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Relations between Stimulation of Mesolimbic Dopamine and Place Conditioning in Rats Produced by Cocaine or Drugs that are Tolerant to Dopamine Transporter Conformational Change

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Abstract

Rationale—Dopamine transporter (DAT) conformation plays a role in the effectiveness of cocaine-like and other DAT-inhibitors. Cocaine-like stimulants are intolerant to DAT conformation changes having decreased potency in cells transfected with DAT constructs that face the cytosol compared to wild-type DAT. In contrast, analogs of benztropine (BZT) are among compounds that are less affected by DAT conformational change.

Methods—We compared the displacement of radioligand binding to various mammalian CNS sites, acute stimulation of accumbens shell dopamine levels, and place-conditioning in rats among cocaine and four BZT analogs with Cl-substitutions on the diphenyl-ether system including two with carboalkoxy substitutions at the 2-position of the tropane ring.

Results—Binding assays confirmed high-affinity and selectivity for the DAT with the BZT analogs which also produced significant stimulation of mesolimbic dopamine efflux. Because BZT analogs produced temporal patterns of extracellular dopamine levels different from those by cocaine (3-10 mg/kg, IP), the place conditioning produced by BZT analogs and cocaine was compared at doses and times at which both the increase in dopamine levels and rates of increase were similar to those produced by an effective dose of cocaine. Despite this equilibration, none of the BZT analogs tested produced significant place conditioning.

Conclusions—The present results extend previous findings suggesting that cocaine-like actions are dependent on a binding equilibrium that favors the outward conformational state of the DAT. In contrast BZT analogs with reduced dependence on DAT conformation have reduced cocaine-like behavioral effects and may prove useful in development of medications for stimulant abuse.

Keywords

dopamine transporter conformation; accumbens shell; drug abuse; dopamine microdialysis; place preference; benztropine analogs; cocaine

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INTRODUCTION

The behavioral effects of cocaine result from its ability to stimulate extracellular DA levels in specific brain areas (Dworkin and Smith 1988; Ikemoto 2002; Pontieri et al. 1995; Tanda et al. 1997a; Wise et al. 1995). Blockade of the dopamine (DA)-transporter (DAT) is the main pharmacologic mechanism responsible for cocaine's psychostimulant effects, and it has been postulated that drugs that bind to the DAT and inhibit DA reuptake will have behavioral effects similar to cocaine (Kuhar et al. 1991; Ritz et al. 1989a; Ritz et al. 1987). However there is growing evidence that not all drugs with high affinity for the DAT fully reproduce the effects of cocaine (Newman and Katz 2008; Runyon and Carroll 2008; Tanda et al. 2009b).

Recent studies have suggested that conformational status of the DAT affects its interactions with different DAT inhibitors. A tyrosine residue in position 335 of the DAT was found critical for regulating isomerization between conformations facing inward toward the cytosol or outward toward the extracellular environment (Loland et al. 2002; Reith et al. 2001; Schmitt et al. 2008). When Y335 is mutated to alanine, the DAT conformational equilibrium is shifted to inward-facing and the potency of cocaine-like DAT inhibitors in cells transfected with this DAT is substantially reduced compared to those with the wild-type DAT (Loland et al. 2002). In contrast, other DAT inhibitors are more tolerant of this same change in DAT conformational equilibrium. For example, several BZT analogs as well as analogs of the sigma (σ) receptor antagonist, rimcazole, show a much smaller change in potency in the Y335A mutant compared to the wild-type DAT (Loland et al. 2008). Interestingly, the tolerance to conformational change, as measured by the Y335A mutant to wild-type ratio of potencies for inhibiting DA transport, is related to the degree to which the DAT inhibitors produce cocaine-like effects *in vivo*. Those compounds that are intolerant to this conformational change are much like cocaine, producing substantial locomotor stimulation as well as cocaine-like discriminative-stimulus effects. On the other hand, those DAT inhibitors with binding affinities less effected by DAT this particular conformational change have substantially reduced effectiveness in stimulating locomotor activity and in producing cocaine-like discriminative-stimulus effects (Loland et al. 2008).

Other studies have suggested that BZT analogs have a slower onset of action than cocaine and several standard DAT inhibitors (Desai et al. 2005; Stathis et al. 1995; Tanda et al. 2005; Tanda et al. 2009a). For example, studies comparing effects on extracellular DA levels of JHW 007, a BZT analog that is relatively tolerant to DAT conformational status induced by the Y335A, with cocaine and its analog, WIN 35,428, showed a slower onset of effects with JHW 007 (Tanda et al. 2009a). A number of previous studies have indicated that a slow onset of effects reduces some of the behavioral effects of cocaine. For example, Balster and Schuster (1973) found that slowing the rate of the injection in a cocaine self-administration procedure had effects similar to decreasing the dose injected. More recently, Samaha et al. (2002; 2004) found that the rate of delivery influenced the degree to which cocaine produced sensitization, blocked DA reuptake, and induced immediate early gene expression. In order to more fully compare actions of drugs with and without tolerance to DAT conformation, and because the drugs had different time courses of effects on DA concentrations, place-conditioning studies were conducted with extended pre-treatment times. This allowed comparisons at times and doses of the BZT analogs at which DA was increased to levels comparable to those produced by cocaine.

The present study compared several DAT inhibitors across doses and times after injection. The behavioral focus of the present study was on reinforcing effects of these drugs. Because drug self-administration is especially dependent on a temporal contingency between responses and injections, we used a respondent place-conditioning procedure, to study

reinforcement because its drug-place pairings can accommodate drugs with slower onsets of effects by adjusting the time between injection and drug-place pairings (De Beun et al. 1992; Li et al. 2005).

The BZT-analogs that were compared to cocaine all had Cl-substitutions on the diphenylether system (See Fig. 1). The 4-Cl, and 4,4-diCl substituted analogs (Newman et al. 1995) were studied along with two 4,4-diCl substituted analogs (MFZ 4-86, MFZ 4-87) that also had carboalkoxy substitutions at the 2-position on the tropane ring (Zou et al. 2006). In addition, both 4-Cl-BZT and MFZ 4-86 are among those that have been assessed and found to be more tolerant than cocaine to DAT conformational status (Loland et al. 2008). The present study also extended the assessment of the selectivity of these compounds by assessing their displacement of radioligands from a wide variety of mammalian CNS binding sites.

MATERIALS AND METHODS

Radioligand binding assays

All of the compounds were screened for activity at a variety of mammalian binding sites by examining their competition with the appropriate radioligands (MDS Panlabs Pharmacology Services, Bothell, Washington). Each compound was tested in duplicate with reference standards for each assay (listed in Table 1). Significant details of the assay procedures are also provided in Table 1.

The H_1 -receptor and σ -receptor binding assays were conducted in more detail with published methods (Garces-Ramirez et al. 2011). For the H_1 -receptor frozen male Sprague-Dawley rat (Taconic Labs) brains (including cerebellum) were homogenized in 20 volumes (w/v) of 50 mM NaK buffer (37.8 mM Na_2HPO_4 , 12.2 mM KH_2PO_4), pH 7.5, at 25°C using a Brinkman Polytron (setting 6 for 20 sec). Homogenates were centrifuged at $20,000 \times g$ for 10 min at 4°C. The pellet was resuspended in buffer and centrifuged again. The final pellet was re-suspended in buffer to a concentration of 200 mg/ml (OWW).

For the σ -receptor assays frozen whole guinea-pig brains (minus cerebellum) were thawed, weighed and homogenized in 10 mM Tris-HCl with 0.32 M sucrose, pH 7.4 (10 ml/g tissue). The homogenate was centrifuged at $800 \times g$ for 10 min at 4°C; the supernatant was collected and spun at $28,000 \times g$ for 15 min at 4°C. The remaining pellet was re-suspended at 3 ml/g (original wet weight, OWW) in the above buffer, vortexed, and incubated at 25°C (water bath) for 15 min. The tissue was then centrifuged ($28,000 \times g$) for 15 min. The remaining pellet was gently re-suspended in buffer to 80 mg/ml (OWW).

Ligand binding experiments were conducted in polypropylene assay tubes containing 0.5 ml of buffer (Tris-HCl buffer for σ receptors; NaK buffer for H_1 receptors) for 120 (σ receptors) or 60 (H_1 receptors) min at room temperature. For σ_1 receptor assays each tube contained 3 nM [3H](+)-pentazocine (Perkin Elmer Life Science, Boston, MA) and 8.0 mg tissue (OWW) with nonspecific binding determined with 10 M haloperidol. For σ_2 receptor assays each tube contained 3 nM [3H]DTG (Perkin Elmer Life Science), 200 nM (+)-pentazocine, and 8.0 mg tissue (OWW) with nonspecific binding determined using 100 M haloperidol. For studies of H_1 receptors, each tube contained 2 nM [3H]mepyramine (Perkin Elmer Life Science, Boston, MA) and 20 mg tissue (OWW) with nonspecific binding determined using 100 M promethazine.

Incubations were terminated by rapid filtration through Whatman GF/B filters, presoaked in 0.5% (σ receptors) or 0.3 % (H_1 receptors) polyethylenimine, using a Brandel R48 filtering manifold (Brandel Instruments, Gaithersburg, MD). The filters were washed twice with 5 ml

cold buffer and transferred to scintillation vials. Beckman Ready Safe (3.0 ml) was added and the vials were counted 24-hr later using a Beckman 6,000 liquid scintillation counter (Beckman Coulter Instruments, Fullerton, CA) at 50% efficiency. For H_1 and σ_1 receptor binding assays, three independent assays were conducted in triplicate.

In vivo microdialysis

These procedures have been described previously (Tanda et al. 2005; Tanda et al. 2008). Briefly, naïve male Sprague Dawley rats (200 to 250g, Charles River, MA) were housed for at least 1 week prior to their use in these experiments in a temperature- and humidity-controlled room, on a 12-h light/dark cycle (lights on from 0700h). All experiments were conducted during the light phase. Food and water were available at all times except during experimental sessions. Concentric dialysis probes, with a dialysing surface limited to the lowest 1.8 mm portion, were prepared with AN69 membranes (Hospal Dasco, Bologna, Italy), as described previously (Tanda et al. 2007; Tanda et al. 1997a). Probes were implanted under ketamine and xylazine (60.0 and 12.0 mg/kg i.p., respectively) anesthesia, and aimed (randomly across subjects) at the right or left NAC shell (uncorrected coordinates from Paxinos and Watson (1998), in mm, were: Anterior = +2.0, and Lateral = ± 1.1 mm from bregma; Dorso-Ventral = -7.9 mm from dura) (Tanda et al. 2005; Tanda et al. 2007; Tanda et al. 2008; Tanda et al. 1997a). See Figure 2 for probe placements.

Experiments started about 23-24 h after probe implant, in the same hemispherical CMA-120 cages (CMA Microdialysis AB, Solna, Sweden) equipped with overhead fluid swivels (375/D/22QM, Instech Laboratories Inc., Plymouth Meeting, PA) where rats recovered from surgery. Ringer's solution (147.0 mM NaCl, 2.2 mM $CaCl_2$ and 4.0 mM KCl) was infused at 1 μ l/min through the probes by a Pump Controller (BAS West Lafayette, IN, USA). Sample collection started after 30 min. 10- μ l samples were taken every 10 min and immediately analysed. After stable (<10% variability) DA values were obtained for at least three consecutive samples (typically after 1 h), the subjects were then injected with saline, or a test compound. Rats were used only once and received only a single treatment. After 2 hours following treatment, samples were taken every 20 min, and after 4 hours every 30 min, but only 10 μ l of dialysates were analyzed. DA was detected and quantified in dialysate samples with a high-performance liquid chromatography apparatus coupled to electrochemical detection (5200a Coulochem II, or a Coulochem III, ESA, Chelmsford, MA), as reported (Tanda et al. 2007; Tanda et al. 2008). Brains were collected at the end of the experiments, and cut on a vibratome in serial coronal slices to confirm probe placement. Data were only used from experiments in which the probes were confirmed to be within the targeted area (Figure 2, rectangles).

Place Conditioning Studies

These procedures have been described previously (Li et al. 2005). Briefly, naïve male Sprague-Dawley rats (Taconic, Germantown, NY), initially weighing 250 g served as subjects. Place conditioning was conducted in acrylic test chambers (40- × 40-cm) housed within a dimly illuminated room. Chambers were comprised of two equally sized (20- × 40-cm) separate compartments (AccuScan Instruments, Inc., Columbus, OH), one constructed of clear and one of black acrylic, and divided by a removable clear acrylic guillotine door. The floor of the clear compartment was constructed of stainless-steel mesh, under which there was Beta-chip bedding. The floor of the black compartment was constructed of stainless-steel rods under which there was no bedding. Each chamber was placed within a monitor that determined the location of the subject by infrared lights directed at light-sensitive detectors spaced 2.5 cm apart along perpendicular walls. Time spent in each compartment was recorded and generated by the VersaMax system (Accuscan Instruments, Inc., Columbus, OH).

Each subject was habituated to handling before the experiment proper, which had three phases. During a pre-conditioning phase, subjects were placed in the chamber close to the divider between the compartments for each of 4 consecutive 15-min daily sessions. The time allocated by the subject to each compartment was recorded. If an individual subject's time in either compartment during the last session of this phase exceeded 10 min it was removed from the study to ensure that the subject pool was without inherent predispositions for occupancy of a particular compartment (an unbiased design), and a relatively similar baseline for all subjects. During the present studies, about 25% of the subjects were removed during this phase.

During the conditioning phase, different doses of cocaine, test compound, or saline injections were administered before alternate 30-min daily sessions in separate groups of subjects. After injection the subjects were placed individually in one compartment with the access to the other compartment blocked. The compartments (black vs. clear) paired with drug were counterbalanced within each treatment group. The compounds that were injected in alternation with vehicle injections are described below. Studies were conducted with compounds injected immediately, as well as 45- and 90-min before the subjects were placed in the chamber for conditioning sessions. During the 45- or 90-min pretreatment intervals subjects remained in their home cages.

The post-conditioning phase was identical in all respects to the pre-conditioning phase and was conducted 24 h after the last conditioning session.

Because these studies were conducted in different shipments of subjects, cocaine (10 mg/kg) and saline groups were concurrently run as positive and negative controls, respectively. There were no appreciable differences among the shipments in the effects of cocaine or vehicles. When possible, these controls were shared between tests of different compounds to minimize animal usage.

Compounds

The compounds tested were: (–)-cocaine HCl (Sigma and NIDA/IRP pharmacy); 4-Cl-BZT [4'-chloro-3 α -(diphenylmethoxy)tropane] HCl, 4,4-diCl-BZT [4'-chloro-4''-chloro-3 α -(diphenylmethoxy)tropane] HCl, MFZ 4-86 [(±)-2 α -carboethoxy-3R-[bis(4-chlorophenyl)methoxy]tropane], and MFZ 4-87 [(±)-2 α -carbomethoxy-3R-[bis(4-chlorophenyl)methoxy]tropane] (Figure 1) were synthesized in the Medicinal Chemistry Section at the NIDA-IRP (Newman et al. 1995; Zou et al. 2006). S(+)-MFZ 4-86 and S(+)-MFZ 4-87 were used in the free base forms for all in vitro experiments. For the in vivo experiments, the racemic mixtures of MFZ 4-86 or MFZ 4-87 were combined with a molar equivalent of D-tartaric acid and dissolved in H₂O at a concentration of 10 mg/mL. These solutions were made fresh before each experiment. Other compounds were dissolved in saline (0.9% NaCl or in sterile water) and were injected i.p. in a volume of 1.0 ml/kg of body weight.

Data analysis

Radioligand binding data were analyzed by using GraphPad Prism software (San Diego, CA). Inhibition constants (K_i values) were calculated as per Cheng and Prusoff (1973), using historical K_d values of the radioligand from MDS Panlabs Pharmacology Services or from studies conducted in this laboratory for H₁ and σ receptor assays.

For microdialysis studies, serial assays of dialysate DA were normalized as percentage of basal DA values (fmol/10 μ l sample) which were the means of the three consecutive samples immediately preceding injections. Results are presented as group means ($\pm$ SEM). Statistical analyses were carried out using two-way analyses of variance (ANOVA) for

repeated measures over time. Changes were considered to be significant when $p < 0.05$. Results from treatments showing significant overall changes were subjected to a *post-hoc* Tukey's test. Microdialysis data for stimulation of NAC shell DA were previously published for 4-Cl-BZT (Tanda et al. 2005) under experimental conditions identical to those described in the present report. The data collected from the experiments with 4-Cl-BZT have been reanalyzed and presented in Figures 6 and 7 in the present manuscript.

Inflection points in the time course of the increases in DA levels for the BZT analogs were visually identifiable and two linear phases of increases and a third phase in which DA levels remained relatively constant or decreased slightly were identified (see Results). The 0- and 45-min pretreatment times chosen for the place conditioning study corresponded to the first and second of these phases, whereas the 90-min pretreatment time was within the latter part of the second phase of increases in DA levels. The increases in NAC shell DA concentrations at the times corresponding to the conditioning session were calculated as a percentage of basal DA values (as described above). Additionally, the rates of increase in DA concentrations during the identified phases of the DA concentration time course were determined as slopes of the linear regression line fitted to the points to yield a %/min value.

For place-conditioning, the time allocated to the compound-paired compartment during the post-conditioning session was expressed as a difference from that during the last pre-conditioning session. The group means were analyzed by two-way ANOVAs followed by Dunnett's multiple comparisons of treatments versus saline controls. When single doses of cocaine were tested concurrently with a BZT analog, the effects were compared with vehicle controls by unpaired Student's *t* tests for independent samples using the Welch correction for potential unequal variances. Effects with calculated $p < 0.05$ were considered statistically significant.

RESULTS

Binding assays

The binding screen showed that across the 40 sites examined, the 2-carboalkoxy BZT analogs (MFZ 4-86 and 4-87) were DAT-selective (Table 2). Notably, the 2-carbomethoxy (MFZ 4-87) or carboethoxy (MFZ 4-86) substituted analogues had lower binding affinities at H_1 and muscarinic receptors compared to 4-Cl-BZT and 4,4-diCl-BZT. Nonetheless, these compounds had moderate affinities at the K^+ hERG channel, resulting in only a ~20-fold selectivity over the DAT. Finally, 2-position substitutions decreased affinity at σ_1 receptors, though affinity for the σ_2 receptor remained within 20-fold of that for the DAT (Table 2).

Cocaine place conditioning and microdialysis

At each dose cocaine produced significant place conditioning, as evidenced by the increases in time allocated to the cocaine-associated compartment (Figure 3, top; $F(3,118)=19.6$, $p < 0.001$). In addition, conditioning with 10 mg/kg of cocaine conducted concurrently with tests of these BZT analogs produced significant increases in the allocation of time to the cocaine-paired compartment during post-conditioning sessions compared with those for saline injections ($t_{11}=3.29$, $p=0.007$; $t_{22}=4.13$, $p=0.0004$; $t_{13}=2.48$, $p=0.028$; $t_{29}=4.32$, $p=0.0002$ for cocaine with each of the BZT analogs). In contrast, saline did not produce a significant change in time allocation after conditioning. The mean value for the change in time from all saline conditions was $5.55 (\pm 12.6)$ sec.

Cocaine, 3 and 10 mg/kg i.p., significantly increased extracellular DA concentrations in the accumbens shell dialysates (Figure 3, bottom). A two-way ANOVA of DA concentrations in dialysates over the 120 min gave a main effect of dose [$F(2,15)=25.3$, $p < 0.001$], time

[F(12,180)=19.2, $p<0.001$], and their interaction [F(24,180)=6.9 $p<0.001$]. Additionally, the average DA concentration increase produced by cocaine during the 30-min period corresponding to the place-conditioning session (Figure 3, top panel filled bars) was also significant (one-way ANOVA F(2,18)=27.9, $p<0.001$). Post-hoc tests ($p<0.05$) indicated the 165% and 281% increases produced by 3.0 and 10.0 mg/kg of cocaine, respectively, were significantly greater than those after vehicle. Finally, rates of increase in DA concentrations up to the maximum at 3.0 and 10.0 mg/kg of cocaine were 4.84 (± 0.359) and 11.4 (± 0.860) %/min, respectively.

Microdialysis with BZT analogs

4,4-diCl-BZT (1, 3, and 10 mg/kg) produced a significant, dose-dependent increase in dialysate DA levels from the NAC shell (Figure 4A). A two-way ANOVA indicated main effects of dose [F(3,20)=27.2, $p<0.001$], time [F(15,300)=15.9, $p<0.001$], and their interaction [F(45,300)=13.6 $p<0.001$]. Administration of the lowest doses of the compound (1.0 and 3.0 mg/kg) did not significantly affect DA levels, while the highest dose produced a significant increase in DA levels lasting greater than 5 hours (Figure 4A). The concentration of DA exceeded 165% of basal values within 20 min after injection of 10 mg/kg with a rate of increase of 4.36 (± 0.375) %/min during the first 30 min. After that time the increase in DA concentrations continued but at a lower rate until about 140 min after injection, after which concentrations of about 350% of basal levels were maintained for the remainder of the five-hr.

The 2-carboethoxy analog, MFZ 4-86, also dose-dependently increased extracellular DA concentrations in dialysates from the NAC shell (Figure 4B). A two-way ANOVA gave main effects of dose [F(3,20)=45.2, $p<0.001$], time [F(18,360)=13.9, $p<0.001$] and their interaction [F(54,360)=8.6, $p<0.001$]. The increases in DA levels at the 10 mg/kg dose exceeded 165% at 20 min after injection, with a rate of increase of 6.74 (± 0.371) %/min. A transition to a lower rate followed, which continued until about 120 min after injection. DA concentrations remained elevated for the entire five-hr session, though there was a modest decline after 120 min. The 3.0 mg/kg dose of MFZ 4-86 also increased DA concentrations, though less than at the higher dose. DA concentrations initially increased at a rate of 3.05 (± 0.270) %/min, and exceeded 165% of basal values at 50 min after injection. The lowest dose of MFZ 4-86 produced increases in DA that did not achieve statistical significance.

The 2-carbomethoxy analog, MFZ 4-87, also significantly increased DA levels in dialysates (Figure 4C). A two-way ANOVA gave main effects of dose [F(3,13)=25.9, $p<0.001$], time [F(18,234)=24.3, $p<0.001$], and their interaction [F(54,234)=11.0, $p<0.001$]. At the 10 mg/kg dose the initial rate of increase in DA concentrations was 5.59 (± 0.712) %/min, and reached a value greater than 250% of basal values within 30 min, which was followed by further, slower increases that were long-lasting. DA values peaked at about 160 min after administration of 10 mg/kg, with maximum stimulation of about 350% of baseline. Within the entire 4-hr period DA concentrations remained elevated. Lower doses of MFZ 4-87 produced increases in dialysate DA concentrations that did not exceed (1.0 mg/kg) or slightly (3.0 mg/kg) exceeded 165% of basal levels. These smaller effects were equally long lasting (Figure 4C).

Place conditioning with BZT analogs

4-Cl-BZT (1 to 10 mg/kg, i.p.) injected immediately before conditioning sessions did not increase the time allocated to the compound-paired compartment on the post-conditioning test (Figure 5A). Because the increases in dialysate DA concentration varied with time after injection, conditioning was also attempted with injections administered at 45 and 90 min before sessions. As with immediate testing, neither the 45-min (Figure 5A, middle panel)

nor the 90-min (Figure 5A, bottom panel) pretreatment times increased the allocation of time to the compound-paired compartment.

Similarly, both 4,4-diCl-BZT and MFZ 4-86 were without significant place-conditioning effects when injections were administered immediately (top panels), 45 min before (middle panels), and 90 min (bottom panels) before conditioning sessions (Figure 5B and 5C). Statistical analyses indicated non-significant effects of dose, time, and their interaction.

The BZT analog, MFZ 4-87, did not produce a change in allocation of time to the compound-paired compartment during the post-conditioning session when administered immediately (Figure 5D, top panel), 45 min (middle panel) or 90 min (bottom panel) before conditioning sessions. With the 90-min pretreatment time, there was a trend towards an increase in the allocation of time to the compound-paired compartment; however those effects were not statistically different from those for saline. Statistical analyses indicated non-significant effects of dose, time, and their interaction.

Comparison of BZT analog- and cocaine-induced stimulation of DA during place-conditioning times

DA concentrations in the NAC shell at times corresponding to the times of conditioning sessions increased as a function of dose (Figure 6). The dashed lines show the 165% of basal DA values ($\pm$ SEM) produced by 3.0 mg/kg of cocaine which produced significant place conditioning. 4-Cl-BZT produced dose-dependent increases in DA levels at all time periods. Although 3.0 mg/kg 4-Cl-BZT did not significantly modify DA values, the 10.0 mg/kg dose increased concentrations of DA beyond that produced by 3.0 mg/kg cocaine at all times, and more so at the later time points. Similarly, the highest dose of 4,4-diCl-BZT increased DA to levels greater than those produced by 3.0 mg/kg of cocaine with effects substantially greater than those of cocaine at the later time points. Each of the 2-substituted analogs, MFZ 4-86 and MFZ 4-87, increased extracellular DA concentrations at 3.0 mg/kg to levels that approximated those of cocaine in all but the first 30 min (Figure 6, bottom panels). The 10.0 mg/kg dose of each of the 2-substituted analogs increased extracellular DA concentrations in the NAC shell to levels that exceeded those of the 3.0 mg/kg dose of cocaine at all pretreatment times.

Not only did the BZT analogs dose-dependently increase concentrations of DA, but the rate of increase was dependent on dose (Figure 7). Each of the compounds produced a rate of increase in DA during the first phase identified in figure 4 that approximated or exceeded 4.84 %/min, the rate of increase produced by the 3.0 mg/kg dose of cocaine that produced significant place conditioning.

DISCUSSION

Most accounts indicate that stimulation of extracellular DA levels is the critical mechanism for the behavioral effects of cocaine that lead to its abuse liability (Dworkin and Smith 1988; Ikemoto 2002; Pontieri et al. 1995; Tanda et al. 1997a; Wise et al. 1995). Further, drugs with affinity for the DAT typically have behavioral effects like those of cocaine primarily differing in potency (Kuhar et al. 1991; Ritz et al. 1989a; Ritz et al. 1987). However, recent studies have suggested that conformational changes of the DAT protein differentially affect its interactions with DAT inhibitors (Loland et al. 2002; Schmitt et al. 2008). The present study examined the effects of several compounds suggested to interact with the DAT differently from cocaine, in order to investigate reinforcing effects of compounds tolerant to changes from outward- to inward-facing conformational state induced by the Y335A mutation of the DAT. Cocaine and similar DA uptake inhibitors have a high affinity for the wild-type DAT that is in an equilibrium shifted to the outward facing conformation. In cells

transfected with a Y335A DAT mutant the conformational equilibrium is shifted toward inward-facing, and the potency of cocaine and like drugs is reduced by about two orders of magnitude (Loland et al. 2002). In contrast, several BZT analogs show a greater tolerance to conformational state than cocaine, with binding affinities that are less affected than those of cocaine-like compounds in DAT mutants with either an inward- or outward-facing conformation. For example, among compounds tested for binding to the Y335A mutant DAT, the potencies of 4,4-diCl-BZT and MFZ 4-86 are respectively only about 40- and 13-fold lower than in cells with the wild-type DAT (Loland et al. 2008).

As has been shown previously for cocaine and 4-Cl-BZT (Tanda et al. 2005; Tanda et al. 1997b), each of the compounds significantly increased DA concentrations in the NAC shell, an area of the brain implicated in the reinforcing effects of drugs (Pontieri et al. 1995; Pontieri et al. 1996; Tanda et al. 1997a). However as was also shown previously for cocaine and 4-Cl-BZT there were differences with regard to changes in levels of DA at different time points as well as the rates of change in DA levels.

In contrast to cocaine none of the compounds were effective in producing a significant place conditioning under the present conditions. The current results extend previous studies with BZT analogs indicating that these compounds typically have reduced reinforcing effects, both in operant-conditioning self-administration procedures (Hiranita et al. 2009; Woolverton et al. 2001) and in respondent, place-conditioning procedures (Li et al. 2011; Li et al. 2005). A number of factors that influence conditioning may have contributed to the present outcomes. For example, conditioning is affected by the intensity of the unconditional stimulus (Bruner 1969; Pavlov 1927) and a lack of effect may be due to insufficient stimulus intensity. However, insufficient unconditional stimulus intensity in the present study is unlikely as a range of doses was studied and the highest doses increased DA concentrations to an extent comparable to that produced by an effective dose of cocaine.

Another influential factor to be considered is the pharmacokinetics of the BZT analogs, which may have compromised the reliable pairing of the drug effect with the set of stimuli uniquely arising from the drug-paired compartment. The various delays between injections and conditioning sessions were intended to offset decreased effectiveness due to delays in achieving sufficient effects of the BZT analogs. Optimization of conditioning at the start of the session is likely dependent on how well the session envelops the actions of the drug. Further, the long-lasting nature of the effects of the BZT analogs entails that the unconditional stimulus is likely present following the conditioning session, during the time in which the subject has been returned to the home cage which occasions another set of stimuli. Mitigating against complications arising from the post-session condition is its non-differential status; the subject is returned to the same home cage after sessions with saline. Further, reliable pairings are likely never perfectly accomplished in any place conditioning procedure using drugs owing to their pharmacokinetics.

Compounds that bind with substantial preference to the outward-facing conformation due to its accessibility generally may show faster association and dissociation rates, as has been demonstrated for the cocaine analog WIN 35,428 compared to JHW 007 (Kopajtic et al. 2010), resulting in the long-lasting stimulation of DA with BZT analogs reported here and previously (Tanda et al. 2005; Tanda et al. 2007). These lasting effects may in turn promote a tonic DA response producing a different expression of behavioral effects compared to a more phasic response. Further studies with enhanced resolution of response over time will undoubtedly help resolve contributions of tonic and phasic DA neurotransmission to the presently observed effects.

In contrast to the present findings, Desai et al. (2010) found that several indirect DA agonists (cocaine, methamphetamine, and GBR 12909) produced full substitution in rats trained to discriminate methamphetamine at doses that increased DA levels to approximately 200 to 400% of control values, increases that were achieved with the BZT analogs in the present study. The relation between elevations in DA that are involved in cocaine discrimination and place conditioning is not known, however one major difference between this and the study by Desai et al. (2010) is the present use of DA uptake inhibitors that are largely independent of DAT conformation. However, it should be noted that it has been reported that the mode of interaction of GBR 12909 with the DAT is also different from that of cocaine (Chen and Reith 2004), and that some novel cocaine analogs that preferentially interact with an outward-facing DAT conformation (induced by a W84L mutation) do not produce a full complement of cocaine-like effects (Reith et al. 2012). Thus conformational status of the DAT may not be a singular determinant of whether compounds acting at that site produce cocaine-like effects. Nonetheless, the present data indicate that BZT analogs that interact with the DAT differently from cocaine can produce elevations in DA in the NAC shell of a magnitude similar to that produced by cocaine and are ineffective in producing the full complement of cocaine-like effects, among which place conditioning is one. Thus, some other pharmacological effect is necessary for, or some aspect of the pharmacology of the BZT analogs interferes with place conditioning.

An alternative explanation for the lack of efficacy of the present BZT analogs might also be found in the ability of these compounds to interact with multiple pharmacologic targets, which might decrease their behavioral effectiveness. The presently reported receptor screen and previous studies provide information on other targets that may contribute to the profile of behavioral effects observed with the BZT analogs. Of the 42 binding sites other than the DAT, 4-Cl-BZT and 4,4-diCl-BZT showed moderate to high binding affinities at histamine H₁ and the muscarinic receptor subtypes M₁, M₂ and M₃. The addition of the 2-carboethoxy substituent in MFZ 4-86 reduced these values to the low micromolar range resulting in a highly selective DAT ligand. Only M₁ binding affinity for MFZ 4-87 remained in the submicromolar range, but this compound was still ~30-fold selective for the DAT compared to any other site assayed. Further, previous studies have determined that effects at muscarinic M₁ and histamine H₁ receptors are not sufficient to antagonize the effects of cocaine when ligands for these sites are administered in combination with cocaine (Campbell et al. 2005; Katz et al. 1999; Tanda et al. 2007; Tanda and Katz 2007). Thus current evidence does not support a supposition that actions at histamine H₁ or at muscarinic M₁ receptors interfere with an otherwise cocaine-like pharmacology of BZT analogs.

A previous study (Li et al. 2011) suggested that activity at σ receptors may oppose the cocaine-like effects of BZT analogs and that the σ -receptor antagonist, rimcazole, that also binds preferentially to the DAT, does not have effects like those of other DAT inhibitors and blocks self-administration of cocaine (Hiranita et al. 2011; Katz et al. 2003). Further studies demonstrated that the effects of rimcazole were related to effects at both the DAT and σ receptors (Hiranita et al. 2011). The 2-substituted BZT analogs show markedly reduced affinity for σ_1 receptors, while their affinities at σ_2 receptors are similar to those of the unsubstituted compounds, but still 10-20 times lower affinity compared to the DAT. In a previous study the non-selective σ -receptor agonist, DTG, and the selective σ_1 -receptor agonist, PRE-084, increased DA levels in the accumbens shell in rats though only the effects of DTG were σ -receptor mediated (Garces-Ramirez et al. 2011). Further studies on the potential contribution of σ receptor actions to the present effects of the BZT analogs are in progress.

In conclusion, in the present report we have compared cocaine, which has effects that are dependent on DAT conformation, with those of BZT analogs that are relatively tolerant to

DAT conformation (Loland et al. 2008). Previous studies using other compounds tolerant to DAT conformational status similarly found them to be ineffective both in producing place conditioning (Li et al. 2011; Li et al. 2005), and in maintaining responding in subjects trained to self-administer cocaine (Hiranita et al. 2009; Woolverton et al. 2001). The present study adds to previous ones by indicating that differences in effects of the BZT analogs and cocaine can be obtained at doses and times at which increases in DA concentration, or rates of increase in DA, are comparable to those produced by cocaine. Thus, those compounds for which the DAT is receptive in both inward and outward conformations have the characteristics of those suggested as candidates for development as pharmacological therapies for cocaine dependence (Newman and Katz 2009; Tanda et al. 2009b). The present lack of significant place conditioning with these compounds suggests a substantially lower, if any, abuse liability. Further studies on combinations of cocaine with these compounds might lead to attenuation of cocaine reinforcing effects, as it has been already shown with other BZT analogs (Hiranita et al. 2009; Tanda et al. 2009a), providing more evidence that this class of compounds may provide leads for a medication that can be used to treat cocaine abuse.

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4,4-diCl-BZT; R=H, X₁=X₂=Cl

4-Cl-BZT; R=H, X₁=Cl, X₂=H

MFZ 4-86 (±); R=COOCH₂CH₃, X₁=X₂=Cl

MFZ 4-87; (±); R=COOCH₃, X₁=X₂=Cl

Figure 1.

Chemical structures of cocaine and the BZT analogs studied.

Figure 2.

Forebrain sections, redrawn from Paxinos and Watson, 1998, showing the limits of the positions of the dialyzing portions of the microdialysis probes (superimposed rectangles) resulting from histological analysis of brain sections. On each section the anterior coordinate (A) (measured from bregma) is indicated. CPU=caudate putamen; shell=nucleus accumbens shell.

Figure 3.

Place conditioning and stimulation of extracellular levels of DA in the NAC shell produced by various doses of cocaine. Ordinates: on the left, time allocated to the compound-paired side during the post-conditioning session expressed as a difference from that during the last preconditioning session. Ordinates on the right also show average changes in extracellular DA levels expressed as a percentage of baseline during the first 30-min after compound injection, corresponding to the time at which place conditioning sessions were conducted with cocaine. Abscissae: dose of compound in mg/kg. The saline and 10.0 mg/kg cocaine data are presented as means and SEM values determined in 52 subjects used over the course of all of the place-conditioning studies. The place conditioning data for the remainder of the

cocaine doses are means and SEM values from six subjects. Basal DA values (fmoles/sample) and group size (n) were: 65.3 ± 8.3 (4), 39.8 ± 6.8 (7), and 58.9 ± 6.1 (6), for saline, 3.0 or 10.0 mg/kg cocaine groups, respectively.

Figure 4.

Time course of effects of systemic administration of 4,4-diCl-BZT (panel A), MFZ 4-86 (Panel B) and MFZ 4-87 (Panel C) on extracellular levels of DA in dialysates from the NAC shell. Results are means, with vertical bars representing SEM, of the amount of DA in 10-min dialysate samples, expressed as percentage of basal values, uncorrected for probe recovery. Basal DA values (fmoles/sample) and group size (n) were: 46.0 ± 1.8 (7), 47.9 ± 8.7 (7), and 51.4 ± 7.6 (8) for 4,4-diCl-BZT doses of 1, 3 and 10 mg/kg, respectively; 48.7 ± 12.3 (6), 51.3 ± 5.1 (8), and 41.9 ± 4.3 (12) for MFZ 4-86 doses of 1, 3 and 10 mg/kg, respectively; 43.4 ± 2.3 (4), 33.3 ± 3.9 (6), and 36.0 ± 4.8 (7) for MFZ 4-87 doses of 1, 3 and 10 mg/kg, respectively.

Figure 5.

Place conditioning results for the chloro-substituted BZT analogs administered to rats at different times before placement in the conditioning chamber. Ordinates: time allocated to the compound-paired side during the post-conditioning session expressed as a difference from that during the last preconditioning session. Abscissae: dose of compound in mg/kg. Panels A, D, G, and K show results from place conditioning when the compounds were given immediately before conditioning sessions. Panels B, E, H, and L show results from the 45-min pretreatment before conditioning sessions. Panels C, F, J, and M show results for the 90-min pretreatment conditioning sessions. Data are presented as means and SEM values of from 8 or 16 subjects. The results of place conditioning with saline or 10.0 mg/kg of cocaine administered immediately before testing are shown by the open and filled bars, respectively. Only the effects of cocaine were statistically significant.

Figure 6.

Extracellular levels of DA as a function of dose of the chloro-substituted BZT analogs, during the place-conditioning time periods. Ordinates: extracellular DA levels in the NAC shell as a percentage of baseline during 30-min periods corresponding to the times after compound administration during which place conditioning sessions were conducted (pretreatment times of 0, 45, or 90 min). Abscissae: dose of compound in mg/kg, log scale. For comparison, the dashed horizontal line shows the effect ($\pm$ SEM) of cocaine on extracellular DA levels as a percentage of baseline at the lowest dose that produced a significant place conditioning (3.0 mg/kg) during the time after injection (0-30 min) at which the positive effect was obtained. Each point represents the average effect ($\pm$ SEM) determined in four to 12 rats.

Figure 7.

Rates of increase in DA extracellular levels in dialysates from the NAC shell produced by the chloro-substituted BZT analogs. Ordinates: rates of increase, percentage/minute in DA levels during the two phases identified on the time course of effects on DA levels described in Figure 4. Abscissae: dose of compound in mg/kg, log scale. For comparison, the dashed horizontal line shows the effect (± SEM) of cocaine on rate of increase in extracellular DA levels during phase 1 at the lowest dose tested that produced significant place-conditioning. Each point represents the average effect (±SEM) determined in four to 12 rats.

Table 1

Summary of assay conditions used for assessing activity at various receptor sites in competition for the specified radioligand. Further details can be found in the published methods (citations) or in MDS Panlabs catalogue (MDS Panlabs Pharmacology Services, 2000).

Assay Target	Ligand	Non-Specific Binding	Tissue	Incubation
Adenosine A ₁	1 nM [³ H]DPCPX	100 μM R(-)-PIA	Human recombinant CHO cells	90 min @ 25°C
Adenosine A _{2A}	0.05 μM [³ H]CGS-21680	50 μM NECA	Human recombinant HEK-293 cells	90 min @ 25°C
Adrenergic α ₁ , non-selective	0.25 nM [³ H]Prazosin	0.1 μM Prazosin	Rat brain	30 min @ 25°C
Adrenergic α _{1A}	0.25 nM [³ H]Prazosin	10 μM Phentolamine	Rat submaxillary gland	60 min @ 25°C
Adrenergic α _{1B}	0.25 nM [³ H]Prazosin	10 μM Phentolamine	Rat liver	60 min @ 25°C
Adrenergic α ₂ , non-selective	0.7 nM [³ H]Rauwolscine	1 μM Yohimbine	Rat cortex	30 min @ 25°C
Adrenergic α _{2A}	1 nM [³ H]MK-912	10 μM WB-4101	Human recombinant insect Sf9 cells	60 min @ 25°C
Adrenergic β, non-selective	0.25 nM [³ H]Dihydroalprenolol	1 μM S(-)-Propranolol	Rat brain	20 min @ 25°C
Adrenergic β ₁	0.03 nM [¹²⁵ I]-Cyanopindolol	100 μM (s)-Propranolol	Human recombinant Rex 16 cells	120 min @ 25°C
Adrenergic β ₂	0.2 nM [³ H]CGP-12177	10 μM ICI-118551	Human recombinant CHO-NBR1 cells	60 min @ 25°C
Ca ⁺⁺ Channel-L, dihydropyridine	0.1 nM [³ H]Nitrendipine	1 μM Nifedipine	Rat cortex	90 min @ 25°C
Dopamine D ₁	1.4 nM [³ H]SCH 23390	10 μM (+)-Butaclamol	Human recombinant CHO cells	120 min @ 37°C
Dopamine D ₂	0.16 nM [³ H]Spiperone	10 μM Haloperidol	Human recombinant CHO cells	120 min @ 25°C
GABA _A , Agonist Site (Muscimol)	1 nM [³ H]Muscimol	100 nM Muscimol	Rat brain (minus cerebellum)	10 min @ 4°C
GABA _A , Benzodiazepine, Central	1 nM [³ H]Flunitrazepam	10 μM Diazepam	Rat brain (minus cerebellum)	60 min @ 25°C
GABA _A , Chloride Channel	3 nM [³ H]TBOB	200 μM Picrotoxin	Rat cortex	20 min @ 25°C
GABA _B , Non-selective	0.6 nM [³ H]CGP-54626	100 nM CGP-54626	Rat brain	20 min @ 25°C
Glutamate, NMDA, phencyclidine	4 nM [³ H]TCP	1 μM MK-801	Rat cortex	45 min @ 25°C
Glutamate, non-selective	3.75 nM [³ H]L-Glutamic acid	50 μM L-Glutamic acid	Rat brain	30 min @ 37°C
Glycine, strychnine-sensitive	10 nM [³ H]Strychnine	1 mM Glycine	Rat spinal cord	10 min @ 4°C
H ₁ receptor	2 nM [³ H]Mepyramine	100 μM Promethazine	Rat brain (including cerebellum)	60 min @ 25°C
Insulin	0.03 nM [¹²⁵ I]Insulin	1 μM Insulin	Rat liver	16 hr @ 4°C

Assay Target	Ligand	Non-Specific Binding	Tissue	Incubation
Muscarinic M ₂	0.29 nM [³ H]N-methylscopolamine (0.8 nM for JHW 013)	1 μM Atropine	Human recombinant insect sf9 cells (Human recombinant CHO cells)	60 min @ 25°C (120 min @ 25°C)
Muscarinic M ₃	0.29 nM [³ H]N-methylscopolamine (0.8 nM for JHW 013)	1 μM Atropine	Human recombinant insect sf9 cells (Human recombinant CHO cells)	60 min @ 25°C (120 min @ 25°C)
Nicotinic Acetylcholine	0.1 nM [¹²⁵ I]Epibatidine	300 μM (–)-Nicotine	Human IMR-32 cells	60 min @ 25°C
Opiate, Non-selective	1.0 nM [³ H]Naloxone	1 μM Naloxone	Rat brain	40 min @ 25°C
Opiate-δ	0.9 nM [³ H]Naltrindole	10 μM Naloxone	Human recombinant CHO cells	120 min @ 25°C
Opiate-κ	0.6 nM [³ H]Diprenorphine	1 μM Naloxone	Human recombinant HEK-293 cells	60 min @ 25°C
Opiate-μ	0.6 nM [³ H]Diprenorphine	10 μM Naloxone	Human recombinant CHO cells	60 min @ 25°C
Phorbol ester	3 nM [³ H]PDBu	1 μM PDBu	Mouse brain	60 min @ 25°C
K ⁺ Channel [K _{ATP}]	5 nM [³ H]Glyburide	1 μM Glyburide	Hamster pancreatic β cells HIT-T15	120 min @ 25°C
K ⁺ Channel hERG	1.5 nM [³ H]Astemizole	10 μM Astemizole	Human recombinant HEK-293 cells	60 min @ 25°C
Progesterone	2 nM [³ H]R-5020	0.41 μM Progesterone	Bovine uterus	16 hr @ 4°C
Serotonin 5-HT ₁ , non-selective	2 nM [³ H]5-HT	10 μM 5-HT	Rat cortex	10 min @ 37°C
Serotonin 5-HT ₂ , non-selective	0.5 nM [³ H]Ketanserin	1 μM Ketanserin	Rat brain	40 min @ 25°C
σ ₁ receptor	3 nM [³ H](+)-Pentazocine	10 μM Haloperidol	guinea-pig brain (minus cerebellum)	120 min @ 25°C
σ ₂ receptor	3 nM [³ H]DTG with 200 nM (+)-pentazocine	100 μM Haloperidol	guinea-pig brain (minus cerebellum)	120 min @ 25°C
Na ⁺ Channel, site 2	5 nM [³ H]Batrachotoxin A 20-α-benzoate	100 μM Veratridine	Rat brain	60 min @ 37°C

NMDA, N-methyl-D-aspartate;

CGP-12177, 4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazol-2-one;

CGP-54626, [S-(R*,R*)]-[3-[[1-(3,4-dichlorophenyl)ethyl]amino]-2-hydroxypropyl](cyclohexylmethyl) phosphinic acid;

CGS 21680, 2-[p-(2-carboxyethyl)phenethylamino]-5'-N-ethylcarboxamidoadenosine;

DPCPX, 8-cyclopentyl-1,3-dipropylxanthine;

ICI 118551, (±)-1-2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy-3-(1-methylethyl)amino-2-butanol;

MK801, (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen;

MK-912, L-657,743, (2S-trans)-1,3,4,5',6,6',7,12b-octahydro-1',3'-dimethyl-spiro[2H-benzofuro[2,3-a]quinolizine-2,4'(1'H)-pyrimidin]-2'(3'H)-one hydrochloride hydrate;

NECA, 5'-N-ethylcarboxamidoadenosine;

PDBu, phorbol-12,13-dibutyrate;

PIA, N⁶-phenylisopropyladenosine;

R-5020, 17α,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione;

SCH 23390, R-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine;

TBOB, *t*-butylbicycloorthobenzoate;

TCP, thienyl cyclohexylpiperidine;

WB-4101, 2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane hydrochloride.

Table 2

K_i values (nanomolar) of BZT Analogues at Various Binding Sites. Unless otherwise specified results are from the receptor screen. All values are in nM^a

Assay Target	4-Cl-BZT	4',4''-diCl-BZT	MFZ 486 (+)	MFZ 487 (+)
DAT	10.5 ± 1.21 ^b	17.5 ± 0.88 ^f	14.6 ± 0.39 ^f	12.6 ± 0.40 ^f
SERT	5120 ± 395 ^c	1640 ± 236 ^c	1560 ± 91 ^f	528 ± 39 ^f
NET	1470 ± 180 ^c	2980 ± 182 ^c	3350 ± 154 ^f	2150 ± 325 ^f
Adenosine A ₁	inactive	inactive	inactive	inactive
Adenosine A _{2A}	inactive	inactive	inactive	inactive
Adrenergic α ₁ , non-selective	NT	137	NT	NT
Adrenergic α _{1A}	<10,000	NT	inactive	inactive
Adrenergic α _{1B}	<10,000	NT	inactive	inactive
Adrenergic α ₂ , non-selective	NT	4140	NT	NT
Adrenergic α _{2A}	<10,000	NT	inactive	inactive
Adrenergic β ₁	inactive	inactive	inactive	inactive
Adrenergic β ₂	inactive	inactive	inactive	inactive
Ca ⁺⁺ Channel-L, dihydropyridine	inactive	4990	inactive	inactive
Dopamine D ₁	<10,000	674	inactive	inactive
Dopamine D _{2L}	NT	inactive	NT	NT
Dopamine D _{2S}	<10,000	NT	inactive	inactive
Estrogen ERα	NT	inactive	NT	NT
GABA _A , Agonist Site (Muscimol)	inactive	inactive	inactive	inactive
GABA _A , Benzodiazepine, Central	inactive	NT	inactive	inactive
GABA _A , Chloride Channel	NT	inactive	NT	NT
Glucocorticoid	NT	inactive	NT	NT
Glutamate, NMDA, phencyclidine	inactive	inactive	inactive	inactive
Glutamate, non-selective	NT	inactive	NT	NT
Glycine, strychnine-sensitive	NT	inactive	NT	NT
Histamine H ₁	39.9 ± 22.6 ^d	122 ± 4.55 ^d	37,600 ± 2990 ^g	27,100 ± 2600
Insulin	NT	inactive	NT	NT
Muscarinic M ₁	4.3 (9) ^e	40.6 (8) ^e	3060 ± 150 ^f	382 ± 37 ^f
Muscarinic M ₂	5.88 ± 0.630	640	3120 ± 50	1220 ± 70
Muscarinic M ₃	0.384 ± 0.043	10.0	1920 ± 229	2390 ± 164
Nicotinic Acetylcholine	inactive	NT	inactive	inactive
Opiate-δ	inactive ^f	inactive	inactive	inactive
Opiate-κ	inactive	inactive	inactive	inactive

Assay Target	4-Cl-BZT	4',4''-diCl-BZT	MFZ 486 (+)	MFZ 487 (+)
Opiate- μ	inactive	3070	inactive	inactive
Phorbol ester	NT	inactive	NT	NT
K ⁺ Channel [K _{ATP}]	inactive	inactive	inactive	inactive
K ⁺ Channel hERG	804 $\pm$ 300	NT	287 $\pm$ 40	335 $\pm$ 12
Progesterone	NT	inactive	NT	NT
Serotonin 5-HT ₁ , non-selective	inactive	inactive	inactive	Inactive
Serotonin 5-HT ₂ , non-selective	<10,000	231	inactive	Inactive
σ_1	451 (379 – 545) ^h	989 (804 – 1210) ^h	8280 (6040 – 11300) ^h	6350 (5130 – 7890) ^h
σ_2	169 (91.4 – 314) ^h	177 (79.3 – 395) ^h	333 (178 – 625) ^h	234 (105 – 521) ^h
Na ⁺ Channel, site 2	449 $\pm$ 42	766	934 $\pm$ 17	1030 $\pm$ 32
Testosterone	NT	inactive	NT	NT

^aIf the displacement at 10,000 nM was greater than 75% "<10,000 nM" was entered. If the displacement at 10,000 nM was less than 75%, "inactive" was entered.

^bNewman and Katz (2009)

^cWoolverton et al., Psychopharmacology (2001)

^dKulkarni et al. Bioorganic & Medicinal Chemistry (2006)

^eKatz et al. Journal of Pharmacology and Experimental Therapeutics (1999) (values in parentheses represent 1SEM)

^fZou et al., J. Med. Chem. (2006)

^gCampbell et al., Journal of Pharmacology and Experimental Therapeutics (2005)

^hThese values are for those of the racemic form of the compound. Values in parentheses are 95% confidence limits.