σ Receptor Effects of N-Substituted Benztropine Analogs: Implications for Antagonism of Cocaine Self-Administration

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ABSTRACT

Several N-substituted benztropine (BZT) analogs are atypical dopamine transport inhibitors as they have affinity for the dopamine transporter (DAT) but have minimal cocaine-like pharmacologic effects and can block numerous effects of cocaine, including its self-administration. Among these compounds, *N*-methyl (AHN1-055), *N*-allyl (AHN2-005), and *N*-butyl (JHW007) analogs of 3 α -[bis(4'-fluorophenyl)methoxy]-tropane were more potent in antagonizing self-administration of cocaine and *d*-methamphetamine than in decreasing food-maintained responding. The antagonism of cocaine self-administration (0.03–1.0 mg/kg per injection) with the above BZT analogs was reproduced in the present study. Further, the stimulant-antagonist effects resembled previously reported effects of pretreatments with combinations of standard DAT inhibitors and σ_1 -receptor (σ_1 R) antagonists. Therefore, the present study examined binding of the BZT analogs to σ Rs, as

well as their in vivo σ R antagonist effects. Each of the BZT analogs displaced radiolabeled σ R ligands with nanomolar affinity. Further, self-administration of the σ R agonist DTG (0.1–3.2 mg/kg/injection) was dose dependently blocked by AHN2-005 and JHW007 but potentiated by AHN1-055. In contrast, none of the BZT analogs that were active against DTG self-administration was active against the self-administration of agonists at dopamine D₁-like [*R*(+)-SKF 81297, ($\pm$)-SKF 82958 (0.00032–0.01 mg/kg per injection each)], D₂-like [*R*(-)-NPA (0.0001–0.0032 mg/kg per injection), (-)-quinpirole (0.0032–0.1 mg/kg per injection)], or μ -opioid (remifentanyl, 0.0001–0.0032 mg/kg per injection) receptors. The present results indicate that behavioral antagonist effects of the *N*-substituted BZT analogs are specific for abused drugs acting at the DAT and further suggest that σ R antagonism contributes to those actions.

Introduction

The reinforcing effects of cocaine result primarily from its inhibition of dopamine (DA) uptake through actions at the presynaptic DA transporter (DAT) (Kuhar et al., 1991). The primary evidence supporting that conclusion is the correlation of DAT affinities of stimulants with their potencies in self-administration procedures (Ritz et al., 1987; Bergman et al., 1989); however, a number of novel compounds have been identified that have high affinity for the DAT but do not produce effects similar to those of cocaine. Moreover, there are several reports of compounds that bind to the DAT and antagonize various effects of stimulant drugs (see review by Reith et al., 2015).

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Among the compounds that have high DAT affinity, and yet have behavioral effects different from those of cocaine, are analogs of benztropine (BZT). Among them, 3 α -[bis(4'-fluorophenyl)methoxy]-tropane (AHN1-055), *N*-allyl-3 α -[bis(4'-fluorophenyl)methoxy]-tropane (AHN2-005), and (*n*-butyl)-3 α -[bis(4'-fluorophenyl)methoxy]-tropane (JHW007) have been widely studied. These BZT analogs have been found to be less effective than cocaine in stimulating the levels of nucleus accumbens DA (Tanda et al., 2005, 2009) and as stimulants of locomotor activity (Katz et al., 1999; Velázquez-Sánchez et al., 2009). Virtually all the BZT analogs examined, across a broad range of doses, have failed to fully substitute in rats trained to discriminate cocaine from saline injections (e.g., Katz et al., 1999, 2004). Further, BZT analogs did not produce place conditioning comparable to cocaine (Li et al., 2005; Velázquez-Sánchez et al., 2009) and were self-administered to a degree only slightly greater than that obtained with vehicle, if at all (Woolverton et al., 2000; 2001; Ferragud et al., 2009; Hiranita et al., 2009).

In studies of combinations of cocaine and BZT analogs, the latter compounds dose dependently decreased the maximal

ABBREVIATIONS: AHN1-055, 3 α -[bis(4'-fluorophenyl)methoxy]-tropane hydrochloride; AHN2-005, *N*-allyl-3 α -[bis(4'-fluorophenyl)methoxy]-tropane oxalate; BD 1008, *N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(1-pyrrolidinyl)ethylamine dihydrobromide; BD 1063, 1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride; BZT, benztropine; CM304, 3-(2-(azepan-1-yl)ethyl)-6-(3-fluoropropyl)benzo[d]thiazol-2(3*H*)-one hydrochloride; DA, dopamine; DAT, dopamine transporter; DTG, 1,3-di-*o*-tolylguanidine; EXT, extinction; FR, fixed ratio; JHW007, *N*-(*n*-butyl)-3 α -[bis(4'-fluorophenyl)methoxy]-tropane hydrochloride; LED, light-emitting diode; *R*(-)-NPA, *R*(-)-10,11-dihydroxy-*N*-*n*-propylinoraporphine hydrochloride; *R*(+)-SKF 81297, *R*(+)-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrobromide; ($\pm$)-SKF-82958, ($\pm$)-6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrobromide (6-chloro-*N*-allyl-SKF-38393 hydrobromide).

self-administration of cocaine (Ferragud et al., 2009; Hiranita et al., 2009; Li et al., 2013) or amphetamines (Ferragud et al., 2014; Hiranita et al., 2014a). Of particular importance, the blockade of stimulant self-administration was obtained at doses that did not affect comparable responding maintained by food reinforcement (Hiranita et al., 2009, 2014a; Li et al., 2013; Ferragud et al., 2014). Additionally, none of the compounds blocked self-administration of the nonstimulant drugs of abuse; (–)-heroin and (±)-ketamine (Hiranita et al., 2014a) further substantiating the specificity of the effect.

The mechanistic hypotheses regarding the unique activity of these so-called atypical DAT inhibitors have been reviewed (Reith et al., 2015). Atypical effects have been suggested to be related to slow association with the DAT, a change in DAT conformation to one open to the cytosol, or activity at other binding sites. In particular, a recent set of studies has suggested that combined activity at σ receptors (σ Rs) and the DAT can block cocaine self-administration (Hiranita et al., 2011; Katz et al., 2016). For example, the selective σ R antagonists, BD1008, BD1047, and BD1063, when combined with standard DA-uptake inhibitors (e.g., methylphenidate, nomifensine, or WIN-35,428), blocked cocaine self-administration and did so selectively, as indicated by a lack of effect on food-reinforced responding. Further, compounds with σ R antagonist effects and DAT affinity (e.g., rimcazole and its analogs) also selectively decreased cocaine self-administration (Hiranita et al., 2011).

Thus, the present study confirmed the antagonism of cocaine self-administration by the *N*-substituted BZT analogs, AHN1-055, AHN2-005, and JHW007, and further assessed whether the compounds function as σ R antagonists *in vivo*. Establishing agonist-antagonist interactions of σ R ligands *in vivo* has been problematic. Studies of the acute toxic effects of cocaine have the potential for differentiating agonist and antagonist effects of σ R ligands (Matsumoto et al., 2014); however, whereas the acute toxicity produced by cocaine may be mediated in part by σ_1 Rs (Lever et al., 2016), the complex pharmacology of cocaine renders these outcomes less than definitive. In a recent study the reinforcing effects of σ R ligands were used to characterize not only agonist-antagonist interactions but σ R subtype selectivity (Katz et al., 2016). The self-administration of DTG, a nonselective σ R agonist, was dose dependently blocked by several σ R antagonists, whereas self-administration of the σ_1 -selective agonists PRE-084 or (+)-pentazocine was blocked only by antagonists with affinity for σ_1 Rs. Consequently, the present study, designed to determine whether the BZT analogs had *in vivo* σ R antagonist effects, directly compared the antagonism of cocaine and DTG self-administration by the BZT analogs.

Materials and Methods

σ_1 and σ_2 Receptor Binding Assays. Male guinea pig brain tissue, excluding cerebella (Pel Freez Biologicals, Rogers, AR), was thawed on ice, homogenized (with a glass and Teflon apparatus) in tissue buffer (10 mM Tris-HCl with 0.32 M sucrose, pH 7.4), and subsequently centrifuged at 800g for 10 minutes at 4°C. The supernatant was collected into a clean centrifuge tube, and the remaining pellet was resuspended by vortex in 10 ml of buffer (tissue) and centrifuged at 800g for 10 minutes at 4°C. The supernatants were pooled and centrifuged at 50,000g for 15 minutes at 4°C. The remaining pellet was resuspended in 10 mM Tris-HCl, pH 7.4. The tissue was resuspended and incubated at 25°C for 15 minutes and then centrifuged at 50,000g for 15 minutes. The supernatant was decanted, and the pellet was gently resuspended in 50 mM Tris-HCl, pH 8.0 (experimental buffer) to 80 mg/ml, original

wet weight (OWW). Incubations were conducted for 120 minutes at room temperature in polypropylene assay tubes containing 0.50 ml of experimental buffer, 3.0 nM [3 H](+)-pentazocine (Perkin-Elmer Life and Analytical Sciences, Waltham, MA) for σ_1 R binding or 3.0 nM [3 H]DTG (Perkin-Elmer) with 200 nM of (+)-pentazocine for σ_2 R binding, 8.0 mg/tube of previously frozen guinea pig brain tissue, and various concentrations of inhibitors. Nonspecific binding was determined with 10 (σ_1 R) or 100 (σ_2 R) μ M haloperidol. Several studies have indicated that [3 H](+)-pentazocine can be used to selectively label σ_1 Rs (de Costa et al., 1989; Bowen et al., 1993). Further, [3 H]DTG has been used to label σ_2 Rs with excess amounts of unlabeled (+)-pentazocine to block σ_1 sites (Weber et al., 1986).

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} value of inhibitors used in the assay and the K_d value of the radioligand previously determined in this laboratory. Hill slopes were calculated from an otherwise unconstrained four-parameter logistic equation fitted to the displacement curves. For the [3 H]DTG binding, the data modeled better for two than for one binding site. A previous report indicated that the high-affinity site represents binding to the σ_2 R (Garcés-Ramírez et al., 2011).

Behavioral Studies. Subjects were 37 male Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing approximately 300 g at the start of the study. Subjects were acclimated to a temperature- and humidity-controlled vivarium for at least 1 week with a 12-hour light/dark cycle (lights on at 07:00 hours); food (Scored Bacon Lover Treats; BIOSERV, Frenchtown, NJ) and tap water were available at all times. After acclimation, body weights were maintained at approximately 320 g by adjusting daily food rations. Tap water continued to be available at all times in the home cages. Care of the subjects was in accordance with the Guidelines of the National Institutes of Health (NIH) and the National Institute on Drug Abuse (NIDA) Intramural Research Program (IRP) Animal Care and Use Program, which is fully accredited by Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

Subjects were surgically prepared under anesthesia (ketamine 60.0 mg/kg i.p. and xylazine 12.0 mg/kg i.p.) in either the right or the left external jugular vein with a chronic indwelling catheter that exited in the midscapular region of the animal's back. Catheters were infused daily with 0.1 ml of a sterile saline solution containing heparin (30.0 IU/ml) and penicillin G potassium (250,000 IU/ml) to minimize the likelihood of infection and the formation of clots or fibroids. All animals were allowed to recover from surgery for approximately seven days before drug self-administration studies were initiated.

Experimental sessions were conducted daily with subjects placed in operant-conditioning chambers (modified ENV-203; Med Associates, St. Albans, VT) that measured 25.5 $\times$ 32.1 $\times$ 25.0 (height) cm and were enclosed within sound-attenuating cubicles equipped with a fan for ventilation and white noise to mask extraneous sounds. On the front wall of each chamber were two response levers, 5.0 cm from the midline and 4.0 cm above the grid floor. A downward displacement of a lever with a force approximating 0.20 N defined a response and always activated a relay mounted behind the front wall of the chamber, producing an audible "feedback" click. Three light-emitting diodes (LEDs) were located in a row 4.5 cm above each lever. A receptacle for the delivery of 20-mg food pellets via a pellet dispenser (model ENV-203-20; Med Associates) was mounted on the midline of the front wall between the two levers and 2.0 cm above the floor. Activation of a syringe infusion pump (model 22; Harvard Apparatus, Holliston, MA) placed above a sound-attenuating cubicle delivered injections at specified volumes from a 10-ml syringe. The syringe was connected by Tygon tubing to a single-channel fluid swivel (375 Series Single Channel Swivels; Instech Laboratories, Inc., Plymouth Meeting, PA), which was mounted on a balance arm above the chamber. Tygon tubing from the swivel to the subject's catheter was protected by a surrounding metal spring and completed the connection to the subject.

Experimental sessions started with illumination of the LEDs above each lever and initially lasted for 120 minutes. Each downward deflection of the right lever turned off the LEDs, produced an audible click, a 20-mg food pellet (Bio-Serv, Frenchtown, NJ), followed by a 20-second timeout period during which LEDs were off and response had no scheduled consequences. The LEDs were illuminated after the timeout, and responding again had scheduled consequences. Responses on the left lever were recorded but had no scheduled consequences. Over the course of several sessions, the number of responses required to produce a food pellet was increased to five (fixed-ratio five-response schedule, FR 5). Subjects were returned to their home cages in the animal-housing facility after each session which ended after delivery of 30 food pellets or 20 minutes.

After subjects were responding at a rate sufficient for the delivery of 30 food pellets within each of three consecutive sessions, cocaine injections (1.0 mg/kg per injection over 10 seconds) replaced food pellets. Once they were relatively stable across sessions, the session was divided into five 20-minute components with adjusted injection volumes and durations in each, allowing assessment of different doses of cocaine. The drug dose per injection was incremented in the sequential components as follows: no injection (also referred to as *extinction*, or EXT, because responses had no scheduled consequences), 0.03, 0.10, 0.32, or 1.0 mg/kg per injection for cocaine. Injection volumes (and durations) were, respectively, 0 μ l (0 seconds), 5.6 μ l (0.32 second), 18.0 μ l (1.0 second), 56.0 μ l (3.2 seconds), and 180 μ l (10.0 seconds) based on a body weight of 0.32 kg. A sample injection of cocaine at the corresponding unit dose and the illumination of stimulus LEDs occurred independently of responding at the end of a 2-minute timeout period that preceded each component with, the exception of the first, during which there were neither injections of cocaine nor illumination of stimulus LEDs. An i.p. injection of saline (1.0 ml/kg) was given approximately 5 minutes before daily sessions as a control for drug pretreatment experiments.

Training continued until responding was maintained individually with less than 20% variation in response rates across three consecutive sessions. Once performances were stable, test sessions were conducted with DTG (0.1–3.2, $n = 17$) substituted for cocaine. The test sessions were single sessions and were conducted only if the preceding 20% criterion was met over the two prior test sessions. All experimental elements for the substitution sessions were identical to the training sessions except for the compound injected. Subsequently, the effects of pretreatments with the BZT analogs AHN1-055, AHN2-005, and JHW007 were assessed on responding maintained by i.v. injections of cocaine or DTG.

The specificity of the effects of the BZT analogs on cocaine self-administration was further assessed by comparing those effects with their effects on various additional self-administered compounds when those compounds were substituted for cocaine in single test sessions. The compounds substituted were *R*(+)-SKF 81297, ($\pm$)-SKF 82958, *R*(-)-NPA, (-)-quinpirole, and remifentanyl. Because of the high rates of remifentanyl self-administration, injection durations of remifentanyl were reduced to 0, 0.24, 0.75, 2.4, or 7.5 seconds to avoid delivery of a total volume of solution within the session that exceeded the capacity of the syringe. All test sessions were separated by a minimum of 72 hours and were studied with a mixed order of drugs and doses.

Response rates on the active lever (responses divided by elapsed times excluding timeouts and injection times) were statistically assessed as a function of dose and pretreatment drug doses by two-way repeated-measures analyses of variance (ANOVA), with post-hoc Bonferroni *t* tests. For all analyses, the criterion for statistical significance was set at $P < 0.05$. As there were few, if any, responses on the inactive lever at any time and on the right lever during timeout periods, these data are not presented.

Drugs. The drugs used in the present study and their salt and enantiomeric forms were as follows: *R*(-)-cocaine HCl (Merck, Whitehouse Station, NJ), *R*(+)-SKF 81297 [*R*(+)-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine HBr; Sigma-Aldrich, St. Louis, MO], ($\pm$)-SKF 82958 [($\pm$)-6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine HBr, Sigma-Aldrich], *R*(-)-NPA

[*R*(-)-10,11-dihydroxy-*N*-*n*-propylnoraporphine HCl, Sigma-Aldrich], (-)-quinpirole HCl (Tocris, Ballwin, MO), remifentanyl HCl (brand name Ultiva; Hospira, Inc., Lake Forest, IL), and the *N*-substituted BZT analogs, AHN1-055 HCl, AHN2-005 oxalate, and JHW007 HCl (synthesized in the Medicinal Chemistry Section of NIDA IRP according to procedures previously published, Newman et al., 1995). All doses of drugs used in the present study were expressed as their salt forms. Saline (0.9% sodium chloride, USP; Hospira Inc.) was used as vehicle for all compounds with mild heat and sonication, as necessary. Self-administered drugs were delivered i.v., whereas the BZT analogs were administered by the i.p. route. Pretreatments with AHN1-055, AHN2-005, and JHW007 were respectively administered at 90, 120, and 150 minutes before sessions, based on previous data (Hiranita et al., 2009, 2014a; Li et al., 2013).

Results

Radioligand Binding Assays. Representative curves for displacement of [3 H](+)-pentazocine by each of the compounds (Fig. 1, top panel) show the variations in affinity, as well as the

Fig. 1. Displacement of radioligands for σ R_s by the BZT analogs studied. Ordinates: Percentage of specific radiotracer bound to membrane preparations as described in *Methods*. Abscissae: Concentration of each competing compound. The top panel shows displacement of [3 H](+)-pentazocine, a specific label for σ_1 R_s. The lower panel shows displacement of [3 H]DTG, a nonspecific σ R label in the presence of excess (+)-pentazocine to mask σ_1 R binding sites. The curves represent the results of a single experiment with vertical bars representing S.E.M.s from averages of results from three samples. The results were selected from at least three independent replications as representative of the binding parameters resulting from a global modeling of all the data.

displacement over an approximate 100-fold range of concentrations. The K_i values for the displacement of radioligand by the compounds ranged from 2.40 nM for JHW007 to 119 for AHN1-055 (Table 1). The Hill slope values for the displacement of radioligand by the compounds were more steep than 1.0 for JHW007 and AHN2-005 (Table 1).

Each of the BZT analogs also displaced [3 H]DTG, with representative displacement curves for each shown in Fig. 1 (bottom panel). Those curves show biphasic displacement over a greater than 100-fold range of concentrations. Hill slope values of the data fitted to the four-parameter logistic equation were significantly less steep than 1.0 (Table 1). A previous study using the same methods (i.e., Garcés-Ramírez et al., 2011) found similar biphasic curves for displacement of [3 H]DTG, with a two-site competition model fitting the data significantly better than a one-site model. In that study, the K_i values for the high-affinity DTG binding site corresponded more closely with published values for the σ_2 R (Garcés-Ramírez et al., 2011). The K_i values of the BZT analogs at the high-affinity site ranged from 12.0 (JHW007) to 78.7 (AHN1-055) nM (Table 1).

Drug Self-Administration. The dose-effect curve for cocaine self-administration was biphasic with maximal responding at a dose of 0.32 mg/kg per injection. The response rate at that dose of cocaine was 0.273 (S.E.M. = 0.056) responses/s (Fig. 2, filled circle above 0.32 mg/kg per injection). In contrast, rates of responding were uniformly low (0.039; S.E.M. = 0.005 responses per second, Fig. 2, open circles) when saline was available. A two-way repeated-measures ANOVA indicated a significant effect of drug versus saline, dose, and the interaction of the two, with post hoc tests indicating significance at 0.1 and 0.32 mg/kg per injection of cocaine (Table 2).

The dose-effect curve for DTG self-administration was similarly biphasic; however, DTG was approximately 3-fold less potent than cocaine. The maximal rate of responding was comparable to that of cocaine but was obtained at a dose of 1.0 mg/kg per injection (Fig. 2, open square above 1.0 mg/kg/injection). A two-way repeated-measures ANOVA indicated significant effects of DTG versus saline, DTG dose, and the interaction, with post hoc tests indicating significance at 0.32 and 1.0 mg/kg per injection of DTG (Table 2).

Treatment with AHN1-055 before sessions dose dependently altered the cocaine self-administration dose-effect curve (Fig. 3A). At the 1.0 mg/kg dose of AHN1-055, the cocaine dose-effect curve was not significantly affected. At 3.2 mg/kg the maximal rates of response were obtained at a 10-fold lower dose than after saline treatment, and those rates were lower than the maximum after saline treatment. At the 10.0 mg/kg dose of AHN1-055, response rates maintained by cocaine were decreased to those obtained during EXT (Fig. 3A) or with saline injection (Fig. 2, open circles). The 3.2 mg/kg dose of AHN1-055 also increased the response rates during the initial EXT

Fig. 2. Substitution of saline or a range of doses of cocaine or DTG in rats trained to self-administer cocaine (0.032–1.0 mg/kg per i.v. injection) under an FR5 response schedule of reinforcement. Ordinates: Responses per second; abscissae: cocaine or DTG unit dose (mg/kg per i.v. injection), log scale, or for saline sequential component of the session. Each point represents the mean $\pm$ S.E.M. of 17 subjects. Substitutions of cocaine (●, 0.032–1.0 mg/kg per injection) include saline (○, first to fifth components) and DTG (□, 0.1–3.2 mg/kg per injection). Effects of substitution for cocaine were determined only once. Note that both DTG and cocaine maintained maximal rates of response greater than those obtained with saline.

component (Fig. 3A). Statistical analyses indicated significant effects of cocaine unit dose, AHN1-055 pretreatment dose, and their interaction (Table 3). Post hoc tests indicated significance of effects at 3.2 or 10.0 mg/kg of AHN1-055 during EXT or at selected cocaine doses per injection (Table 3).

Presession injections of AHN2-005 or JHW007 each dose dependently decreased maximal rates of responding maintained by cocaine injections, without indications of the leftward shift in the cocaine dose-effect curve observed with AHN1-055 (Fig. 3, B and C). At the highest doses of each BZT analog, response rates maintained by cocaine were decreased to those obtained during EXT or with saline injection (Figs. 2 and 3, B and C). Statistical analyses indicated significant effects of unit dose of self-administered cocaine, pretreatment dose of either BZT analog and their interactions (Table 3). Post hoc tests indicated significance of effects at 1.0 (AHN2-005 only), 3.2, or 10.0 mg/kg pretreatment doses at cocaine doses of 0.1 and 0.32 mg/kg per injection (Table 3).

The specificity of the effects of the BZT analogs was assessed by comparing their effects on self-administration of the DA direct agonists or the μ -opioid receptor agonist remifentanyl when those drugs were substituted for cocaine. The dose-effect curves for self-administration of each of the compounds, as seen with cocaine, were biphasic, with maximal rates of

TABLE 1

K_i values and Hill slopes derived from inhibition of binding of radioligand labeling σ_1 and σ_2 receptors

Values in parentheses are 95% confidence limits. The value below the name of each compound is the K_i value at the DAT for displacement of [3 H]WIN35,428 obtained in a previous study (Katz et al., 2004). For the [3 H]DTG assay, only data for the high-affinity site are displayed.

Compound	σ_1 R K_i Value [3 H](+)-Pentazocine	Hill Slope	σ_2 R K_i Value [3 H]DTG	Hill Slope
	nM		nM	
JHW007 (DAT: 12.0 nM)	2.40 (2.07–2.80)	1.38 (1.19–1.56)	12.0 (10.0–14.4)	0.759 (0.646–0.873)
AHN2-005 (DAT: 8.82 nM)	15.5 (13.2–18.3)	1.75 (1.48–2.01)	28.5 (23.6–34.4)	0.680 (0.584–0.776)
AHN1-055 (DAT: 4.09 nM)	119 (98.9–142)	0.980 (0.819–1.14)	78.7 (50.6–122)	0.630 (0.535–0.724)

TABLE 2

Statistical analyses of dose-effect curves for cocaine or substitution of various compounds for cocaine compared with saline availability as shown in Fig. 2 and 4.

Comparisons were made in rates of responses on the active lever maintained during each corresponding component using a two-way repeated-measures ANOVA followed by post hoc Bonferroni *t* tests with results shown if effects were statistically significant.

Treatment	Drug (vs Saline)	Dose	Interaction	Post-Hoc Test
Cocaine versus saline substitution	$F_{1,64} = 22.5$; $P < 0.001$	$F_{4,64} = 18.9$; $P < 0.001$	$F_{4,64} = 19.7$; $P < 0.001$	0.10 mg/kg per inj ($t = 5.01$, $P < 0.001$) 0.32 mg/kg per inj ($t = 8.84$, $P < 0.001$)
DTG versus saline substitution	$F_{1,64} = 28.8$; $P < 0.001$	$F_{4,64} = 22.8$; $P < 0.001$	$F_{4,64} = 23.8$; $P < 0.001$	0.32 mg/kg per inj ($t = 4.97$, $P < 0.001$) 1.0 mg/kg per inj ($t = 10.1$, $P < 0.001$)
<i>R</i> (+)-SKF 81297 versus saline substitution	$F_{1,20} = 14.4$; $P = 0.013$	$F_{4,20} = 14.1$; $P < 0.001$	$F_{4,20} = 13.2$; $P < 0.001$	1.0 μ g/kg per inj ($t = 5.40$, $P < 0.001$) 3.2 μ g/kg inj ($t = 6.12$, $P < 0.001$)
($\pm$)-SKF 82958 versus saline substitution	$F_{1,20} = 16.3$; $P = 0.010$	$F_{4,20} = 11.5$; $P < 0.001$	$F_{4,20} = 13.1$; $P < 0.001$	1.0 μ g/kg per inj ($t = 5.29$, $P < 0.001$) 3.2 μ g/kg per inj ($t = 6.54$, $P < 0.001$)
<i>R</i> (-)-NPA versus saline substitution	$F_{1,20} = 7.25$; $P = 0.043$	$F_{4,20} = 7.27$; $P < 0.001$	$F_{4,20} = 6.92$; $P = 0.001$	0.32 μ g/kg per inj ($t = 3.11$, $P = 0.006$) 1.0 μ g/kg per inj ($t = 5.02$, $P < 0.001$)
(-)-Quinpirole versus saline substitution	$F_{1,20} = 5.04$; $P = 0.075$	$F_{4,20} = 3.55$; $P = 0.024$	$F_{4,20} = 3.50$; $P = 0.025$	32 μ g/kg per inj ($t = 4.10$, $P < 0.001$)
Remifentanil versus saline substitution	$F_{1,20} = 7.64$; $P = 0.040$	$F_{4,20} = 6.34$; $P = 0.002$	$F_{4,20} = 6.69$; $P = 0.001$	0.32 μ g/kg per inj ($t = 3.70$, $P = 0.002$) 1.0 μ g/kg per inj ($t = 4.55$, $P < 0.001$)

Inj, injection.

response ranging from 0.234 (S.E.M. = 0.038) responses per second, obtained with *R*(+)-SKF 81297 to 0.577 (S.E.M. = 0.126) responses per second, with remifentanil (Fig. 4, filled points). Statistical analyses indicated significant differences between substitution of each of the drugs compared with saline, dose of the self-administered drug, and their interaction (Table 2). Post hoc tests indicated significance compared with saline substitution with at least two doses per injection for each compound, with the exception of (-)-quinpirole, which was only significant at one dose (Table 2).

In contrast to cocaine, self-administration of each of these drugs was largely insensitive to the BZT analogs. Most importantly, the dose-related decreases in maximum self-administration observed with cocaine self-administration

were uniformly absent with self-administration of the DA agonists and remifentanil (Fig. 4, compare open and filled points). Nonetheless, statistical analyses indicated significant effects (as expected) with dose of the self-administered drug, as well as with pretreatment dose and the interaction terms (Table 4). Post hoc analyses for those instances in which there was a significant effect of pretreatment dose showed that, for AHN1-055, the statistical significance was largely attributed to increases in response during EXT and at the lowest doses per injection (Fig. 4, left column; Table 4). Those increases were less evident with remifentanil self-administration. The response rates during EXT and at the lowest remifentanil dose were increased by 3.2 mg/kg of AHN1-055; however, those effects did not achieve statistical significance

Fig. 3. Effects of pre-session treatments with the *N*-substituted BZT analogs on self-administration of cocaine under an FR 5-response schedule of reinforcement. Ordinates: Responses per second; abscissae: cocaine unit dose (mg/kg per i.v. injection), log scale. Each point represents the mean $\pm$ S.E.M. of response rates on the active lever in six subjects. A dose of 0 mg/kg of each test compound ($\bullet$) indicates pre-session treatment with saline injection. The other pretreatment doses are as shown in the key. AHN1-055, AHN2-005, and JHW007 were administered i.p. at 90, 120, and 150 minutes before sessions, respectively. (A) Effects of AHN1-055. (B) Effects of AHN2-005. (C) Effects of JHW007. Note that the *N*-substituted BZT analogs dose dependently decreased the maximal rates of response maintained by cocaine injections (0.32 mg/kg per injection).

TABLE 3

Statistical analyses of effects of pretreatments on self-administration of cocaine as shown in Fig. 3

Comparisons were made in the rates of responses on the active lever maintained during each corresponding component in six subjects using a two-way repeated-measures ANOVA followed by post-hoc Bonferroni *t* tests. The post hoc results are shown only if effects were statistically significant. The Δ values, in responses/second, were calculated as a subtraction of response rates maintained by cocaine after pretreatment with the BZT analog from those obtained after saline pretreatment.

Pretreatment	Cocaine Unit Dose	Pretreatment Dose	Interaction	Post-Hoc Tests
AHN 1-055	$F_{4,60} = 5.91$; $P = 0.003$	$F_{3,60} = 7.56$; $P = 0.003$	$F_{12,60} = 8.57$; $P < 0.001$	3.2 mg/kg (i.p.) at EXT, $t = 3.08$, $P = 0.009$ ($\Delta = +0.080$) 3.2 mg/kg (i.p.) at 0.032 mg/kg per inj (i.v.), $t = 4.41$, $P < 0.001$ ($\Delta = +0.114$) 3.2 mg/kg (i.p.) at 0.10 mg/kg per inj (i.v.), $t = 3.54$, $P = 0.002$ ($\Delta = -0.092$) 3.2 mg/kg (i.p.) at 0.32 mg/kg per inj (i.v.), $t = 6.08$, $P < 0.001$ ($\Delta = -0.157$) 10 mg/kg (i.p.) at 0.10 mg/kg per inj (i.v.), $t = 4.64$, $P < 0.001$ ($\Delta = -0.120$) 10 mg/kg (i.p.) at 0.32 mg/kg per inj (i.v.), $t = 5.85$, $P < 0.001$ ($\Delta = -0.151$)
AHN 2 -005	$F_{4,60} = 13.7$; $P < 0.001$	$F_{3,60} = 14.4$; $P < 0.001$	$F_{12,60} = 11.5$; $P < 0.001$	1.0 mg/kg (i.p.) at 0.10 mg/kg per inj (i.v.), $t = 3.91$, $P = 0.001$ ($\Delta = -0.087$) 3.2 mg/kg (i.p.) at 0.10 mg/kg per inj (i.v.), $t = 7.10$, $P < 0.001$ ($\Delta = -0.158$) 3.2 mg/kg (i.p.) at 0.32 mg/kg inj (i.v.), $t = 7.14$, $P < 0.001$ ($\Delta = -0.159$) 10 mg/kg (i.p.) at 0.10 mg/kg per inj (i.v.), $t = 7.22$, $P < 0.001$ ($\Delta = -0.161$) 10 mg/kg (i.p.) at 0.32 mg/kg/inj (i.v.), $t = 9.37$, $P < 0.001$ ($\Delta = -0.208$)
JHW 007	$F_{4,60} = 7.31$; $P < 0.001$	$F_{3,60} = 8.52$; $P = 0.002$	$F_{12,60} = 7.01$; $P < 0.001$	3.2 mg/kg (i.p.) at 0.10 mg/kg/inj (i.v.), $t = 4.87$, $P < 0.001$ ($\Delta = -0.127$) 3.2 mg/kg (i.p.) at 0.32 mg/kg per inj (i.v.), $t = 5.23$, $P < 0.001$ ($\Delta = -0.137$) 10 mg/kg (i.p.) at 0.10 mg/kg per inj (i.v.), $t = 5.08$, $P < 0.001$ ($\Delta = -0.133$) 10 mg/kg (i.p.) at 0.32 mg/kg per inj (i.v.), $t = 6.91$, $P < 0.001$ ($\Delta = -0.180$)

Inj, injection.

(Table 4). Occasional significant post hoc analyses (e.g., AHN1-055 with quinpirole self-administration, AHN2-005 with *R*(-)-NPA self-administration, and JHW007 with *R*(+)-SKF 81297, (+)-SKF 82958, and *R*(-)-NPA self-administration, Table 4) were obtained at other dose combinations. These effects were relatively small compared with the decreases observed with cocaine self-administration (compare Fig. 3 and Fig. 4; see also Δ values in Table 4).

As described above, when DTG was substituted for cocaine, self-administration was maintained in a manner similar to that seen with cocaine, although with 3-fold lower potency (Figs. 2 and filled symbols in Fig. 5). Pretreatments with AHN 1-055 produced a dose-related, leftward shift in the DTG self-administration dose-effect curve (Fig. 5A). The 0.32-mg/kg dose was largely ineffective, whereas doses of 1.0 and 3.2 mg/kg decreased maximal response rates maintained by DTG, and the 3.2 mg/kg dose increased response rates at 0.1 mg/kg/injection of DTG. There was also a trend, although not statistically significant, for increases in response rates during EXT (Fig. 5A). Statistical analysis indicated a significant effect of DTG dose per injection, AHN1-055 dose, and the interaction of the two, with post hoc tests confirming the specific changes at DTG doses detailed above (Table 5).

In contrast, both AHN2-005 (Fig. 5B) and JHW007 (Fig. 5C) produced dose-related decreases in the maximal self-administration of DTG. Both compounds decreased maximal response rates maintained by DTG injections, without indications of a leftward shift as obtained with AHN1-055. The decreases in maximal rates of response maintained by DTG were dose-dependent; at the highest doses of each BZT analog, response rates maintained by DTG were decreased to those obtained during EXT (Fig. 5, B and C) or with saline injection (Fig. 2, open circles). Statistical analyses indicated significant effects of unit dose of self-administered DTG, pretreatment dose of either BZT analog, and their interactions (Table 5). Post hoc tests indicated the significance of effects at 1.0- and 3.2-mg/kg pretreatment doses at the 1.0 mg/kg per injection dose of DTG that maintained maximal response rates (Table 5).

The potencies of each of the BZT analogs in decreasing the maximal self-administration of the various compounds studied are shown in Fig. 6. Each of the BZT analogs produced

dose-related decreases in the maximal response rates maintained by cocaine and DTG. In contrast, the compounds were inactive in decreasing the maximal response rates maintained by the DA agonists or remifentanyl (Fig. 6, open symbols). The decreases in DTG self-administration were obtained at doses approximately threefold lower than those that decreased cocaine self-administration (Fig. 6).

Discussion

Each of the BZT analogs examined in the present study had affinity for both σ_1 and σ_2 receptors. As these compounds have also been shown to antagonize cocaine self-administration (Hiranita et al., 2009; Velázquez-Sánchez et al., 2009; Li et al., 2013), the present study sought to assess the contribution of in vivo σ R antagonist actions to this antagonism. Unfortunately, there is no commonly accepted assay demonstrating in vivo σ R antagonist activity (see Katz et al., 2017 for a review); however, recent studies of σ R agonist self-administration have provided a means to distinguish σ R agonists from antagonists, as well as σ R subtype selectivity (Hiranita et al., 2010; Katz et al., 2016). Using blockade of DTG self-administration, the present study demonstrated that each of the BZT analogs had dose-related effects on both cocaine and DTG self-administration. Further, consistent with previous studies, the BZT analogs were effective in blocking the self-administration of cocaine (Hiranita et al., 2009), but they were inactive in blocking self-administration of selective DA agonists or the μ -opioid agonist, remifentanyl. This selectivity suggests that the DAT, an initial target of cocaine but not the other compounds, is critically involved in the actions of the BZT analogs in blocking stimulant self-administration.

The similarity of the effects of the BZT analogs in blocking cocaine and DTG self-administration and the previous report of cocaine's σ R affinity (Sharkey et al., 1988) prompt a question of what action of cocaine is antagonized by the BZT analogs. Previous studies with selective σ R antagonists have found little, if any, effect on cocaine self-administration (Martin-Fardon et al., 2007; Hiranita et al., 2010, 2011, 2013; Katz et al., 2016); however, σ R antagonists that also have DAT affinity can selectively block cocaine self-administration, suggesting that actions at both proteins is sufficient for a blockade of stimulant

Fig. 4. Effects of presession treatments with the *N*-substituted BZT analogs on self-administration of the direct DA D_1 -like [*R*(+)-SKF 81297 and ($\pm$)-SKF 82958], D_2 -like [*R*(-)-NPA and (-)-quinpirole] or μ -opioid (remifentanyl) receptor agonists in rats trained to self-administer cocaine (0.032–1.0 mg/kg per i.v. injection) under an FR 5-response schedule of reinforcement. Each point represents the mean $\pm$ S.E.M of response rates on the active lever in six subjects. Details are as in Fig. 3. (A) Effects of AHN1-055 on *R*(+)-SKF 81297 substitution for cocaine. (B) Effects of AHN2-005 on *R*(+)-SKF 81297 substitution for cocaine. (C) Effects of JHW007 on *R*(+)-SKF 81297 substitution for cocaine. (D) Effects of AHN1-055 on ($\pm$)-SKF 82958 substitution for cocaine. (E) Effects of AHN2-005 on ($\pm$)-SKF 82958 substitution for cocaine. (F) Effects of JHW007 on ($\pm$)-SKF 82958 substitution for cocaine. (G) Effects of AHN1-055 on *R*(-)-NPA substitution for cocaine. (H) Effects of AHN2-005 on *R*(-)-NPA substitution for cocaine. (I) Effects of JHW007 on *R*(-)-NPA substitution for cocaine. (J) Effects of AHN1-055 on (-)-quinpirole substitution for cocaine. (K) Effects of AHN2-005 on (-)-quinpirole substitution for cocaine. (L) Effects of JHW007 on (-)-quinpirole substitution for cocaine. (M) Effects of AHN1-055 on remifentanyl substitution for cocaine. (N) Effects of AHN2-005 on remifentanyl substitution for cocaine. (O) Effects of JHW007 on remifentanyl substitution for cocaine. Note that, in contrast to cocaine self-administration (Fig. 3), none of the doses of *N*-substituted BZT analogs appreciably antagonized self-administration of each direct DA or μ -opioid receptor agonist.

self-administration (Hiranita et al., 2011), with more recent studies pointing toward molecular bases for σ_1 R and DAT interactions (see below).

The effects of AHN1-055 differed from those of the other BZT analogs, with leftward shifts in both cocaine and DTG dose-effect curves. AHN1-055 also increased response rates under the extinction conditions for many of the drugs, suggesting

that these changes may have been simply response-rate increasing effects; however, the effects with DTG were greater than those during extinction, indicating specific leftward shifts for DTG and possibly cocaine. The differences in the effects of AHN1-055 compared with the other BZT analogs seem unlikely to be due to differences among the compounds in their affinity at DAT, as those values are relatively similar, differing at most by 3-fold

TABLE 4

Statistical analyses of pretreatments with BZT analogs on self-administration of $R(+)$ -SKF 81297, $(\pm)$ -SKF 82958, $R(-)$ -NPA, $(-)$ -quinpirole, and remifentanyl as shown in Fig. 4. Comparisons were made in rates of responses on the active lever maintained during each component compared with corresponding components after pretreatments with saline using a two-way repeated-measures ANOVA followed by post-hoc Bonferroni t tests. The post-hoc results are shown only if effects were statistically significant. The Δ values, in responses/second, were calculated as a subtraction of response rates maintained by cocaine after pretreatment with the BZT analog from those obtained after saline pretreatment.

Treatment	Self-Administered Drug Unit Dose	Pretreatment Dose	Interaction	Post Hoc Tests
AHN 1-055 pretreatments (i.p.) $R(+)$ -SKF 81297 self-administration	$F_{4,60} = 12.1$; $P < 0.001$	$F_{3,60} = 5.17$; $P = 0.012$	$F_{12,60} = 3.70$; $P < 0.001$	3.2 mg/kg (i.p.) at EXT, $t = 4.15$, $P < 0.001$ ($\Delta = +0.109$) 3.2 mg/kg (i.p.) at 0.32 μ g/kg per inj (i.v.), $t = 4.47$, $P < 0.001$ ($\Delta = +0.118$)
$(\pm)$ -SKF 82958 self-administration	$F_{4,60} = 12.0$; $P < 0.001$	$F_{3,60} = 3.44$; $P = 0.044$	$F_{12,60} = 3.27$; $P = 0.001$	3.2 mg/kg (i.p.) at EXT, $t = 3.54$, $P = 0.002$ ($\Delta = +0.156$) 3.2 mg/kg (i.p.) at 0.32 μ g/kg per inj (i.v.), $t = 4.20$, $P < 0.001$ ($\Delta = +0.185$)
$R(-)$ -NPA self-administration	$F_{4,60} = 6.60$; $P = 0.001$	$F_{3,60} = 9.48$; $P < 0.001$	$F_{12,60} = 8.33$; $P < 0.001$	3.2 mg/kg (i.p.) at EXT, $t = 6.18$, $P < 0.001$ ($\Delta = +0.101$) 3.2 mg/kg (i.p.) at 0.10 μ g/kg per inj (i.v.), $t = 5.97$, $P < 0.001$ ($\Delta = +0.097$)
$(-)$ -Quinpirole self-administration	$F_{4,60} = 3.64$; $P = 0.022$	$F_{3,60} = 5.48$; $P = 0.010$	$F_{12,60} = 4.80$; $P < 0.001$	3.2 mg/kg (i.p.) at EXT, $t = 4.61$, $P < 0.001$ ($\Delta = +0.169$) 3.2 mg/kg (i.p.) at 0.32 μ g/kg per inj (i.v.), $t = 4.35$, $P < 0.001$ ($\Delta = +0.159$)
Remifentanyl self-administration	$F_{4,60} = 6.87$; $P = 0.001$	$F_{3,60} = 1.59$; $P = 0.234$	$F_{12,60} = 3.81$; $P < 0.001$	10 mg/kg (i.p.) at 32 μ g/kg per inj (i.v.), $t = 3.41$, $P = 0.003$ ($\Delta = -0.125$) 3.2 mg/kg (i.p.) at 0.32 μ g/kg per inj (i.v.), $t = 3.18$, $P = 0.007$ ($\Delta = -0.096$)
AHN 2-005 pretreatments (i.p.) $R(+)$ -SKF 81297 self-administration	$F_{4,60} = 13.3$; $P < 0.001$	$F_{3,60} = 2.28$; $P = 0.121$	$F_{12,60} = 4.08$; $P < 0.001$	3.2 mg/kg (i.p.) at 1.0 μ g/kg per inj (i.v.), $t = 4.28$, $P < 0.001$ ($\Delta = -0.129$) 10 mg/kg (i.p.) at 1.0 μ g/kg per inj (i.v.), $t = 3.68$, $P = 0.002$ ($\Delta = -0.111$)
$(\pm)$ -SKF 82958 self-administration	$F_{4,60} = 12.7$; $P < 0.001$	$F_{3,60} = 2.38$; $P = 0.110$	$F_{12,60} = 3.84$; $P < 0.001$	1.0 mg/kg (i.p.) at 3.2 μ g/kg per inj (i.v.), $t = 4.87$, $P < 0.001$ ($\Delta = +0.045$) 10 mg/kg (i.p.) at 1.0 μ g/kg per inj (i.v.), $t = 3.94$, $P = 0.001$ ($\Delta = -0.037$)
$R(-)$ -NPA self-administration	$F_{4,60} = 6.82$; $P = 0.001$	$F_{3,60} = 4.85$; $P = 0.015$	$F_{12,60} = 5.74$; $P < 0.001$	1.0 mg/kg (i.p.) at 3.2 μ g/kg per inj (i.v.), $t = 3.41$, $P = 0.003$ ($\Delta = -0.024$) 10 mg/kg (i.p.) at 1.0 μ g/kg per inj (i.v.), $t = 3.97$, $P = 0.001$ ($\Delta = -0.028$)
$(-)$ -Quinpirole self-administration	$F_{4,60} = 3.81$; $P = 0.018$	$F_{3,60} = 3.19$; $P = 0.054$	$F_{12,60} = 2.65$; $P = 0.007$	1.0 mg/kg (i.p.) at 0.32 μ g/kg per inj (i.v.), $t = 5.85$, $P < 0.001$ ($\Delta = -0.029$) 3.2 mg/kg (i.p.) at 1.0 μ g/kg per inj (i.v.), $t = 5.61$, $P < 0.001$ ($\Delta = +0.028$)
Remifentanyl self-administration	$F_{4,60} = 6.78$; $P = 0.001$	$F_{3,60} = 3.17$; $P = 0.055$	$F_{12,60} = 2.92$; $P = 0.003$	1.0 mg/kg (i.p.) at 32 μ g/kg per inj (i.v.), $t = 2.74$, $P = 0.023$ ($\Delta = -0.061$) 1.0 mg/kg (i.p.) at 0.32 μ g/kg per inj (i.v.), $t = 3.05$, $P = 0.011$ ($\Delta = -0.077$)
JHW 007 pretreatments (i.p.) $R(+)$ -SKF 81297 self-administration	$F_{4,60} = 13.5$; $P < 0.001$	$F_{3,60} = 8.89$; $P = 0.001$	$F_{12,60} = 6.88$; $P < 0.001$	1.0 mg/kg (i.p.) at 1.0 μ g/kg per inj (i.v.), $t = 3.19$, $P = 0.007$ ($\Delta = -0.081$) 3.2 mg/kg (i.p.) at 1.0 μ g/kg per inj (i.v.), $t = 2.97$, $P = 0.013$ ($\Delta = -0.076$) 10 mg/kg (i.p.) at 1.0 μ g/kg per inj (i.v.), $t = 5.28$, $P < 0.001$ ($\Delta = -0.134$)

(continued)

TABLE 4—Continued

Treatment	Self-Administered Drug Unit Dose	Pretreatment Dose	Interaction	Post Hoc Tests
(±)-SKF 82958 self-administration	$F_{4,60} = 12.4$; $P < 0.001$	$F_{3,60} = 6.83$; $P = 0.004$	$F_{12,60} = 4.98$; $P < 0.001$	10 mg/kg (i.p.) at 3.2 µg/kg per inj (i.v.), $t = 4.81$, $P < 0.001$ ($\Delta = -0.049$)
				1.0 mg/kg (i.p.) at 1.0 µg/kg per inj (i.v.), $t = 4.99$, $P < 0.001$ ($\Delta = +0.036$)
R(-)-NPA self-administration	$F_{4,60} = 7.51$; $P < 0.001$	$F_{3,60} = 3.70$; $P = 0.036$	$F_{12,60} = 4.56$; $P < 0.001$	10 mg/kg (i.p.) at 3.2 µg/kg per inj (i.v.), $t = 4.94$, $P < 0.001$ ($\Delta = -0.036$)
				1.0 mg/kg (i.p.) at 0.32 µg/kg per inj (i.v.), $t = 2.83$, $P = 0.036$ ($\Delta = -0.015$)
				3.2 mg/kg (i.p.) at 0.32 µg/kg per inj (i.v.), $t = 4.37$, $P < 0.001$ ($\Delta = -0.023$)
				10 mg/kg (i.p.) at 0.32 µg/kg per inj (i.v.), $t = 3.35$, $P = 0.008$ ($\Delta = -0.018$)
(–)-Quinpirole self-administration Remifentanyl self-administration	$F_{4,60} = 3.51$; $P = 0.025$	$F_{3,60} = 2.11$; $P = 0.142$	$F_{12,60} = 1.72$; $P = 0.086$	10 mg/kg (i.p.) at 1.0 µg/kg per inj (i.v.), $t = 4.38$, $P < 0.001$ ($\Delta = -0.023$)
	$F_{4,60} = 7.08$; $P = 0.001$	$F_{3,60} = 2.42$; $P = 0.106$	$F_{12,60} = 1.95$; $P = 0.046$	1.0 mg/kg (i.p.) at 1.0 µg/kg per inj (i.v.), $t = 3.48$, $P = 0.006$ ($\Delta = -0.098$)
				3.2 mg/kg (i.p.) at 1.0 µg/kg per inj (i.v.), $t = 4.22$, $P < 0.001$ ($\Delta = -0.119$)
				10 mg/kg (i.p.) at 1.0 µg/kg per inj (i.v.), $t = 3.55$, $P = 0.005$ ($\Delta = -0.100$)

(Katz et al., 2004). Larger differences were obtained in their affinities at σ_1 Rs, with AHN1-055 much less potent than the other BZT analogs, which differed from each other by only 6-fold. Possibly more important is the ratio of σ_1 R to DAT affinity. There was an approximate 30-fold difference in these affinities with AHN1-055, whereas that ratio was less than 2-fold for the other compounds. Interestingly, rimcazone and two of its analogs that blocked cocaine self-administration differed from each other in affinities at σ_1 Rs by about 50-fold, and yet each compound had a less than 10-fold difference in DAT and σ_1 R affinities (Hiranita et al., 2011). Thus, past and present results suggest that there may be a critical relation between these affinities for antagonism, although further studies are needed to confirm this suggestion.

The antagonism of the self-administration of cocaine and DTG by AHN2-005 and JHW007 was characterized by a decrease in maximal self-administration rather than a rightward shift in the dose-effect curve. Whether that change is surmountable is not presently clear. It is noteworthy that the interaction with DTG self-administration was similar to that of cocaine with *selective* σ_1 R antagonists combined with DAT inhibitors (Katz et al., 2016). As self-administration dose-effect curves are typically biphasic, it is likely that multiple mechanisms are involved (see, e.g., Katz, 1989). Previous studies have indicated that a surmountable interaction with only the ascending limb of the biphasic dose-effect curve results in an interaction characterized by a decrease in the maximum (Collins et al., 2005).

Interestingly, the Hill slopes obtained for the binding of JHW007 and AHN2-005 to σ_1 Rs were significantly steeper than 1.0, suggesting positive cooperativity. It is possible that the positive cooperativity results from actions at another protein. A number of studies have suggested protein-protein interactions involving σ_1 Rs (Tsai et al., 2012). For example, effects of cocaine on DA D1- or D2-mediated intracellular cascades were absent in tissue from mice with genetic deletions of σ_1 Rs (Navarro et al., 2010, 2013). The authors suggested DA and σ_1 R heteromers as accounting for these effects. How those in vitro interactions translate to in vivo effects is not presently clear, and the present study found no evidence of that interaction with the reinforcing effects of either D1- or D2-like direct-acting DA agonists.

On the other hand, there is growing evidence of interactions of σ_1 Rs and the DAT that may relate to the present findings. For example, Lin et al. (2012) found that activation of σ_1 Rs blocked a DAT-mediated increase in the firing of DA neurons after methamphetamine administration. Patch-clamp and amperometry in cells and in vivo chronoamperometry revealed a σ_1 R-mediated decrease in methamphetamine-stimulated DA efflux. Another study (Hong et al., 2017) showed that the σ_1 R agonists (+)-pentazocine and PRE-084 increased the $B_{\max}$ values of [3 H] WIN35,428 in radioligand binding assays both in cultured cells and rat striatal synaptosomes. Further, coimmunoprecipitation and bioluminescence resonance energy-transfer assays indicated an interaction between σ_1 R and DAT in transfected cells. Moreover, cysteine accessibility assays showed that exposure to σ_1 R agonists induced a change in conformation equilibrium favoring an outward facing DAT conformation, which would facilitate the effects of cocaine. Finally, (+)-pentazocine decreased, whereas the selective σ_1 R antagonist CM304 increased, σ_1 R multimerization, suggesting that the oligomerization status of σ_1 Rs modulates the interaction with membrane-bound proteins such as the DAT (Hong et al., 2017).

Fig. 5. Effects of precession treatments with the *N*-substituted BZT analogs on self-administration of a nonselective σ R agonist DTG in rats trained to self-administer cocaine (0.032–1.0 mg/kg/injection, i.v.) under an FR 5-response schedule of reinforcement. Each point represents the mean $\pm$ S.E.M. of response rates on the active lever in six subjects. Details are as in Fig. 3. (A) Effects of AHN1-055 on DTG substitution for cocaine. (B) Effects of AHN2-005 on DTG substitution for cocaine. (C) Effects of JHW007 on DTG substitution for cocaine. Note that, as with cocaine self-administration (Fig. 3, B and C), AHN2-005 and JHW007 dose dependently decreased the maximal rate of responses maintained by DTG injections (1.0 mg/kg per injection). In contrast to cocaine self-administration (Fig. 3A), AHN1-055 uniformly left-shifted the dose-effect curves of DTG self-administration.

These data together support a hypothesis of protein-protein interactions between σ_1 Rs and the DAT, which serve as a mechanistic basis for their interactions and may underlie the effects reported here.

Reith et al. (2015) proposed three pharmacologic aspects of DAT inhibitors that may contribute to atypical effects differing from those of cocaine. Studies of in vivo and in vitro effects of BZT analogs showed that rates of DAT occupancy were retarded compared with those of cocaine (Desai et al., 2005a,b; Kopajtic et al., 2010). Further, the amount of cocaine-induced locomotor stimulation was disproportionately greater than that predicted by occupancy, suggesting that rate of association was involved in its behavioral effects (Desai et al., 2005a,b). A slow association with DAT may enable efficacy of compensatory responses dampening behavioral effects that would accrue from an unmitigated cascade of events consequent to DAT binding and DA overflow

(Kohut et al., 2014). Other studies, however, have identified atypical DAT inhibitors with a relatively fast onset of behavioral actions (Li et al., 2011; Reith et al., 2012), indicating that a slow association rate may be sufficient, but not necessary, for atypical effects of DAT inhibitors.

Several studies using substituted-cysteine accessibility methods have indicated that standard DA-uptake inhibitors shift DAT conformation equilibrium toward accessibility from extracellular space (outward-open), whereas several atypical DAT inhibitors shift that equilibrium toward cytosol-open conformation (Reith et al., 2001; Loland et al., 2008; see review by Reith et al., 2015); however, several DAT inhibitors promote outward-facing conformations but retain atypical effects (Reith et al., 2012; Hiranita et al., 2014b; Hong et al., 2016). In the study by Hong et al. (2016), several 3β -aryltropanes derivatives with DAT affinity had behavioral effects distinct from those of

TABLE 5

Statistical analyses of effects of pretreatments on self-administration of DTG as shown in Fig. 5

Comparisons were made in the response rates of responses on the active lever maintained during each component compared with corresponding components after pretreatments with saline using a two-way repeated-measures ANOVA followed by post hoc Bonferroni *t* tests. The post hoc results are shown only if effects were statistically significant. The Δ values, in responses/second, were calculated as a subtraction of response rates maintained by cocaine after pretreatment with the BZT analog from those obtained after saline pretreatment.

Pretreatment	Self-Administered DTG Unit Dose	Pretreatment Dose	Interaction	Post-Hoc Tests
AHN 1-055	$F_{4,60} = 6.30$; $P = 0.002$	$F_{3,60} = 5.83$; $P = 0.008$	$F_{12,60} = 5.85$; $P < 0.001$	1.0mg/kg (i.p.) at 1.0 mg/kg/inj (i.v.), $t = 4.03$, $P < 0.001$ ($\Delta = -0.304$)
				3.2 mg/kg (i.p.) at 0.1 mg/kg/inj (i.v.), $t = 5.13$, $P < 0.001$ ($\Delta = +0.388$)
				3.2 mg/kg (i.p.) at 1.0 mg/kg/inj (i.v.), $t = 4.47$, $P < 0.001$ ($\Delta = -0.338$)
AHN 2-005	$F_{4,60} = 9.41$; $P < 0.001$	$F_{3,60} = 8.20$; $P = 0.002$	$F_{12,60} = 6.13$; $P < 0.001$	1.0mg/kg (i.p.) at 1.0 mg/kg/inj (i.v.), $t = 4.63$, $P < 0.001$ ($\Delta = -0.180$)
				3.2 mg/kg (i.p.) at 1.0 mg/kg/inj (i.v.), $t = 7.82$, $P < 0.001$ ($\Delta = -0.303$)
JHW 007	$F_{4,60} = 10.2$; $P < 0.001$	$F_{3,60} = 8.03$; $P = 0.002$	$F_{12,60} = 7.48$; $P < 0.001$	1.0mg/kg (i.p.) at 1.0 mg/kg/inj (i.v.), $t = 5.27$, $P < 0.001$ ($\Delta = -0.196$)
				3.2 mg/kg (i.p.) at 1.0 mg/kg/inj (i.v.), $t = 8.12$, $P < 0.001$ ($\Delta = -0.302$)

Inj, injection.

Fig. 6. Comparison of potency of pre-session treatments with various compounds tested for decreasing the maximal rates of responses maintained by injections of DTG (1.0 mg/kg per injection), cocaine (0.32 mg/kg per injection), *R*(+)-SKF 81297 (0.0032 mg/kg per injection), (±)-SKF 82958 (0.0032 mg/kg per injection), *R*(-)-NPA (0.001 mg/kg per injection), (-)-quinpirole (0.032 mg/kg per injection), or remifentanyl (0.001 mg/kg per injection) from Figs. 3–5. Ordinates: Response rates as percentage of control response rates (sessions before drug tests), which averaged 0.372 (S.E.M. = 0.066, *n* = 12), 0.244 (S.E.M. = 0.050, *n* = 12), 0.219 (S.E.M. = 0.055, *n* = 6), 0.253 (S.E.M. = 0.065, *n* = 6), 0.247 (S.E.M. = 0.092, *n* = 6), 0.578 (S.E.M. = 0.296, *n* = 6), and 0.583 (S.E.M. = 0.214, *n* = 6) responses/second, respectively, for the maximal rates of responses maintained by injections of DTG, cocaine, *R*(+)-SKF 81297, (±)-SKF 82958, *R*(-)-NPA, (-)-quinpirole, or remifentanyl; abscissae: mg/kg of test compounds administered i.p., log scale. Each point represents the mean ± S.E.M. (*n* = 6). No significant difference was found in the control rates across the drug groups ($F_{6,47} = 1.46$; $P = 0.214$, one-way ANOVA). AHN1-055, AHN2-005, and JHW007 were administered i.p. 90, 120, and 150 minutes before sessions, respectively. A dose of 0 mg/kg of each test compound indicates vehicle injections. (A) Effects of AHN1-055. (B) Effects of AHN2-005. (C) Effects of JHW007.

cocaine, whereas cysteine-accessibility assays suggested the induction of an outward-facing conformation. Further, molecular dynamics of *in silico* inhibitor-DAT complexes suggested that the compounds reached equilibrium in the binding pocket in a manner like that of cocaine (Hong et al., 2016). Thus, an outward-facing DAT-conformation equilibrium shift may also be sufficient but unnecessary for atypical DAT effects.

Results of the present study suggest that σ R antagonist effects of the BZT analogs combined with their actions at the DAT, contribute to their atypical DAT-inhibitor activity. The mechanisms by which actions at σ Rs alter stimulant self-administration may relate to the effects on DAT conformation discussed here. However, as not all atypical DAT inhibitors have high affinity for σ Rs (e.g., Hiranita et al., 2014b), σ R antagonist actions may represent another condition sufficient for atypical DAT inhibitor effects and the blockade of stimulant self-administration.

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Institutes of Health and the National Institute on Drug Abuse Intramural Research Program Animal Care and Use Program, which is fully accredited by AAALAC International.

Authorship Contributions

Participated in research design: Hiranita, Kopajtic, Katz.
Conducted experiments: Hiranita, Kopajtic.
Performed data analysis: Hiranita, Kopajtic, Katz.
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