BZT analogs fully substituted for cocaine at any studied doses or times between injection and testing (Fig. 7, top row, open symbols). These compounds were examined across a range of doses from those that had no effects to those that produced substantial decreases in response rates (data not shown).

Pretreatments with each of the BZT analogs produced leftward shifts in the cocaine dose-effect curves (Fig. 7, bottom row). For GA 1-69, GA 2-50, and GA 2-99, the shifts were approximately two- to threefold and did not depend linearly on pretreatment dose. JHW013 was inactive at the lowest dose and shifted the cocaine dose-effect curve leftward at higher doses. Similar effects were obtained with each of the BZT analogs when administered 60 minutes before cocaine (data not shown).

Cocaine Self-Administration. The average response rates maintained by cocaine were a bitonic function of dose,

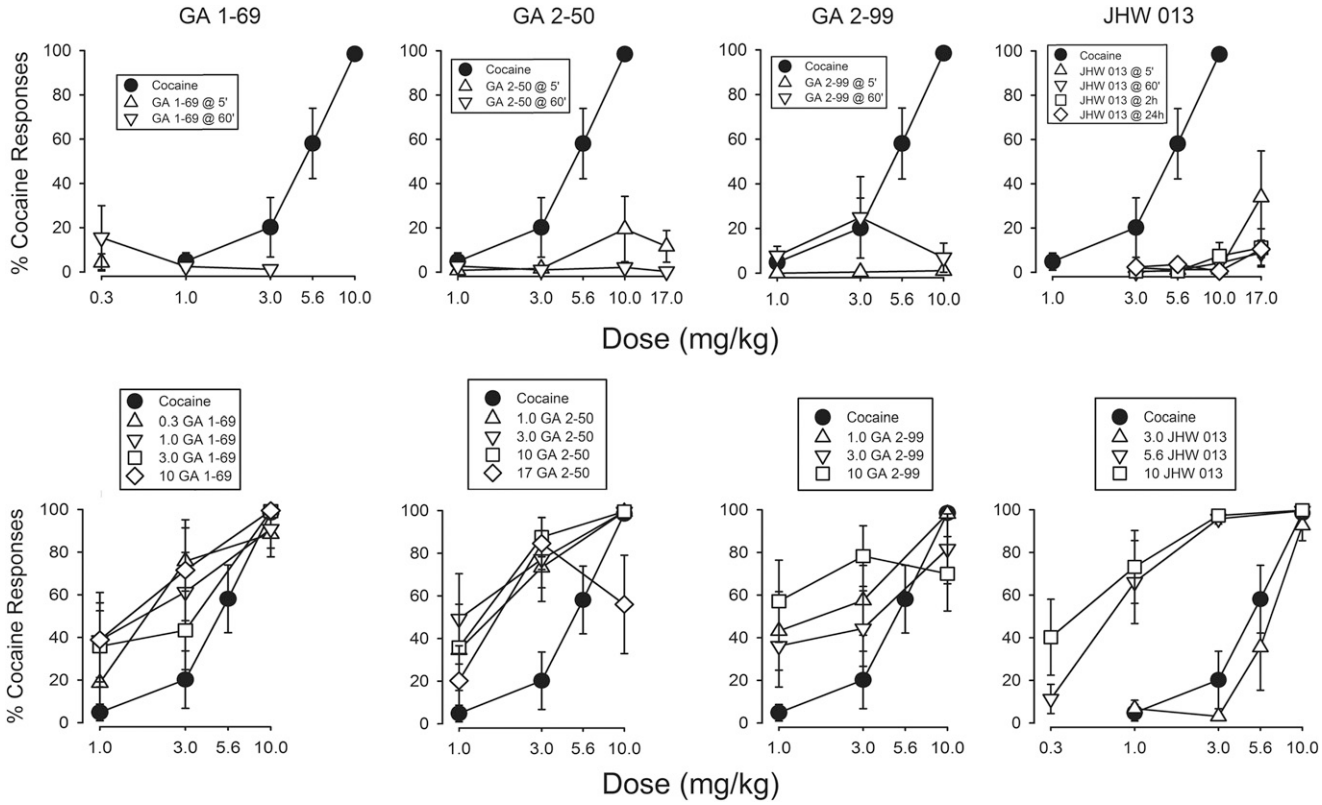

Fig. 7. Effects of various doses of cocaine and *N*-substituted BZT analogs in rats trained to discriminate injections of cocaine (10 mg/kg) from saline at various times after injection. Ordinates: Percentage of responses on the cocaine-appropriate key. Abscissae: drug dose in milligrams per kilogram (log scale). Each point represents the effect in six rats. Top row shows effects of each *N*-substituted BZT analog administered alone compared with effects of cocaine. Filled circles, reproduced in each panel of the figure, represent the effects of cocaine. Open triangles show effects obtained with the BZT analog injected 5 (triangles up) or 60 minutes (triangles down) before testing. Bottom row shows effects of each *N*-substituted BZT analog administered in combination with cocaine. Filled circles, reproduced in each panel of the figure, represent the effects of cocaine administered alone. Open symbols show effects obtained with combinations of cocaine and each of the BZT analogs at various doses.

with a maximum at 0.94 μmol/kg/injection (Fig. 8A, filled circles). The maximal rates obtained were significant and nearly 10-fold greater than the approximate 0.03 responses/second occurring in the first EXT component or with saline (Fig. 8A). One-way repeated-measures ANOVA indicated a significant effect of component (or dose) on response rate ($F_{4,20} = 35.0$, $P < 0.001$). The self-administration of WIN35,428 was similar to that obtained with cocaine though with a 10-fold greater potency (Fig. 8A, triangles). One-way repeated-measures ANOVA indicated a significant effect of component/dose on response rate ($F_{4,20} = 8.69$, $P < 0.001$).

In contrast, no dose of any of the *N*-substituted BZT analogs maintained response rates comparable with those maintained by cocaine (Fig. 8B). No effect of dose/component was obtained with GA1-69, whereas dose/component was significant with each of the other compounds ($F_{4,20} \geq 5.36$; P values ≤ 0.004). Post hoc tests indicated that the effects of dose/component for JHW013 were due to a decrease in response rates below those obtained in EXT. Furthermore, increases in response rates

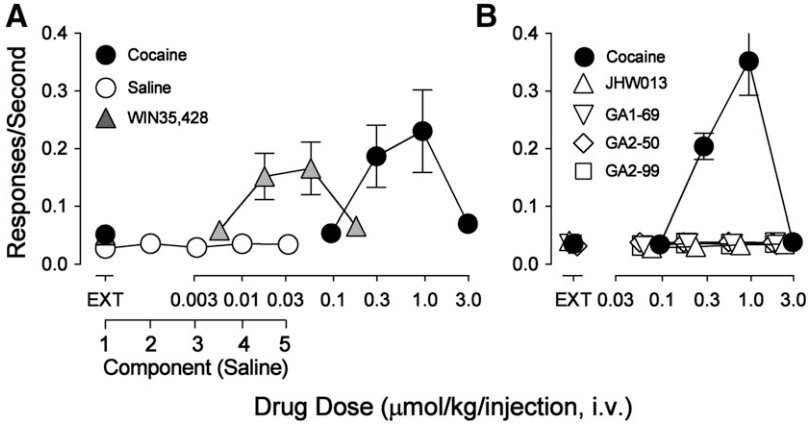

Fig. 8. Substitution of different doses of WIN35,428, BZT analogs, or saline in rats trained to self-administer cocaine [0.09–2.94 μmol/kg/injection]. Ordinates, responses per second. Abscissae, injection dose in milligrams per kilogram. Points above EXT (extinction) represent response rates when responses had no consequences. Each point represents the mean (with bars showing S.E.M., $n = 6$). (A) WIN35,428 or saline substitution for cocaine. (B) GA1-69, GA2-50, GA2-99, JHW013, or saline substitution for cocaine.

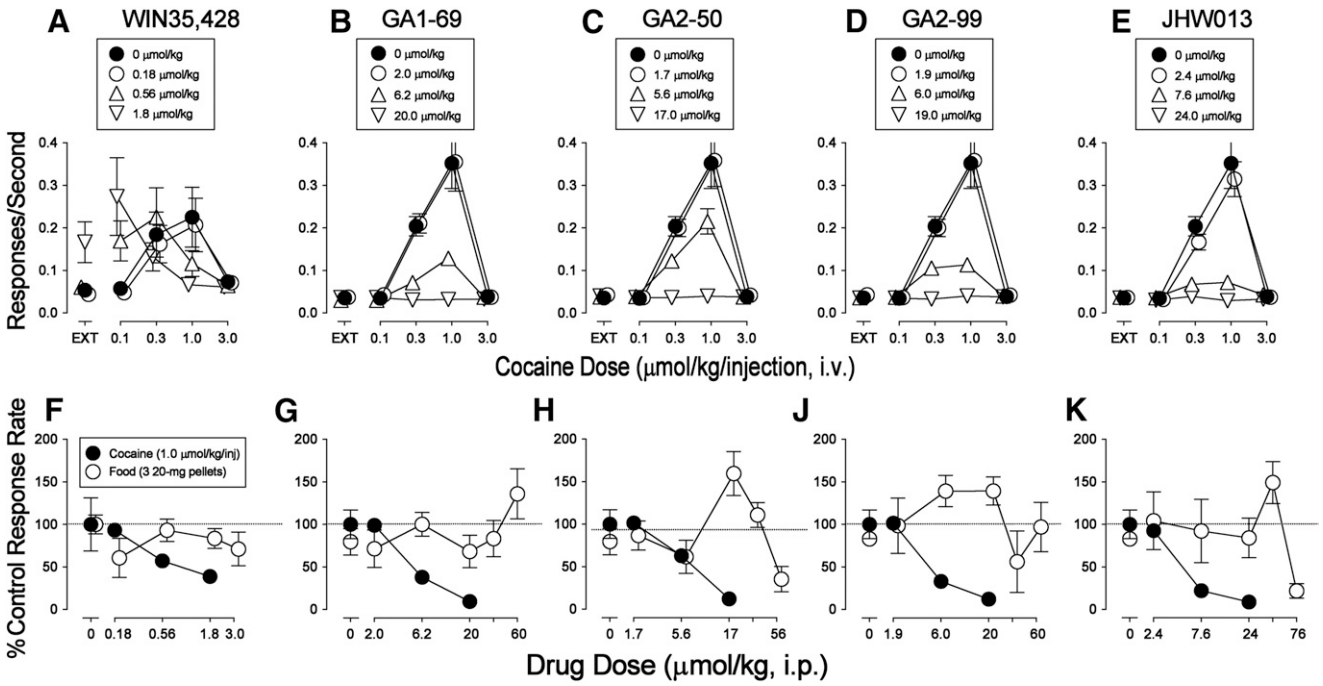

Fig. 9. Effects of pre-session treatments with WIN35,428 or BZT analogs in rats trained to self-administration cocaine [0.09–2.94 μmol/kg/injection]. Each point represents the mean ± S.E.M. Top row: effects of test compounds [WIN35,428 (A), GA1-69 (B), GA2-50 (C), GA2-99 (D), JHW013 (E)] on self-administration of cocaine (n = 6). Ordinates, responses per second. Abscissae, injection dose of cocaine in micromoles per kilogram. Points above EXT (extinction) represent response rates when responses had no consequences. Bottom row: effects of pre-session treatments with test compounds [WIN35,428 (F), GA1-69 (G), GA2-50 (H), GA2-99 (J), JHW013 (K)] on responding maintained by cocaine injections or food presentations. Ordinates, response rates as percentage of control response rates (sessions before drug tests). Abscissae, micromoles per kilogram of the test compounds (i.p.), log scale. Rates of responding were from the fourth 20-minute component of the session (see *Methods*). All test compounds were administered 5 minutes before sessions. Rates of responding maintained by food reinforcement averaged 0.549 ± 0.059 responses/s (n = 18), whereas those maintained by cocaine averaged 0.287 ± 0.050 responses/s (n = 12). Significant difference was found in the control rates across the groups ($F_{1,28}$ = 9.84; P = 0.04, one-way ANOVA).

with GA2-50 or GA2-99 were only 123% or 103% of those occurring during EXT, respectively.

Pre-session treatments with the standard DAT inhibitor WIN35,428 decreased response rates at the 0.94 μmol/kg/injection dose of cocaine that maintained the highest response rates and prominently shifted the cocaine self-administration dose-effect curve leftward (Fig. 9A). The leftward shifts were dose related, with the lowest dose inactive and doses of 0.56 and 1.8 μmol/kg progressively shifting leftward the cocaine dose-effect curve by 3- and 10-fold, respectively. The highest dose of WIN35,428 increased response rates during EXT. A two-way repeated-measures ANOVA indicated a significant effect (P values ≤ 0.002) of cocaine dose ($F_{4,60}$ = 6.52), pre-session WIN35,428 dose ($F_{3,60}$ = 7.88), and their interaction ($F_{12,60}$ = 6.44).

In contrast to the effects of WIN35,428, each of the *N*-substituted BZT analogs produced a dose-dependent decrease in the maximal response rates maintained by 0.94 μmol/kg/injection of cocaine (Fig. 9, B–E), with no evidence of a leftward shift in the cocaine dose-effect curve. At the highest doses of the *N*-substituted BZT analogs, no dose of cocaine maintained responding at levels above those maintained in EXT (Fig. 9, B–E, inverted triangles). Statistical analysis of the effects on response rates of each of the compounds indicated significant effects of cocaine dose ($F_{4,60}$ values ≥ 31.8, P values < 0.001), pre-session treatments ($F_{3,60}$ values ≥ 38.3, P values < 0.001), and the interactions of the two ($F_{12,60}$ values ≥ 27.8, P values < 0.001). Post hoc tests indicated that the highest two doses of each of the compounds decreased rates of responding maintained by cocaine at doses of 0.29 and 0.94 μmol/kg/injection (q values ≥ 6.26, P values < 0.001). Doses of the *N*-substituted BZT analogs that decreased response rates maintained by cocaine at its maximally effective dose [0.94 μmol/kg/injection] were lower than those that decreased response rates maintained by food reinforcement (Fig. 9, G–K, compare filled with open circles). For each compound, there was at least one dose that decreased response rates maintained by cocaine without decreases in rates of responding maintained by food reinforcement.

Discussion

Previous in vitro studies showed that DAT-binding affinities of the present BZT analogs were relatively high, and the compounds were DAT selective compared with a variety of additional sites, including other monoamine transporters (Li et al., 2011). In the present study, all except GA1-69 produced DAT-occupancy levels in vivo approximating those obtained with behaviorally effective cocaine doses, indicating that DAT occupancy alone is insufficient to explain the atypical DAT-inhibitor profile. Future studies will examine DA levels produced by uptake inhibition, although previous results using place conditioning (Tanda et al., 2013) or cocaine discrimination (Kohut et al., 2014) suggest that those data are similarly inadequate to solely account for the effects of atypical DAT inhibitors. As suggested by Reith et al. (2015), at present no single factor explains the atypical DAT-inhibitor pharmacological profile. It is likely that a number of different factors can

contribute, possibly in different combinations producing variants of atypical DAT-inhibitor profiles.

Other hypotheses for atypical DAT-inhibitor effects include actions at off-target sites and slow onsets of action, allowing compensatory mechanisms to mitigate standard DAT-inhibitor effects (Reith et al., 2015). Antagonism of sigma receptors was previously identified as a mitigating off-target action (Katz et al., 2017); however, only GA2-50 and JHW013 had sigma-receptor affinities comparing favorably to their DAT affinities (Li et al., 2011). The DAT apparent-association rates for the compounds were slower than that for cocaine, whereas rapid onsets of effects were reported previously. However, those effects were decreases in locomotor activity rather than increases seen with standard DAT inhibitors. The rapid onsets of distinct behavioral effects with relatively slow DAT associations suggest off-target actions. Nonetheless, broad-spectrum screens of these compounds failed to identify a singular candidate for those off-target effects (Li et al., 2011).

The DAT occupancy without cocaine-like effects suggests potential antagonism of cocaine. As suggested, the BZT analogs blocked the locomotor-stimulant effects and self-administration of cocaine. One hypothesis for blockade postulates enhanced stereotypy interfering with behavioral activation. Indeed, doses of JHW013 and GA2-99 that blocked the cocaine-induced locomotion also enhanced stereotypy. However, GA1-69 blocked cocaine-induced locomotion without enhancing stereotypy, whereas GA2-50 presented the contrapositive case of increases in stereotypy without locomotor-stimulant antagonism. Importantly, WIN35,428 increased both cocaine-induced stereotypy and locomotion. Together these findings challenge the widely disseminated premise that increased stereotypy decreases stimulant-induced locomotion and indicate that response competition is an inadequate explanation for the present cocaine antagonism (Li et al., 2013).

The decreases in maximal cocaine self-administration (also Hiranita et al., 2009, Li et al., 2013), as opposed to antagonism characterized by rightward shifts in dose-effect curves, have suggested a behavioral mechanism of action in which BZT analogs induce "satiety" for stimulant drugs. That interpretation was supported in recent behavioral-economic analyses (Zanettini et al., 2018). Furthermore, methadone pretreatment or food prefeeding produce similar decreases in maximal opioid-agonist self-administration or food-reinforced responding, respectively, under procedures like those of the present study (Hiranita et al., 2014b). Satiation was suggested previously as explanation for the clinical efficacy of methadone in treating opioid abuse (Dole and Nyswander, 1967).

Curiously, the BZT analogs shifted cocaine-discrimination dose-effect curves leftward (also Katz et al., 2004), an effect ostensibly contrasting with effects on cocaine self-administration. However, leftward shifts might be expected from satiation-like effects. Prefeeding rhesus monkeys trained to discriminate levels of food deprivation has discriminative-stimulus effects like decreased food deprivation (Corwin et al., 1990). An agent that satiates cocaine self-administration might be expected to share some of its discriminative-stimulus effects.

Two methods assessed the outward- or inward-facing DAT conformation upon compound binding, hypothetically related to the behavioral effects of standard or atypical DAT inhibitors, respectively. Simultaneous cysteine replacement of Thr-316 and alanine replacement of Cys-306 yielded a DAT construct retaining functional DA uptake and inhibitor binding. T316C resides on the inner half of TM6a, near the S1 DAT inhibitor-binding pocket. Previous data showed that in T316C/C306A hDAT, T316C was the only site that reacted with the membrane-impermeant maleimide-PEO_2-biotin from the extracellular side, whereas C90 was not involved (Hong and Amara, 2010). T316C accessibility serves as a sensor, with its increased or decreased labeling, suggesting outward- or inward-facing DAT conformations, respectively.

Cocaine clearly increased T316C accessibility, indicating stabilization of an outward-facing DAT conformation. GA2-99 showed a trend to increase T316C accessibility. In contrast, BZT, GA1-69, and JHW013 decreased T316C accessibility, suggesting stabilization of inward-facing DAT conformations. While inward-facing structures of eukaryotic DAT remain unresolved, the inward-facing $LeuT_{Aa}$ structure (Fig. 2B) exhibits substantial conformational rearrangement of several domains, compared with the outward-facing dDAT structure (Fig. 2C). Particularly, the inner-half of TM6a becomes inaccessible from the extracellular side. Assuming similarity of inward-facing hDAT and LeuT, such structural insight provides a mechanistic explanation of the present biochemical observations.

Previously, DA-uptake inhibitor potency of GA2-99 in WT hDAT transfected cells was approximately 50-fold greater than in Y335A DAT transfected cells; the Y334A construct is believed to assume an inward-facing conformation (Loland et al., 2008). Additionally, potency ratios in Y335A cells, compared with WT DAT, categorically predicted atypical or standard DAT-inhibitor effects. Recently, a GA2-99 analog, JJC7-043, with atypical behavioral effects, bound a DAT Y156F mutant with lower potency than WT DAT (Zou et al., 2017). That potency differential was suggested to indicate a binding preference for inward-facing DAT conformation (Zou et al., 2017). In that same study, two isoxazol derivatives of cocaine showed Y156F:WT potency ratios similar to JJC7-043, despite previous cysteine-accessibility assays indicating both inducing outward-facing DAT conformations, although only one producing atypical DAT-inhibitor effects (Hiranita et al., 2014a). Finally, Hong et al. (2016) found a series of cocaine analogs with atypical effects stabilized cocaine-like outward DAT conformations. Thus, previous and present results taken together indicate that various methods of assessing inward- versus outward-facing DAT conformation are neither in complete agreement nor predictive of standard or atypical DAT-inhibitor effects.

The second method examining DAT conformation involved short (nanosecond) MD simulations of inhibitors bound within the DAT S1 pocket. Conformations open to the extracellular space or cytosol were inferred from the respective absence or presence of a "salt bridge" ionic interaction between outer-gate residues R85 (TM1) and D476 (TM10) (Pedersen et al., 2014; Cheng and Bahar, 2015). The presence of that interaction is consistent with an intramolecular side-chain distance of 3 to 4 Å. After an initial 250 ns of fluctuation with the BZT-DAT complex, R85-D476 distance remained approximately 3 Å (Fig. 3, top row), consistent with effects of BZT in cysteine-accessibility assays (Hong et al., 2016). GA1-69 behaved similarly to BZT in this biochemical assay, and its MD pattern also reflected an intact salt bridge, stabilizing at 200 ns.

In contrast, the cocaine- and GA2-99-DAT complexes never established a stable direct R85-D476 bond (Fig. 3, top row). The bond appeared to form intermittently during the cocaine-DAT

simulations, but R85 and D476 were more often separated by 6 to 7 Å. MD simulations with GA2-99, the analog that did not induce an inward-facing conformation in alkylation assays, displayed particularly constant R85-D476 separations of 6 to 7 Å, never achieving the 3 to 4 Å separation needed for the intramolecular ionic bond seen with BZT-DAT complexes. Although the larger separation precludes direct ionic interactions, R85 and D476 side chains could conceivably connect through a third side chain, water molecules, or ions within the extracellular vestibule. Those arrangements might occlude the substrate permeation pore while providing space sufficient for the alkylating agent, accounting for the observed GA2-99 pharmacology and cysteine-accessibility assay results. Curiously, GA2-99's S1 pocket orientation differed from that of the other inhibitors, because neither of its two positively charged tropane nitrogen atoms interacted ionically with D79. Instead, S1 ligand docking appeared to be driven by sequestration of one of its 4-fluorophenyl rings by a hydrophobic pocket consisting of L150 (TM3) and the TM6 residues F320, F326, and V328.

Caveats in comparing cysteine-accessibility and MD-simulation outcomes regarding DAT-inhibitor complexes arise from their different methods. The cysteine-accessibility assay requires several minutes and nonphysiological (4°C) temperatures to form necessary covalent bonds between T316C and the alkylating agent. Furthermore, cysteine alkylation itself could conceivably stabilize DAT conformation. The MD simulations, in contrast, are conducted at 37°C, but over nanosecond time courses, probably too brief for R85 and D476 side chains to reach equilibrium in response to ligand binding. Additionally, more fluctuation with MD simulations may be expected, as no covalent bonds are formed, allowing conformational sampling. Formation of a T316C-maleimide-PEO_2-biotin covalent bond precludes further fluctuations providing discrete outcomes.

In summary, several atypical DAT inhibitors were examined from pharmacological, behavioral, and molecular perspectives. Each compound exhibited slower in vivo DAT association than cocaine, suggesting an unidentified action responsible for previously reported fast-onset behavioral effects, and interfering with common DAT-inhibitor effects. Results from biochemical and computational studies add to previous results, suggesting that DAT conformation alone is insufficient to predict atypical actions. Certainly, additional biochemical and electrophysiological contributions might clarify the relation of DAT conformation to atypical pharmacology. Nonetheless, these findings provide further approaches for sorting actions of atypical DAT inhibitors, although resolving the binding dynamics reliably, distinguishing standard and atypical DAT inhibitors, remains elusive. That resolution would likely facilitate a rational-design approach in discovery of medical treatments for psychomotor-stimulant abuse.

Acknowledgments

We thank Patty Ballerstadt and Maryann Carrigan for administrative assistance, Bettye Campbell and Dawn French-Evans for technical assistance. Jianjing Cao and Amy H. Newman kindly provided the compounds. Animal husbandry and all other care were in accordance with NIDA institutional animal care and use guidelines and the Guide for the Care and Use of Laboratory Animals (1996) Institute for Laboratory Animal Research, National Research Council, National Academy Press, Washington, DC.

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